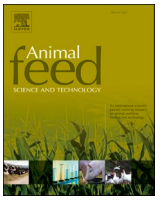
Animal Feed Science and Technology 295 (2023) 115526

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



The effects of exogenous fibrolytic enzymes on the *in vitro* release of xylooligosaccharides and monosaccharides varies across six varieties of wheat

Tom Dale [a], Michael R. Bedford [b], Julie King [a], Gregory A. Tucker [a], John M. Brameld [a], Tim Parr [a,*]

[a] School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK
[b] AB Vista, Woodstock Court, Marlborough, Wiltshire SN8 4AN, UK

ARTICLE INFO

Key words:
Wheat. Xylanase. Xylooligosaccharide. Prebiotic. Feed

ABSTRACT

Wheat is a widely used cereal grain for pig and poultry feeds globally. Despite this, there are noticeable differences in its nutritive value, potentially due to varying characteristics like inherent non-starch polysaccharide (NSP) content and composition. Fibrolytic enzymes help degrade NSP and thereby improve feed efficiency in livestock. However, it has been suggested that these enzymes have different effects dependent upon the characteristics of the wheat variety used in a feed. This study investigated the efficacy of different enzyme-wheat variety combinations, by quantifying the release of NSP-derived xylooligosaccharides (XOS) and monosaccharides from six varieties of wheat (Maris Huntsman, Highbury, Paragon, Sinuelo, Chinese Spring and Pavon 76) over a 24 h *in vitro* incubation with commercially available fibrolytic enzymes (Econase XT, Econase MP1000 or Barley P700). Complete non-starch acid hydrolysis showed there were differences between varieties in their total monosaccharide contents ($P < 0.001$). There were significant wheat variety x enzyme x incubation time interactions for the release of xylobiose, galactose and glucose (all $P < 0.001$) and significant enzyme x wheat variety interactions for the release of xylotriose ($P = 0.022$), xylose ($P < 0.001$) and arabinose ($P = 0.028$). Clear differences in release of XOS were observed between the different combinations of enzyme and wheat variety. Econase XT increased xylotriose release from Highbury wheat, and xylobiose release from Sinuelo, with both wheat varieties showing comparable release of xylose. These findings suggest that the fibrolytic enzymes tested have some specificity for the wheat varieties. Hence it might be possible to optimise the combinations of wheat variety and enzyme used in animal feeds, to help maximise the feed efficiency of livestock.

Abbreviations: ×g, times gravity; AME, apparent metabolisable energy; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; BU/g, beta-units per gram; BXU/g, beta-xylanase units per gram; ECU/g,, endo-cellulase units per gram; HPAEC-PAD,, high-performance anion-exchange chromatography with pulsed amperometric design; MNU/g, mannanase units per gram; NSP, non-starch polysaccharide; SNSP, water soluble non starch polysaccharide; TFA, trifluroacetic acid; XOS, xylooligosaccharide.

* Corresponding author.
E-mail address: tim.parr@nottingham.ac.uk (T. Parr).

https://doi.org/10.1016/j.anifeedsci.2022.115526
Received 10 August 2022; Received in revised form 14 November 2022; Accepted 17 November 2022
Available online 19 November 2022

1. Introduction

Wheat is the most common energy source used in European poultry feeds (Choct et al., 1999). This is in large part due to its high content of storage polysaccharides encapsulated within the cereal grain (del Alamo et al., 2008). Wheat contributes to over 600 g/kg as fed of starter feed formulations for broilers, and up to 750 g/kg as fed in finisher feed formulations (Austin et al., 1999). There is, however, large variation in the apparent metabolisable energy (AME) of wheat-based feeds, largely attributable to their relatively high non-starch polysaccharide (NSP) contents (120–180 g/kg as fed) (Slominski et al., 2000).

NSP present in the wheat plant cell walls are known to have anti-nutritional effects in broilers, increasing the viscosity of digesta, resulting in reduced mixing and movement of digesta. This limits the potential for both exogenous and endogenous enzymes to reach their target sites, reducing digestibility (Choct and Annison, 1992; Bedford, 2000). Given that broilers do not possess the necessary endogenous enzymes to sufficiently break down plant cell walls, the encapsulation of starch and proteins within cell walls is thought to act as a direct barrier to these nutrients being utilised (Bedford and Autio, 1996), further reducing the AME of wheat-based feeds.

Arabinoxylan is the predominant plant cell wall polysaccharide present in wheat, with total arabinoxylans (AX) accounting for 590 g/kg of NSP, comprising mainly of xylose, β-glucans, cellulose and glucose, with galactose, uronic acids, mannose, rhamnose and fucose making up the remainder (De Keyser et al., 2018). Hence, enzymes such as xylanases, β-glucanases and cellulases, which target arabinoxylan, β-glucan and cellulose respectively, have the potential to increase digestibility of NSP in the intestinal tracts of broilers consuming wheat-based feeds (Masey O'Neill et al., 2014). Our previous research (Dale et al., 2022) showed clear differences in the effects of exogenous enzymes on the sugars released from different cereals *in vitro.* The three enzymes studied varied in their dominant activity, but all contained xylanase. The enzymes were tested on four different cereals (barley, maize, oats and wheat) and showed clear differences in the release of XOS. This is likely due to the divergence in properties of the xylanases present in each preparation and shows cereal type and enzyme characteristics interact to determine the XOS released.

The same *in vitro* model (Dale et al., 2022) is used here to quantify the monosaccharides and XOS released from different varieties of wheat during a 24 hr incubation with different fibrolytic enzymes. The mean retention time in the small intestine of broilers is suggested as being between 5 and 6 h (Svihus and Itani, 2019), compared with 2.5–4 h in pigs (Wilfart et al., 2007). However others have described most of a digestion marker was excreted by 12 hrs in growing chicks (Tuckey et al., 1958). The digestions described in this study were done in the absence of any other enzymes, therefore we assumed the rates of digestion are likely to be slower than those observed *in vivo*, so we monitored the release of sugars over a 24 hr time course, which was when most of the changes occurred in our previous study (Dale et al., 2022), but does mean that the times are unlikely to represent the *in vivo* situation. The data generated from this study should help inform future *in vivo* research to identify the optimal xylanase for use in wheat-based broiler feeds, by tailoring specific enzymes to specific wheat varieties (Maisonnier-Grenier et al., 2006; Lafond et al., 2011). Wheat was chosen as it released more monosaccharides and XOS in response to the different enzymes in the previous *in vitro* study (Dale et al., 2022).

The aim therefore was to compare the effects of the three different fibrolytic enzymes on the *in vitro* release of XOS and monosaccharides from six different varieties of wheat over a 24 h incubation, particularly focussing on the arabinoxylan (AX) component of wheat.

2. Materials and methods

2.1. Wheat varieties

The six varieties of wheat used were Maris Huntsman, Highbury, Paragon, Sinuelo, Pavon 76 and Chinese Spring, obtained from the Biotechnology and Biological Sciences Research Council (BBSRC) Wheat Research Centre at the University of Nottingham School of Biosciences, Loughborough, UK. They were selected for their differing characteristics and geographical location of use. Maris Huntsman (Foulkes et al., 2006) and Highbury are both winter wheat varieties with the latter being used for bread making (Payne et al., 1987); Paragon is a spring wheat variety which is also used in bread making (Milec et al., 2012), and all of these have been or are currently used in the UK. Sinuelo is a variety adapted for use in the harsh environment found in Brazil and shows resistance to mycotoxin (Fusarium) growth (Machado et al., 2017); Pavon 76 is grown in Mexico and is relatively resistant to leaf rust (Puccinia) (Singh et al., 1998); whilst Chinese Spring originates from China and can tolerate high levels of humidity and temperature (Warner et al., 2000). The 6 wheat varieties were originally sourced from different places, but the seeds used in this work were all potted in the same compost (John Innes No. 2) and multiplied in an onsite glasshouse under a regime of 16 h light and 8 h dark, with a day time temperature of 22–23 °C and night time of 16–17 °C. Hence any variability observed between varieties is unlikely to be due to differences in growing conditions/ environment.

2.2. Total hydrolysis of non-cellulosic polysaccharides

Total sugar contents in the 6 wheat varieties were determined by total hydrolysis of their non-cellulosic polysaccharides with Trifluroacetic acid (TFA), as previously described (Fry, 1988). The aim was to determine the baseline contents of xylose and arabinose and possibly galactose and starch derived glucose. TFA is often used in the analysis of plant cell walls, as it is milder than the more commonly employed Seaman hydrolysis (Seaman, 1945) and as such does not tend to degrade the cellulosic component of the wall. Briefly, wheat samples were ground to a fine powder (0.5 mm screen) and suspended at 10 mg/ml in 2 M TFA in triplicate. Tubes were sealed and heated to 120 °C for 1 hr in an autoclave, then allowed to cool to room temperature before being centrifuged at 2236 *g* for 10 min at room temperature. The supernatant was then diluted 1:100 with 10 mM NaOH and transferred to 2 ml clear vials for sugar

analysis. The release of 4 monosaccharides (arabinose, galactose, glucose and xylose) was quantified using high-performance anion-exchange chromatography/pulsed amperometric detection (HPAEC-PAD), with the sum of these 4 monosaccharides taken to be the total sugar content.

2.3. *In vitro digestion with 3 different enzymes*

The following 3 commercial enzyme preparations were provided by AB Vista (Marlborough, UK) and used in *in vitro* digestions with the 6 varieties of wheat:

i. Econase XT – a xylanase with β-1, 4 endo-xylanase activity (160,000 BXU/g). The enzyme was used at its suggested dose of 100 mg/kg of feed.
ii. Econase MP 1000 – a mannanase enzyme cocktail reported to contain mannanase (1,000,000 MNU/g), β-glucanase (300,000 BU/g) and endo-xylanase (200,000 BXU/g) activities. It was used at the recommended dose of 100 mg/kg of feed.
iii. Econase Barley P 700 - an enzyme cocktail prepared from a strain of *Trichoderma reesei*, designed for use in barley feeds. Its main activity is β-glucanase (700,000 BU/g), but it also contains endo-cellulase (165,000 ECU/g) and endo-xylanase (190,000 BXU/g) activities. It was used at the recommended dose of 40 mg/kg of feed.

The 6 varieties were individually ground to a fine powder (0.5 mm) and 0.2 g of each added to 40mls of 50 mM sodium citrate buffer (pH 5.2) containing one of the following (in triplicate):

a) Wheat samples only – no enzyme added.
b) Wheat samples + Econase XT at 100 μg/g.
c) Wheat samples + Econase MP 1000 at 100 μg/g.
d) Wheat samples + Econase Barley P 700 at 40 μg/g.

The buffer was chosen to represent the average pH of the broiler digestive tract (Mabelebele et al., 2014). Digestion reactions were placed in a shaking incubator at 150RPM at a temperature of 41 °C for up to 24 hrs. At each time point (0, 3, 6, 9, 12 and 24hrs), 1 ml of digest was removed and added to 9 ml of 10 mM NaOH at room temperature, then mixed, centrifuged at 2236g for 10 min at room temperature and frozen at − 20 °C prior to sugar analysis.

2.4. *Identification and quantification of released sugars using HPAEC-PAD*

The concentrations (g/100 g) of arabinose, galactose, glucose and xylose, as well as the XOS, present in each sample were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the method of Xu et al. (2013). Each sample was assayed in triplicate. Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3 mm x 150 mm) and CarboPac PA20 Guard (3×30mm) for the monosaccharide analysis. A CarboPac PA200 column (3 mmx 250 mm) and CarboPac PA200 guard (3 mm x 50 mm) were used for the oligosaccharide (XOS) analysis. An injection volume of 10 μl was used throughout for all standards and samples. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS standards (xylo-biose, -triose and –tetraose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25 g/L for monosaccharides and 0.5, 0.25, 0.125 and 0.0625 g/L for XOS) were made fresh for each batch of analyses.

For the monosaccharides, a single eluent, containing 10 mM NaOH solution, was used as the mobile phase at 0.5 ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1 M sodium hydroxide (Solution A) and 0.1 M NaOH containing 0.5 M sodium acetate (solution B) in standard quadruple waveform, as described by Xu et al. (2013). The gradient program used for XOS determination was 100% solution A at 0 min, rising to 80% solution A and 20% solution B at 25 min, before returning to 100% solution A after 25 min. Both eluents were stored in plastic pressurised bottles with inert nitrogen gas at 6–9 psi. Data were collected with Dionex Chromeleon software (Version 6.7).

2.5. *Data and statistical analysis*

A single source was used for each wheat variety and all analyses (digestions and Dionex analysis) were carried out in batches of each enzyme-wheat variety combination in triplicate. Standards for the four monosaccharides or XOS were run at the start and end of each batch and standard curves generated from the areas under the curve and are presented as g/100 g of wheat variety. Data were processed in excel (Microsoft, 2013) and expressed as means ± standard error of differences (SED).

Data was analysed by one- (wheat variety), two- (wheat variety x time) or three- (enzyme x wheat variety x time) way ANOVA, as appropriate, using Genstat statistical software (VSN International 2019), with blocking for batch and digestion tube. Post hoc Bonferroni tests were used for significant effects/ interactions, with $P < 0.05$ taken as statistically significant.

3. Results

3.1. Total hydrolysis of non-cellulosic polysaccharides

The total sugar contents of each wheat variety were first determined by TFA hydrolysis and taken to be the sum of the arabinose, galactose, glucose and xylose monosaccharides. There was a significant difference between varieties, with Maris Huntsman containing the highest total sugars, and Paragon containing the lowest (Table 1, $P < 0.001$). As expected, the main sugar present was glucose, so it is not surprising that the wheat varieties order of glucose content was the same as the total sugar content (Table 1, $P < 0.001$). Galactose was present in low amounts in all varieties (0.6–1.0 g/100 g) and followed a similar pattern to the glucose content (Table 1, $P < 0.001$). Maris Huntsman also had the highest arabinose and xylose contents (Table 1). It is assumed that the vast majority of arabinose and xylose were present as arabinoxylan, hence the total arabinoxylan (AX) content was also highest in Maris Huntsman (Table 1, $P < 0.001$). As well as differences in the total AX content, the ratio of arabinose: xylose (A:X) was significantly different between wheat varieties, with the highest being in Sinuelo (Table 1, $P < 0.001$). Since the A:X ratio can influence the susceptibility of an AX to hydrolysis by an endo-xylanase, the range in A:X ratios suggested there might be differences in the oligosaccharides released from the 6 wheat varieties by different xylanase enzymes.

3.2. Release of XOS and monosaccharides from different wheat varieties in the absence of any exogenous enzymes

As in the previous study (Dale et al., 2022), some sugars were released in the absence of any exogenous enzyme (the control incubations), indicating the presence of enzymes either in the cereals themselves or from microbes present in the system, since no preservatives or antibiotics were included in the incubation buffer.

In the current study, there was no xylotriose released in the control incubations by any of the 6 wheat varieties. There were no significant wheat variety x time interactions for any of the other sugars measured, but they all showed significant independent effects of wheat variety and time (Fig. 1, $P < 0.001$ for both). Xylobiose release increased in a linear manner throughout the 24 hr time course in all 6 wheat varieties (Fig. 1). Maris Huntsman and Paragon released the most xylobiose, with Highbury, Chinese Spring and Pavon 76 all releasing similar quantities, and Sinuelo releasing the least.

Interestingly, although Sinuelo contained the lowest total quantity of xylose (Table 1), it released the largest amount of xylose over the 24 hr incubation compared to the other varieties. Paragon, Chinese Spring and Pavon 76 all released similar amounts of xylose, while Highbury released the least (Fig. 1). Xylose was mainly released during the initial (0–9 hr) incubation period, and then tended to plateau (9–24 hr). Overall, the release of xylose from the 6 wheat varieties was relatively small, amounting to only ~1 g/100 g of the total xylose available (Table 1).

As was observed for the total quantity of arabinose following TFA hydrolysis (Table1), Maris Huntsman released the most arabinose over the 24 hr incubation. The Highbury and Paragon varieties were similar, but slightly lower than Huntsman, with Sinuelo, Chinese Spring and Pavon 76 all releasing the lowest amounts of arabinose. Interestingly, arabinose release did not begin until 3 hr of incubation and increased rapidly during the 3–6 hr time period, before increasing more gradually to 24 hr. Only about 1 g/100 g of the total arabinose available was released at 24 hr (Table 1).

Release of galactose was much more variable (Fig. 1), but the greatest release was from 0 to 3 hr with very little further release from 6 to 24 hr. Paragon and Sinuelo varieties released slightly more galactose than the other varieties, even though they tended to be the varieties with the lower total galactose content following TFA hydrolysis (Table 1). Unlike xylose and arabinose, the proportion of total galactose released was much higher, with ~25 g/100 g of the total galactose available being released (Table 1).

Glucose release was greatest between 0 and 3 hr but then continued to rise more slowly to 24 hr. The profile of glucose release was

Table 1
Monosaccharide compositions, total arabinoxylan (AX) contents and arabinose: xylose (A:X) ratios of the six varieties of wheat determined by Trifluroacetic acid hydrolysis.

	Wheat Variety						SED[a]	P-Value[b]
Sugar (g/100 g)[c]	Maris Huntsman	Highbury	Pavon 74	Sinuelo	Chinese	Paragon		
Total Sugars[d]	65.04^{a}	54.37^{b}	52.94^{bc}	50.77^{c}	50.31^{c}	41.88^{d}	0.738	**< 0.001**
Arabinose	4.87^{a}	3.52^{b}	3.50^{b}	3.18 ^{cd}	2.95^{d}	3.30^{bc}	0.073	**< 0.001**
Galactose	0.99^{a}	0.71^{b}	0.67^{bc}	0.65^{bcd}	0.61 ^{cd}	0.59^{d}	0.018	**< 0.001**
Glucose	54.26^{a}	46.39^{b}	45.18^{bc}	44.09^{c}	43.41^{c}	34.67^{d}	0.556	**< 0.001**
Xylose	4.92^{a}	3.75^{b}	3.59^{bc}	2.85^{d}	3.34^{c}	3.33^{c}	0.095	**< 0.001**
Total AX[e] (g/100g)	9.79^{a}	7.27^{b}	7.09^{bc}	6.03^{e}	6.29^{de}	6.62 ^{cd}	0.1673	**< 0.001**
A:X Ratio[f]	0.99^{b}	0.94^{c}	0.98^{b}	1.09^{a}	0.88^{d}	0.99^{b}	0.005	**< 0.001**

[a,b,c,d] Mean values with different superscript letters were significantly different from each other ($P < 0.05$, *post hoc* Bonferroni test).
[a] SED, Standard Error of Difference.
[b] One-way ANOVA indicated significant differences between wheat varieties for each sugar.
[c] Values are expressed as mean grams of each monosaccharide per 100 g of wheat for biological triplicates.
[d] Total Sugars is the sum of the quantities of arabinose, galactose, glucose and xylose.
[e] Total AX is the sum of the arabinose and xylose.
[f] A:X values represent grams of arabinose per 100 g divided by grams of xylose per 100 g.

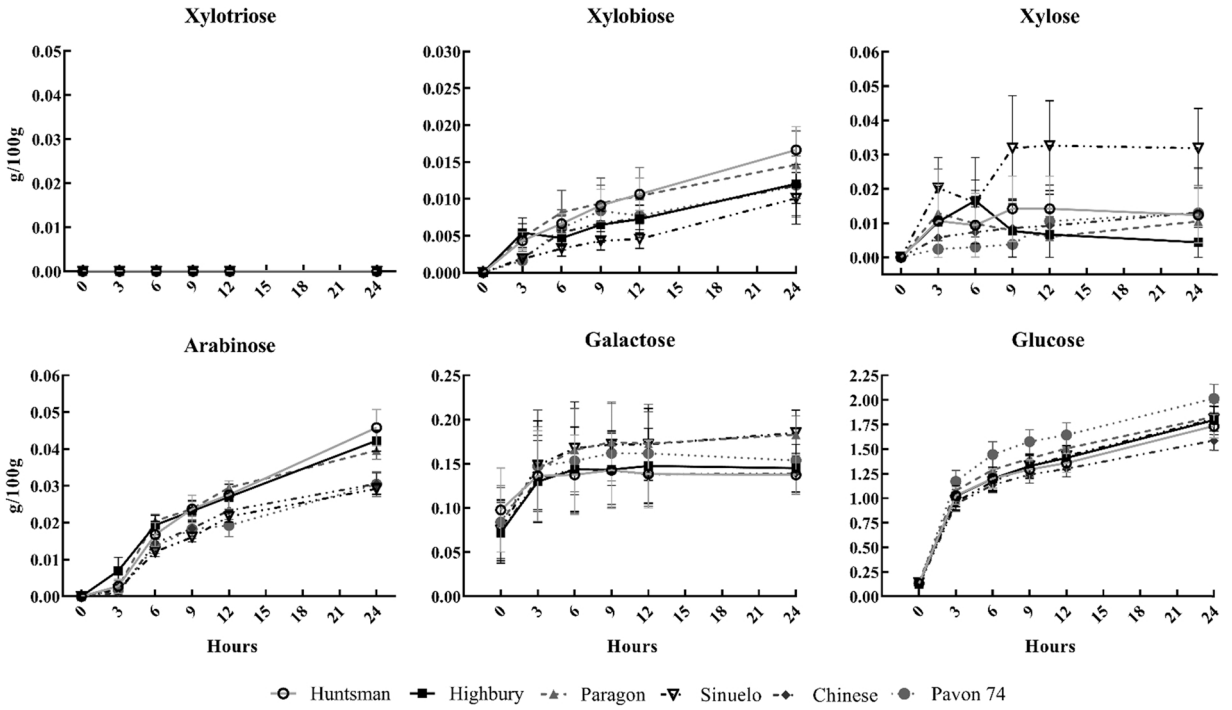


Fig. 1. Release of monosaccharides and xylooligosaccharides from the six varieties of wheat in the absence of any enzymes. Mean values (n = 3) are expressed as total g/100 g biomass released during *in vitro* incubation in sodium citrate buffer (pH5.2) over 24 h at 41 °C. Error bars represent the standard error of the mean (SEM). There was no detectable Xylotriose released in the absence of any exogenous enzyme. Two-way ANOVA indicated no significant variety x time interactions, but did indicate significant effects of both variety and time (both $P < 0.001$) for the other sugars.

similar for all 6 varieties, although Pavon 76 released slightly more than the others. Although the quantity of glucose released was greater than the other monosaccharides, it was only ~5 g/100 g of the total glucose available (Table 1).

3.3. Release of monosaccharides and XOS from different wheat varieties by the different exogenous xylanase enzymes

Further incubations were then done in the presence of each of the 3 exogenous enzymes, with the release during control (no enzyme) incubations being subtracted in order to determine effects of the enzymes. There was no release of xylotetraose observed from any of the wheat varieties by any of the enzymes used (data not shown).

For xylotriose release, significant enzyme x variety ($P = 0.022$), enzyme x time ($P < 0.001$) and variety x time ($P = 0.011$) interactions were observed (Fig. 2). Econase XT showed the greatest variation in response between varieties compared to the other 2 enzymes, particularly at 24hrs. Econase BP700 induced a rapid initial release of xylotriose, followed by a decline, whereas Econase MP1000 resulted in a gradual release of xylotriose up to 12 hr, before plateauing. Sinuelo generated the most xylotriose with all three enzymes.

For xylobiose release there was a significant enzyme x variety x time interaction ($P < 0.001$, Fig. 2). As for xylotriose, Econase XT showed the greatest variation in response between varieties compared to Econase BP700 and Econase MP1000, which both gave much more consistent and gradual release across the 6 wheat varieties, as well as releasing only about half the quantity of xylobiose compared to Econase XT (Fig. 2). In contrast to the xylotriose results, Econase XT released the most xylobiose from Highbury and the least from both Paragon and Sinuelo, with the other varieties in between.

For xylose release, a significant enzyme x variety interaction was observed (Fig. 2, $P < 0.001$). Once again, Econase XT showed the greatest variation in response between varieties, particularly at later timepoints (6–24hrs). Indeed, Econase XT released twice as much xylose from Maris Huntsman and Highbury (Fig. 2) than the other two enzymes, but similar amounts of xylose were released by all 3 enzymes from the other four varieties. At 24hrs, all 3 enzymes released ~4 g/100 g of the total xylose available (Table 1), but Econase XT released more (~8 g/100 g of the total xylose available) in Maris Huntsman and Highbury varieties (Table 1).

For arabinose release, significant enzyme x variety ($P = 0.028$), enzyme x time ($P < 0.001$) and variety x time ($P < 0.001$) interactions were observed (Fig. 3). Once again, Econase XT showed the greatest variation in response between varieties, with Maris Huntsman and Highbury giving the greatest release, and Sinuelo and Paragon releasing the least (Fig. 3). However the amount of arabinose released was much lower than that for xylose. At 24hrs all 3 enzymes released ~1.5% of the total arabinose available (Table 1).

There was a significant enzyme x variety x time interaction ($P < 0.001$) for galactose release (Fig. 3). Incubations with Econase BP700 or Econase MP1000 were not different to controls, as shown by the flat lines (Fig. 3). Econase XT resulted in greater variation in

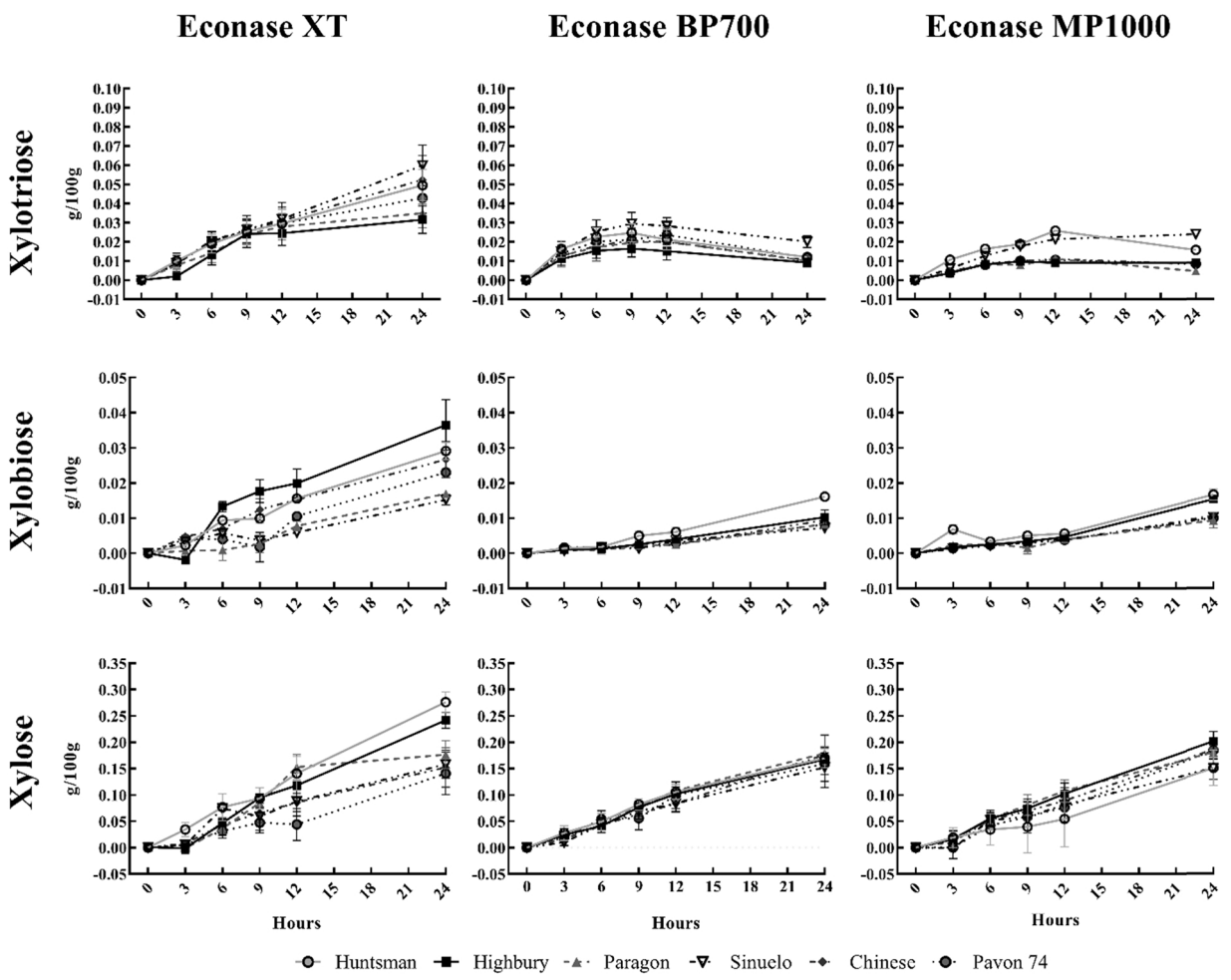


Fig. 2. Effect of three exogenous enzymes on the release of xylotriose, xylobiose and xylose from the six varieties of wheat. Mean values (n = 3) are expressed as total xylooligo- or mono- saccharide (g/100 g biomass) released (enzyme minus the control, no enzyme) during *in vitro* digestion in sodium citrate buffer (pH5.2) over 24 h at 41 °C. Error bars represent the standard error of the mean (SEM). Three-way (enzyme x variety x time) ANOVA statistical analyses were performed for each sugar. For xylotriose there were significant enzyme x variety ($P = 0.022$), enzyme x time ($P < 0.001$) and variety x time ($P = 0.011$) interactions. For xylobiose there was a significant enzyme x variety x time interaction ($P < 0.001$). For xylose there was a significant enzyme x variety interaction ($P < 0.001$).

galactose release (some of which was actually negative), but any additional release of galactose was relatively small compared to that released in the control incubations (Fig. 1).

There was also a significant enzyme x variety x time interaction ($P < 0.001$) for glucose release (Fig. 3), with all 3 enzymes increasing the release, but this was again relatively small at ~1 g/100 g of the total glucose available (Table 1). This was the only instance where all 3 enzymes resulted in clear differences in sugar release from the different varieties of wheat. Econase XT gave similar increases in release over time in most of the wheat varieties, with highest release observed from Maris Huntsman, but a markedly lower release from Sinuelo. Econase BP700 resulted in the most variation in glucose release across the time course (Fig. 3), with greatest release from Sinuelo and lowest release from Maris Huntsman, opposite that observed for Econase XT. Econase MP1000 released similar amounts of glucose from Sinuelo and Maris Huntsman, and the lowest amounts from Highbury and Paragon varieties (Fig. 3).

4. Discussion

To our knowledge, this is the first study to compare the effects of different commercial exogenous enzymes on the *in vitro* release of mono- and oligosaccharides from different varieties of wheat. Hence, apart from our previous study on different cereals (Dale et al., 2022) there is little published literature to compare results to. A key finding is that some sugars were released in the control incubations with no enzymes added, with the majority of xylobiose, galactose and glucose released. However greater release was observed in the presence of exogenous enzymes for xylotriose (no release in control), xylose (~10x higher with enzymes) and arabinose (~2x higher).

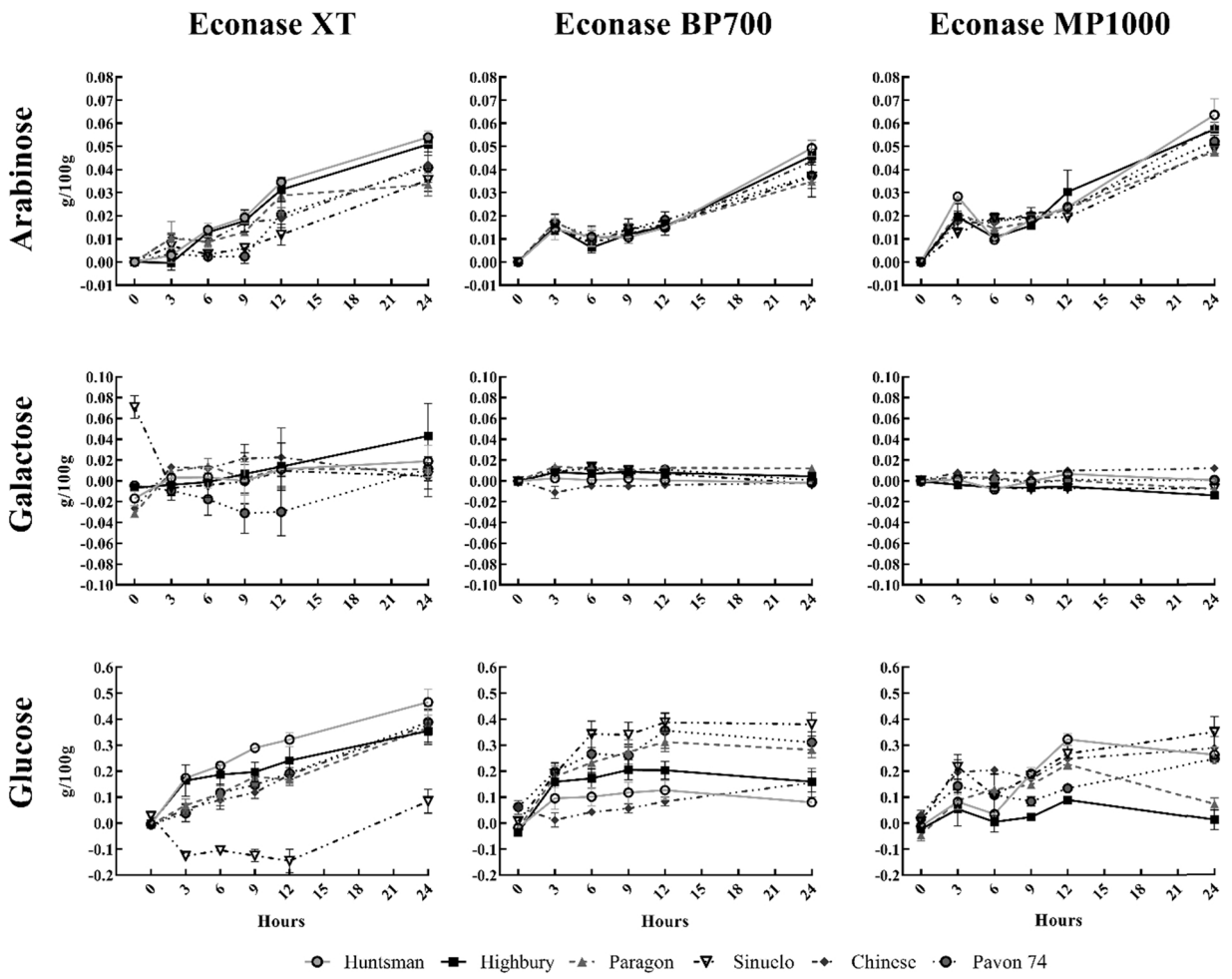


Fig. 3. Effect of three exogenous enzymes on the release of arabinose, galactose and glucose from the six varieties of wheat. Mean values (n = 3) are expressed as total monosaccharide (g/100 g biomass) released (enzyme minus the control, no enzyme) during *in vitro* digestion in sodium citrate buffer (pH5.2) over 24 h at 41 °C. Error bars represent the standard error of the mean (SEM). Three-way (enzyme x variety x time) ANOVA statistical analyses were performed for each sugar. For Arabinose there were significant enzyme x variety ($P = 0.028$), enzyme x time ($P < 0.001$) and variety x time ($P < 0.001$) interactions. For Galactose and Glucose there were significant enzyme x variety x time interactions ($P < 0.001$ for both).

The main finding was that the three enzymes appeared to have very different specificities for the different varieties of wheat, at least in these *in vitro* single enzyme incubations, with some enzymes producing greater variation in release of monomeric products and XOS than others. This agrees with our previous study (Dale et al., 2022), where we also observed that the same 3 enzymes had differing specificities for the 4 main cereals (barley, oats, maize and wheat).

In general, Econase XT released more xylotriose, xylobiose and xylose than the other 2 enzymes but the release was more variable between wheat varieties; whereas there were lower amounts released and there was less variability between varieties for BP700 and MP1000 enzymes. In contrast, the monosaccharides were released in similar amounts when comparing the 3 enzymes, but there were some clear differences between the wheat varieties, particularly in relation to glucose release (by all 3 enzymes), as well as arabinose and galactose release by Econase XT. This suggests that it might be possible to optimise the combination of enzyme and wheat variety used in animal feeds.

The present study found glucose to be 80–85 g/100 g of the total sugar contents (sum of arabinose, galactose, glucose and xylose) observed in the 6 different wheat varieties, which agrees with previous findings (Knudsen, 1997). Total AX contents and A:X ratios determined in the 6 wheat varieties were also in line with those described previously by Pritchard et al. (2011), who found AX to range from 2 to 11 g/100 g and A:X to be 0.4–1.3 across the 211 wheat varieties studied. Interestingly, the AX and total sugar contents in the different varieties were found to be positively correlated ($R^2 = 0.70$). The wheat varieties had a wide range of total sugars, with the varieties used in the UK (Maris Huntsman, Highbury and Paragon) having higher values than the others, bred for other climates. Generally, the carbohydrate (sucrose and starch) content of wheat is known to be reduced in drought stress (Balla et al., 2011), whilst protein increases (Flagella et al., 2010). However we did not analyse the non-starch polysaccharide nor protein contents of the varieties, but it is highly unlikely that the protein content was increased significantly to compensate and possibly reflects differences in

cellulose contents.

As in the previous study (Dale et al., 2022), all of the wheat varieties released both monosaccharides and some XOS when incubated in the absence of exogenous enzymes (the control incubations). This is likely due to the presence of endogenous enzymes either in the wheat varieties themselves or from microbes present in the system, since no preservatives or antibiotics were included in the incubation buffer. We have also observed (unpublished data) that autoclaving cereals removes this exogenous enzyme activity. For most of the monosaccharides, the amount released was relatively low as a proportion of the total available, the exception being galactose, which suggests some galactosidase activity. Overall, greater amounts of galactose and glucose were released in the absence of any exogenous enzyme, with only limited release of arabinose and xylose. Interestingly, although the Sinuelo variety had the lowest total xylose content it actually released the most xylose in the absence of exogenous enzyme, suggesting some endogenous xylosidase activity in that variety.

As expected, the main sugar released after incubation with the enzymes was glucose, as wheat is known to have a high starch content (Holm et al., 1986), highlighting the potential capacity for exogenous enzymes to improve the release of nutrients. In the present study, Sinuelo released the least glucose with Econase XT supplementation, but showed the greatest release of all 6 varieties with BP700 + MP1000 enzymes. This may relate to the glycosidase activity present in those 2 enzymes, but lacking in Econase XT.

In agreement with our findings, Lafond et al. (2015) found that wheat varieties with the highest AX contents released significantly more arabinose and xylose in an *in vitro* digestion, compared to the varieties with the lowest AX content. Similarly, the Maris Huntsman variety had the highest total AX content and released the most arabinose and xylose when incubated with Econase XT. While the amount of arabinose and xylose released in the presence of enzyme was greater than the control incubations, it was relatively low compared to the total AX values obtained from TFA hydrolysis. This can partly be explained by the fact that the AX values from TFA hydrolysis represent a total of both soluble and insoluble fractions, whereas the release was measured in the digestion supernatant, and therefore only relates to the soluble fraction.

Both Econase BP700 and Econase MP1000 contain endo-xylanase activity. Their ability to generate xylose over the 24 hr was similar to Econase XT, but there was a clear difference between these two enzymes and Econase XT in their interaction with different varieties, with Econase XT resulting in a much greater range of xylose release. It was also clear that Econase XT resulted in the greatest release of XOS *in vitro,* but was again variable and dependent on the wheat variety, especially for xylobiose. This suggests that the endo-xylanase activity in Econase XT is sensitive to the arabinoxylan structure within the wheat variety. Highbury had a relatively high total xylose content and one of the lowest A:X ratios in the study, but had the lowest xylotriose and the highest xylobiose release following incubation with Econase XT. This is in contrast to the Sinuelo variety, which had the lowest total xylose content and the highest A:X ratio and released more xylotriose but the least xylobiose when incubated with Econase XT, the opposite of that seen with Highbury. Both varieties released comparable amounts of xylose though, which indicates that complete degradation was similar, albeit through slightly different hydrolysis pathways, thereby generating different XOS intermediary products. This could relate to where arabinose was substituted on the AX backbone. A:X ratios suggest the likely number of arabinose units per xylose unit, but it is possible that there are regions of the backbone with no arabinose substitutions and others which are heavily substituted. Therefore, Sinuelo may have more regions with longer stretches of unsubstituted xylose, which allow more xylotriose to be released. This could be important, since the release of XOS are thought to impact on the gut microbiome and feed efficiency (De Maesschalck et al., 2015; Craig et al., 2020; Akter and Akter, 2021), suggesting that optimum enzyme-wheat variety combinations may be possible.

The generation of monosaccharides and XOS increased over time, especially following incubation with Econase XT. This could be important, as different time points could hypothetically correspond to delivery to different sections of the digestive tract. For instance, later timepoints could possibly relate to delivery to the hindgut where these soluble sugars and XOS may then be metabolised by the microflora in the caeca, producing volatile fatty acids (VFA) (Yang et al., 2009) and potentially impacting on performance. Indeed, delivery of XOS to the hindgut may be beneficial for the microbiome composition of the caeca in birds (De Maesschalck et al., 2015; Craig et al., 2020; Akter and Akter, 2021). Although the quantities released in the present study may not be sufficient to fuel significant VFA production, they could be enough to signal hindgut bacteria to change metabolism towards AX fibre degradation. Indeed, one hypothesis (Bedford, 2018) suggests that supplementation with fibrolytic enzymes in the feed of broilers results in greater release of XOS, that then act as prebiotics inducing adaptation of the microbiome, greater fibre degradation *via* increased endogenous xylanase production by the microbes, which leads to even greater AX degradation and increased substrate availability for fermentation in the hindgut. It is therefore the greater capacity for fermentation of dietary fibre, rather than the XOS generated by the enzyme that allows for AX hydrolysis to increase, challenging the traditional prebiotic hypothesis, where the XOS are thought to act as a prebiotic (Bedford, 2018).

Previous studies (Aulrich and Flachowsky, 2001) have shown how an *in vitro* digestion model can be used for rapid and cost effective screening for activity of NSP degrading enzymes on wheat varieties, with the combination of β1,4-xylanase plus β1,4-glucanase activities shown to increase the release of both arabinose and xylose. The release of arabinose, xylose and XOS in the present study reinforces the idea that insoluble plant cell wall polysaccharides can be hydrolysed into soluble fractions by enzymatic action. It is worth noting that the incubation with exogenous enzymes may have produced other oligosaccharides / polymers of higher molecular weight or greater complexity of branching (*e.g.*, Arabinoxylo-oligosaccharides), but we do not know, as only xylose to xylotetraose (X1-X4) were quantified. One limitation of the study is that these incubations are in the absence of the endogenous digestive enzymes normally present in the digestive tract, so are likely to under-estimate the quantities and rates of digestion.

We must also acknowledge that the results described might be different to those observed *in vivo.* An *in vitro* model of digestion shows the capability of the enzymes tested to release sugars of varying molecular mass. However, enzyme efficacies can be modulated by the varying conditions present in the digestive tract, including other endogenous enzyme activities, composition of the gut microflora, nutrient absorption/ utilisation, differing pH levels and moisture contents. It is also possible that gut microbes might

produce arabinosidase, xylanase and/or acetyl esterase enzymes that could interfere with or enhance the exogenous enzyme activities. We previously (Dale et al., 2020) compared the *in vivo* (in broiler chickens) and *in vitro* effects of Econase XT on the release of monosaccharides and XOS from a wheat based diet using this same simplified *in vitro* digestion model. We observed similar results for the release of X4 (increased release by Econase XT), X3, galactose and glucose (no change with Econase XT) in both, but also some differences (increased release of X2 and arabinose *in vitro* compared with increased xylose release *in vivo*), suggesting that the *in vitro* model works for some sugars but not for others. Hence there are limitations of the simplified *in vitro* system used here, indicating further developments in the *in vitro* model as well as comparisons with *in vivo* measures of digestibility are required.

5. Conclusions

Although all the commercial enzymes used had endo-xylanase activity, they had variable effects on the 6 wheat varieties studied, especially Econase XT which only has endo-xylanase activity. Hence, the impact of exogenous enzymes on the release of XOS and monosaccharides appears to be wheat variety dependent and may relate to the different arabinoxylan contents and/or arabinose: xylose ratios of the 6 varieties of wheat. Importantly, the variation in xylooligosaccharides released may subsequently impact on feed efficiency. Future research should further develop the simplified *in vitro* model used and then examine how the *in vitro* observations compare to *in vivo* studies, including assessment of the xylooligosaccharides and monosaccharides released both with and without fibrolytic enzyme supplementation. Ultimately, it might be possible to optimise the combination of wheat variety and enzyme used in animal feeds, to help maximise the feed efficiency of livestock.

Funding source and role of the funding source

This work was financially supported by AB Vista Feed Ingredients Ltd, Woodstock Court, Marlborough, UK and the University of Nottingham, UK. The funding sources had no involvement in the study design.

CRediT authorship contribution statement

Tom Dale: Investigation, Methodology, Formal analysis, Visualisation, Writing – original draft. **Michael Bedford:** Conceptualisation, Resources, Writing – review & editing. **Julie King:** Resources, Writing – review & editing. **Gregory Tucker:** Visualisation, Supervision, Writing – review & editing. **John Brameld:** Conceptualisation, Resources, Visualisation, Supervision, Writing – review & editing. **Tim Parr:** Conceptualisation, Resources, Visualisation, Supervision, Writing – review & editing.

Declaration of Competing Interest

MRB is an employee of AB Vista who sell the exogenous enzymes being tested. His contribution is clearly indicated in the CRediT authorship contribution statement.

Acknowledgements

The authors thank Jim Craigon (University of Nottingham, UK) for his assistance with statistical analyses.

References

Akter, M., Akter, N., 2021. Effect of xylo-oligosaccharides (XOS) on growth performance, blood biochemistry and total viable count in ileum and caecum of broiler chickens from day 13-26. Int. J. Poult. Sci. 20, 158–164. https://doi.org/10.3923/ijps.2021.158.164.

Aulrich, K., Flachowsky, G., 2001. Studies on the mode of action of non-starch-polysaccharides (NSP)-degrading enzymes *in vitro*. 2. Communication: effects on nutrient release and hydration properties. Arch. fur Tierernahr. 54, 19–32.

Austin, S.C., Wiseman, J., Chesson, A., 1999. Influence of non-starch polysaccharide structure on the metabolisable energy of UK wheat fed to poultry. J. Cereal Sci. 29, 77–88.

Balla, K., Rakszegi, M., Li, Z., Bekes, F., Bencze, S., Veisz, O., 2011. Quality of winter wheat in relation to heat and drought shock after anthesis. Czech J. Food Sci. 29, 117–128.

Bedford, M., Autio, K., 1996. Microscopic examination of feed and digesta from wheat-fed broiler chickens and its relation to bird performance. Poult. Sci. 75, 1–14.

Bedford, M.R., 2000. Exogenous enzymes in monogastric nutrition — their current value and future benefits. Anim. Feed Sci. Technol. 86, 1–13.

Bedford, M.R., 2018. The evolution and application of enzymes in the animal feed industry: the role of data interpretation. Br. Poult. Sci. 59, 486–493.

Choct, M., Annison, G., 1992. Anti-nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. Br. Poult. Sci. 33, 821–834.

Choct, M., Hughes, R., Annison, G., 1999. Apparent metabolisable energy and chemical composition of Australian wheat in relation to environmental factors. Aust. J. Agric. Res. 50, 447–452.

Craig, A.D., Khattak, F., Hastie, P., Bedford, M.R., Olukosi, O.A., 2020. Xylanase and xylo- oligosaccharide prebiotic improve the growth performance and concentration of potentially prebiotic oligosaccharides in the ileum of broiler chickens. Br. Poult. Sci. 61 (1), 70–78. https://doi.org/10.1080/00071668.2019.1673318.

Dale, T., Hannay, I., Bedford, M.R., Tucker, G.A., Brameld, J.M., Parr, T., 2020. The effects of exogenous xylanase supplementation on the *in vivo* generation of xylooligosaccharides and monosaccharides in broilers fed a wheat-based diet. Br. Poult. Sci. 61 (4), 471–481. https://doi.org/10.1080/00071668.2020.1751805.

Dale, T., Bedford, M.R., Tucker, G.A., Brameld, J.M., Parr, T., 2022. The effects of exogenous fibrolytic enzymes on the *in vitro* generation of xylooligosaccharides and monosaccharides is dependent upon cereal type. Anim. Feed Sci. Technol. 292, 115406 https://doi.org/10.1016/j.anifeedsci.2022.115406.

De Keyser, K., Dierick, N., Kuterna, L., Maigret, O., Kaczmarek, S., Rutkowski, A., Van der Beke, E., 2018. Non-starch polysaccharide degrading enzymes in corn and wheat-based broiler diets: dual activity for major substrates. J. Agric. Sci. Technol. A 8 (2), 76–88.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B., Van Immerseel, F., 2015. Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Appl. Environ. Microbiol. 81, 5880–5888. https://doi.org/10.1128/AEM.01616-15.

del Alamo, A.G., Verstegen, M., Den Hartog, L., De Ayala, P.P., Villamide, M., 2008. Effect of wheat cultivar and enzyme addition to broiler chicken diets on nutrient digestibility, performance, and apparent metabolizable energy content. Poult. Sci. 87, 759–767.

Flagella, Z., Giuliani, M.M., Giuzio, L., Volpi, C., Masci, S., 2010. Influence of water deficit on durum wheat storage protein composition and technological quality. Eur. J. Agron. 33, 197–207.

Foulkes, M.J., Paveley, N.D., Worland, A., Welham, S.J., Thomas, J., Snape, J.W., 2006. Major genetic changes in wheat with potential to affect disease tolerance. Phytopathology 96, 680–688.

Fry, S.C., 1988. The Growing Plant Cell Wall: Chemical and Metabolic Analysis. The Blackburn Press, Caldwell, USA, pp. 1–333. Reprint Edition.

Holm, J., Bjork, I., Drews, A., Asp, N.G., 1986. A rapid method for the analysis of starch. Starch-Stärke 38, 224–226.

Knudsen, K.E.B., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67, 319–338.

Lafond, M., Bouza, B., Eyrichine, S., Bonnin, E., Crost, E.H., Geraert, P.A., Giardina, T., Ajandouzel, H., 2011. An integrative in vitro approach to analyse digestion of wheat polysaccharides and the effect of enzyme supplementation. Br. J. Nutr. 106, 264-73.

Lafond, M., Bouza, B., Eyrichine, S., Rouffineau, F., Saulnier, L., Giardina, T., Bonnin, E., Preynat, A., 2015. *In vitro* gastrointestinal digestion study of two wheat cultivars and evaluation of xylanase supplementation. J. Anim. Sci. Biotechnol. 6, 5.

Mabelebele, M., Alabi, O., Ngambi, J., Norris, D., Ginindza, M., 2014. Comparison of gastrointestinal tracts and pH values of digestive organs of Ross 308 broiler and indigenous Venda chickens fed the same diet. Asian J. Anim. Vet. Adv. 9, 71–76.

Machado, F.J., Santana, F.M., Lau, D., Del Ponte, E.M., 2017. Quantitative review of the effects of triazole and benzimidazole fungicides on Fusarium head blight and wheat yield in Brazil. Plant Dis. 101, 1633–1641.

Maisonnier-Grenier, S., Clavurier, K., Saulnier, L., Bonnin, E., Geraert, P.A., 2006. Biochemical characteristics of wheat and their relation with apparent metabolisable energy value in broilers with or without non-starch polysaccharide enzyme. J. Sci. Food Agric. 86, 1714–1721.

Masey O'Neill, H.V., Smith, J.A., Bedford, M.R., 2014. Multicarbohydrase enzymes for non-ruminants. Asian-Australas. J. Anim. Sci. 27, 290–301.

Milec, Z., Tomkova, L., Sumikova, T., Pankova, K., 2012. A new multiplex PCR test for the determination of Vrn-B1 alleles in bread wheat (Triticum aestivum L.). Mol. Breed. 30, 317–323.

Payne, P.I., Nightingale, M.A., Krattiger, A.F., Holt, L.M., 1987. The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. J. Sci. Food Agric. 40, 51–65.

Pritchard, J.R., Lawrence, G.J., Larroque, O., Li, Z., Laidlaw, H.K., Morell, M.K., Rahman, S., 2011. A survey of β-glucan and arabinoxylan content in wheat. J. Sci. Food Agric. 91, 1298–1303.

Singh, R.P., Mujeeb-Kazi, A., Huerta-Espino, J., 1998. Lr46: A gene conferring slow-rusting resistance to leaf rust in wheat. Phytopathology 88, 890–894.

Slominski, B., Gdala, J., Boros, D., Campbell, L., Guenter, W., Jones, O., 2000. Variability in chemical and nutritive composition of Canadian wheat and the potential for its minimization by enzyme use. Proceedings of the XXI World Poultry Congress, Montreal, QC, Canada, 20–24 August 2000.

Svihus, B., Itani, K., 2019. Intestinal passage and its relation to digestive processes. J. Appl. Poult. Res 28 (3), 546–555. https://doi.org/10.3382/japr/pfy027.

Tuckey, R., March, B., Biely, J., 1958. Diet and the rate of food passage in the growing chick. Poult. Sci. 37, 786–792. https://doi.org/10.3382/ps.0370786.

Warner, R.L., Kudrna, D.A., Spaeth, S.C., Jones, S.S., 2000. Dormancy in white-grain mutants of Chinese Spring wheat (Triticum aestivum L.). Seed Sci. Res. 10, 51–60.

Wilfart, A., Montagne, L., Simmins, H., Noblet, J., van Milgen, J., 2007. Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. Livest. Sci. 109, 27–29. https://doi.org/10.1016/j.livsci.2007.01.032.

Xu, Y., Fan, L., Wang, X., Yong, Q., Yu, S.Y., 2013. Simultaneous separation and quantification of linear xylo-and cello-oligosaccharides mixtures in lignocellulosics processing products on high-performance anion-exchange chromatography coupled with pulsed amperometric detection. BioResources 8, 3247–3259.

Yang, Y., Iji, P., Choct, M., 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World'S. Poult. Sci. J. 65, 97–114.