The effects of exogenous fibrolytic enzymes on the *in vitro* generation of xylooligosaccharides and monosaccharides is dependent upon cereal type

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ABSTRACT

Fibrolytic enzymes are routinely added to non-ruminant livestock feeds to help degrade the non-starch polysaccharide (NSP) contents and thereby improve feed efficiency. This study investigated the range of xylooligosaccharides (XOS) and monosaccharides produced from four cereal samples (barley, maize, oats and wheat) over a 72 hour *in vitro* incubation using 3 commercially available (AB Vista, Marlborough, Wiltshire, United Kingdom) fibrolytic enzymes, Econase XT, Econase MP1000 and Barley P700, all containing endo-xylanase with other combinations of enzymes. Complete non-starch acid hydrolysis showed there were differences between cereals in the total monosaccharide ($P<0.01$). There was a cereal x enzyme x incubation time 3-way interaction in the generation of XOS (xylotetraose, xylotriose and xylobiose) ($P<0.01$) indicating the generation of XOS varies dependent on both the cereal and the enzyme used. The enzymes failed to generate any detectable xylose from maize. For xylose there was also a cereal x enzyme x incubation time 3-way interaction ($P<0.05$). Econase XT generated the greatest quantity of xylose, with 38% of available xylose from wheat being released after 72 h, 11% from barley and 9% from oats, whilst no xylose was detected from maize using any of the 3 commercial enzyme preparations. For arabinose and galactose production there was a cereal x enzyme x incubation time 3-way interaction ($P<0.01$), whilst glucose release was only significantly affected by cereal ($P<0.05$) or time ($P<0.05$). These findings suggest that the fibrolytic enzymes tested have some specificity for certain cereals and therefore it might be possible to optimise the combinations used in animal feeds, to help maximise the feed efficiency of livestock.

Key words: Cereal; Xylanase; Xylooligosaccharide; in vitro digestion.

Abbreviations: ×g, times gravity; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; BU/g, beta-units per gram; BXU/g, beta-xylanase units per gram; DP, degree of polymerisation; 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; XOS, xylooligosaccharide; SNSP, water soluble non starch polysaccharide; TFA, trifluroacetic acid.

1. Introduction

There is an increasing literature on the use of non-starch polysaccharide (NSP) degrading enzymes in non-ruminant diets to improve feed efficiency in pigs and poultry (Classen et al., 1985, Wyatt et al., 1997, Bedford and Morgan, 2007). The main feed ingredients used in non-ruminant diets are cereals, particularly wheat, maize, oats and barley. Their NSP content can vary widely between and within (varietal) cereals (Knudsen, 1997). The main polysaccharides present in cereal cell walls are arabinoxylans (AX) (Bastawde, 1992, Theander et al., 1993), with the major components being the pentose sugars, arabinose and xylose (Choct, 1997). The hydrolysis of AX requires a mix of enzyme activities, since β-1,4 endoxylanases cleave linkages of the xylan backbone, whilst β-d-xylosidases release xylose from susceptible polymeric and oligomeric xylooligosaccharides (XOS). Other enzymes such as arabino-furanosidases, cleave the substituent sugars, clearing the xylan backbone for greater access by the endo-xylanases.

High molecular weight polysaccharides such as some AX are anti-nutritive, as their high water-soluble fractions increase viscosity in the digestive tract and form a barrier between the substrate and enzyme, impairing digestibility (Bedford and Classen, 1992, Misir and Marquardt, 1978). More viscous feed ingredients such as barley, wheat and oats have higher concentrations of water soluble non starch polysaccharide (SNSP) and are therefore thought to respond well to carbohydrase supplementation. Indeed, xylanase supplementation of wheat-based diets released arabinose and xylose in the ileum, jejunum and duodenum of broiler chickens (Zhang et al., 2014), likely due to the hydrolysis of AX. Addition of xylanase has been shown to depolymerise high molecular weight arabinoxylans and this was accompanied with reduced viscosity of digesta. The xylanase supplementation was associated with improved nutrient digestion, absorption and thus, animal performance (Zhang et al., 2014). However, the mechanism by which non-starch NSP degrading enzymes affect animal growth is much debated (Aftab and Bedford, 2018). Maize is the most common feed ingredient in global poultry production, but the potential for Xylanase enzymes to positively influence maize based feeds may be limited, since maize is considered highly digestible (Kocher et al., 2003) as there is 50% less total NSP in maize compared to wheat (Chesson, 1993), most of which is not soluble therefore not a viscosity risk. Despite this, several studies have reported benefits of incorporating xylanases and carbohydrase mixtures in maize based diets (Cowieson and Ravindran, 2008, Cowieson et al., 2010).

Interestingly, Bedford and Cowieson (2012) showed that the effects of xylanase on oligosaccharide size, degree of substitution and quantity could differ if maize or wheat was used as the main substrate, given their different resistances to complete hydrolysis. In addition to the potential effects of xylanase on viscosity, the generation of fermentable oligosaccharides, including XOS, and their

prebiotic effect on the gut microbiota could be a key mechanism for the improved feed efficiency seen with enzyme supplementation (De Maesschalck et al., 2015). In agreement with this, Arabinoxylan oligosaccharides (AXOS) derived from wheat-bran were shown to significantly improve feed efficiency of both wheat and maize based diets (Courtin et al., 2008). Decreased feed intake and increased bodyweight gain and therefore improved feed efficiency was observed in both studies (Courtin et al., 2008), but importantly the AXOS had prebiotic effects on the caecal microbiota. A number of recent studies in chickens have similarly demonstrated beneficial effects of arabinoxylo-, galacto- or xylo-oligosaccharides (AXOS, GOS or XOS) on growth (Richards et al, 2020), feed conversion ratio (Akter and Akter, 2021; Craig et al, 2020; De Maesschalck et al, 2015), gut morphology (De Maesschalck et al, 2015; Richards et al, 2020), short chain fatty acid (SCFA) production in the caecum (Craig et al, 2020; Singh et al, 2021) and/or changes in gut microbiome (Akter and Akter, 2021; De Maesschalck et al, 2015; Richards et al, 2020).

These findings suggest a need to characterise the differential effects of commercially available (AB Vista, Marlborough, Wiltshire, United Kingdom) exogenous enzyme preparations, consisting of a range of NSP degrading enzymes, on common cereal feed ingredients. We decided to perform simple in vitro digestions in the absence of any of the endogenous enzymes present *in vivo*, using three commercial enzyme preparations (AB Vista, Marlborough, Wiltshire, United Kingdom) in order to investigate any differences in the profiles of XOS and monosaccharides released from the four cereals (barley, maize, oats and wheat). Although the total tract retention time of broilers is suggested as being between 5-6 hours (Svihus and Itani, 2019), others have described most of the marker is excreted by 12 hrs (Tuckey et al., 1958), but can still be detected up to 72 hrs after feeding (Duke et al., 1968). Since we decided to perform digestions in the absence of any other enzymes, the rates of digestion are likely to be slower than those observed *in vivo*, so we monitored the release of sugars over a 72hr time course.

2. Materials and methods

2.1 Total hydrolysis of non-cellulosic polysaccharides

An indication of the xylose, arabinose, galactose and glucose composition of the 4 cereals (barley, maize, oats and wheat) was determined by hydrolysis with Trifluroacetic acid (TFA), as previously described by Fry (1988). The aim of this analysis was to simply 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. All cereals were obtained from a commercial feed manufacturer (Target Feeds, UK). The release of 4 sugars (arabinose, galactose, glucose and xylose) was quantified, but the XOS contents were not, as they were not detectable prior to enzyme hydrolysis in the current experiment, as determined by using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD), as described below. The 4 cereals (barley, maize, oats and wheat) were ground to a fine powder (0.5mm) and suspended to 10mg/ml in 2M 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 10mM NaOH and transferred into 2ml clear vials for sugar analysis.

2.2 In-vitro digestion of cereals with 3 different enzymes.

The following 3 commercial enzyme preparations were provided by AB Vista (Marlborough, UK) and used in digestions with the 4 cereals:

i. Econase XT (AB Vista, Marlborough, Wiltshire, United Kingdom) – a xylanase with β-1,4 endo-xylanase activity (160,000 BXU/g). The enzyme was used at its suggested dose of 100mg/kg of feed.

ii. Econase MP 1000 (AB Vista, Marlborough, Wiltshire, United Kingdom) – 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 100mg/kg of feed.

iii. Econase Barley P 700 (AB Vista, Marlborough, Wiltshire, United Kingdom) - 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 40mg/kg of feed.

These enzymes were taken from commercial grade preparations for agricultural use in animal feeds. The specific enzyme activities and thereby their characteristics, as defined by the Carbohydrate-Active enZYmes database (CAZy database, http://www.cazy.org), was not disclosed by the company.

The 4 cereals (barley, maize, oats and wheat) were individually ground to a fine powder (0.5mm), 0.2g of each cereal was suspended (in triplicate) in 40mls 50 mM sodium citrate buffer (pH 5.2) containing one of the following enzymes:

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

The four enzyme preparations were added separately (in triplicate) along with 40ml of 50 mM sodium citrate buffer (pH 5.2). The buffer used has previously been described as representing the average pH of the broiler digestive tract (Mabelebele et al., 2014). Digestion reactions were then placed in a shaking incubator at 150RPM, with a temperature of 41°C for 72 h, with 1ml samples taken at 0, 3, 6, 9, 12, 24, 36, 48, 60 and 72 h. At each time point, 1ml of each digest sample was removed and added to 9ml of 10 mM NaOH at room temperature, mixed, centrifuged and then frozen at -20°C prior to sugar analysis.

2.3 Identification and quantification of sugars using HPAEC-PAD

The sample concentrations of arabinose, galactose, glucose and xylose, as well as the XOS, were determined using High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD) following the method of Xu et al. (Xu et al., 2013). Analysis was carried out using a Dionex ICS-3000 with a Dionex CarboPac PA20 Column (3mm x 150mm) and CarboPac PA20 Guard (3x30mm) for the monosaccharide analysis. A CarboPac PA200 column (3mmx250mm) and CarboPac PA200 guard (3mm x 50mm) were used for the oligosaccharide analysis. An injection volume of 10µl was used throughout for both samples and standard solutions. Monosaccharide standards (arabinose, galactose, glucose and xylose) were purchased from Sigma-Aldrich, UK and XOS standards (xylo-biose, -triose, –tetraose and -pentaose) from Megazyme, Ireland. Serial dilutions for each standard (2.0, 1.0, 0.5 and 0.25g/L for monosaccharides and 0.5, 0.25, 0.125 and 0.0625g/L for XOS) were made fresh for each batch of analyses.

For the monosaccharides, a single eluent, containing 10mM NaOH solution, was used as the mobile phase at 0.5ml/min for 14 min. For oligosaccharides, 2 eluents were used in a gradient for the mobile phase, 0.1M sodium hydroxide (Solution A) and 0.1M NaOH containing 0.5M 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 minutes, rising to 80% solution A and 20% Solution B at 25 minutes, before returning to 100% Solution A after 25 minutes elapsed. 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).

2.4 Data and statistical analysis

One source was used for each cereal and all analyses (digestions and Dionex analysis) were carried out in batches of each enzyme-cereal combination in triplicate and the data processed in Excel (Windows 10, Microsoft Corp., Redmond, Washington, USA) and expressed as means and standard deviations (SD). Using this level of replication, the preliminary experiments which sought to determine monosaccharide concentrations gave an average co-efficient variance for detection of the monosaccharides of 3.3% (+/- 0.2 (standard deviation). Based on this experiment with F-statistic of 9.75 it was calculated (G*Power3.1, University of Dusseldorf, Germany) that for 4 cereal groups the Power = 1 for total sample size of 8 (n=2 per group). Therefore, for it was deemed that an n=3 per group was sufficient.

Standards for the four monosaccharides or XOS were run at the start and end of each batch, standard curves generated from the areas under the curve, and results are presented as g/100g of cereal. Data was then analysed by one (cereal-only) or three (enzyme x cereal x time) way ANOVA, as appropriate, using Genstat (2018) statistical software (19th Edition), with blocking for batch and digestion tube. The two standard errors of the difference of the means (SED) obtained from the three-way ANOVA were used as error bars in the figures. Error bar 'A' represents the SED for comparing control and enzyme treated mean values on the same graph, whilst error bar 'B' represents the SED for comparing different times within the same treatment (control or enzyme) on the same graph. A Tukey post-hoc test was used to identify significant differences between cereals following a significant 1-way ANOVA. No post hoc tests were possible for the enzyme digestion analyses since significant 3- or 2-way interactions were observed for all sugars. $P<0.05$ was taken as statistically significant.

3. Results

3.1 Total hydrolysis of non-cellulosic polysaccharides

Sugar contents determined by TFA hydrolysis were similar for all 4 cereals (Table 1). As expected, the main monosaccharide present in all 4 cereals was glucose, mainly from starch, and the order of glucose content was the same as that for total sugar content (maize> wheat= oats> barley) ($P<0.001$). It is important to note that the monosaccharide concentrations determined after total acid hydrolysis represent the total reduction of monosaccharide, oligosaccharide and polysaccharides present in the sample. As expected, the concentration of xylose, arabinose and galactose was much lower than glucose but the relative range of concentrations of each monosaccharide across cereals was greater. (Table 1).

It is assumed that the vast majority of xylose and arabinose were present as arabinoxylan, and as such the total content and arabinose to xylose ratio of the arabinoxylan was determined. Barley had the highest xylose content (barley> maize> wheat= oats) ($P<0.001$), whereas wheat had the highest arabinose content (wheat> barley> maize> oats) ($P<0.001$). Galactose was only present in low amounts in 3 of the 4 cereals, but the content was significantly different ($P<0.001$) (oats> maize> wheat), with no detectable galactose found in barley. These total hydrolysis values for each sugar were subsequently used to calculate the proportion that was released during the *in vitro* digestions with and without the different enzymes, except in the case of galactose from barley digestions, where no comparison could be made as no galactose was detected after total non-cellulosic hydrolysis, but was present after enzyme digestion. This could be due to the fact that galactose is susceptible to acid hydrolysis which would lower the observed amount in the acid digestion, but would still be released by the enzyme.

Total arabinoxylan (AX) content was highest in barley (Table 2, barley> wheat= maize> oats) ($P<0.001$), whereas the arabinose:xylose (A:X) ratio was highest in wheat (Table 2, wheat> barley= maize> oats) ($P<0.001$).

3.2 In-vitro digestion of cereals with various enzymes – generation of xylooligosaccharides (XOS)

There were no XOS generated from maize in any of the control incubations nor by any of the enzymes studied (data not shown). There was also no generation of xylopentaose from any of the cereals by any of the enzymes used (data not shown), but there were significant enzyme x cereal x time interactions (all $P<0.01$) for the generation of the other 3 XOS measured (xylotetraose, xylotriose and xylobiose).

For xylotetraose (Figure 1), Econase XT had a small effect on barley and wheat; Econase MP1000 had a much bigger effect, but only in barley; while Barley P700 had small effects on barley, oats and wheat (in that order) (enzyme x cereal x time 3-way interaction, P=0.002). Interestingly, a rapid initial generation, followed by decline was seen for barley-Econase MP1000, barley-Barley P700 and oats-Barley P700 combinations, suggesting initial generation then potential further digestion (Figure 1).

There was virtually no generation of xylotriose for most of the cereal-enzyme combinations (Figure 2). However, there was a significant 3-way enzyme x cereal x time interaction (P=0.003). Only oats incubated with Econase MP1000 resulted in generation of xylotriose above that of the control, with the peak again suggesting an initial generation followed by further digestion (Figure 2).

Although there was a significant 3-way enzyme x cereal x time interaction (P=0.002), there was very little xylobiose generated for most of the cereal-enzyme combinations (Figure 3). Econase XT generated xylobiose from barley, wheat and oats (in that order), whereas Econase MP1000 had no effect on any of the cereals and Barley P700 only released small amounts from barley and wheat, but not oats (Figure 3).

3.3 In-vitro digestion of cereals with various enzymes – release of monosaccharides

Surprisingly, there was virtually no release of xylose, galactose or arabinose from maize in any of the control incubations nor by any of the enzymes studied, with only glucose being released.

There was a significant enzyme x cereal x time (P=0.042) interaction for the release of xylose (Figure 4). There was no detectable release of xylose from maize. The greatest xylose release was when the 3 cereals (barley, oats and wheat) were incubated with Econase XT or Econase MP1000, whereas Barley P700 had a much smaller effect in barley and wheat and no effect on oats. The xylose release over time was highest for wheat (P=0.042, Figure 4).

The next greatest release of monosaccharide was arabinose (Figure 5). Once again there was no arabinose released from maize, but there was a significant enzyme x cereal x time interaction (P=0.007) indicating that the different enzymes had different substrate specificities. Econase XT increased the release of arabinose from barley and wheat to similar extents, but less so from oats (Figure 5). Econase MP1000 had similar effects on all 3 cereals, whereas Barley P700 released more arabinose from wheat than barley, with no effect on oats (Figure 5).

There was also a significant enzyme x cereal x time interaction (P=0.007) for the release of galactose (Figure 6). While small amounts were released from control incubations for all 3 cereals

(Figure 6), only oats incubated with Econase XT or Econase MP1000 enzymes showed consistent increases above the controls.

There were no significant interactions for glucose release. However, glucose release from the 4 cereals significantly increased with time (P=0.036) and there was a significant effect of cereal (P=0.047). There was a trend for an interaction between cereal and time (P=0.090). All 3 enzymes gave similar increases in glucose release from barley, whereas only Barley P700 increased glucose release above controls for barley and oats, with no apparent effect on wheat and maize, however this 2-way enzyme x cereal interaction was only a trend (P=0.090, Figure 7).

4. Discussion

To our knowledge, this is the first study to compare the effects of commercially available (AB Vista) exogenous enzyme preparations on the release of mono- and oligo-saccharides from different cereals *in vitro* over time. Hence there is very little published literature to compare our results to. The values obtained here for total sugar content of the four cereals (using the TFA method) are generally in line with previous studies, except the arabinose, xylose and A/X ratio differ from those reported by Knudsen (1997) using the Englyst method. For example we calculated a wheat A/X ratio of 1.02 whereas Knudsen (1997) reported a value of 0.62. We suggest that this might partly be due to differences in methods used (TFA vs Englyst method). The Englyst method is rather complex involving separation into starch, non-starch, soluble and insoluble fractions followed by compositional analysis of each fraction, all of which will have errors associated with each measurement. In contrast, the TFA method allows a simple one step determination of the xylose and arabinose contents and avoids any potential errors from generating and analysing multiple fractions. When comparing the 2 studies in terms of the contents of the 2 sugars (arabinose and xylose), the values for xylose are almost identical (3.8g/100g for Knudsen and 3.75g/100g here), with the major difference being the arabinose contents (2.2g/100g in Knudsen and 3.82g/100g here). These differences are likely due to different varieties of wheat being studied, since Knudsen analysed a range of feedstocks (available in the 1990's), whereas a single source of wheat was analysed here. The wheat varieties currently in commercial use are likely to have changed since the 1990s and this is something we are currently investigating further.

Interestingly a number of 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. The main finding from this study is that the 3 enzymes appeared to have differing specificities for the 4 cereals, at least in this simple *in vitro* digestion system, suggesting that it might be possible to optimise the enzyme-cereal combinations used in animal feeds.

4.1 Barley

Interestingly, while barley had the lowest total glucose content after TFA hydrolysis, it had the highest xylose and total AX contents, but there was no galactose detected (Tables 1 and 2). This agrees with previous studies in terms of arabinose and xylose contents of barley. When 6 varieties of barley were assessed for monosaccharide contents by (Åman and Newman, 1986), the average total arabinose and xylose contents were 2.5% and 3.6% of total biomass respectively, which is lower than the 3.2% and 5.2% in the present study. This can be explained by the fact that two thirds

of samples used in the Aman and Newman (1986) study were hulless, therefore lower in AX. AX is considered a good indicator for responsiveness to carbohydrase enzymes and certainly all 3 enzymes increased the release of most sugars from barley, with the only exceptions being xylotriose and galactose. However, despite barley having the highest total AX content, more arabinose and xylose was released from wheat, highlighting that the amount of sugars released by enzyme digestion does not necessarily relate to their inherent content, at least not *in vitro*, as observed with maize in the current experiment.

Interestingly, there were clear differential effects of the 3 enzymes on the generation of xylotetraose from barley (Figure 1), with a short, sharp generation observed around 12hrs that subsequently decreased, presumably due to it being rapidly broken down into smaller oligos and monomers. Econase MP1000 induced the biggest peak, followed by Barley P700, with Econase XT showing a smaller, but more prolonged release across the 72hr time course. There was very little effect of any of the enzymes on xylotriose release (Figure 2) and only Econase XT induced appreciable generation of xylobiose (Figure 3). All 3 enzymes increased the glucose generated from barley to a similar extent (Figure 7), despite there being appreciable glucose release in the absence of any enzyme. Similarly, some xylose and arabinose were released in the absence of any enzyme (Figures 4 and 5) and all 3 enzymes increased the release, although Econase XT tended to release the most and Barley P700 the least. Surprisingly, these findings suggest that Econase XT, and possibly Econase MP1000, might be better for use in barley-based diets, both of which released more sugars than barley P700, although this obviously needs *in vivo* confirmation.

4.2 Maize

Maize has often been perceived as being less responsive to NSPase treatments due to its low NSP content and low viscosity (Choct, 1997). During the *in vitro* digestions the detection of glucose in maize was predominantly found in the absence of any enzyme, so was likely coming from starch dissolution rather than cellulose or β-glucan. Importantly, all 4 cereals released glucose to a similar extent in the absence of any enzyme and all 3 enzymes increased the glucose release to a similar extent from barley and oats, whereas there were no apparent effects of Barley P700 on glucose release from wheat or maize.

There was no release of any of the XOS from maize, nor any of the other monosaccharides measured (xylose, arabinose and galactose). This would therefore agree with the idea that maize is almost exclusively insoluble and relatively unresponsive to the exogenous enzymes (for anything other than glucose release). Importantly, this was not due to a lack of those sugars in maize, since TFA hydrolysis showed that the monosaccharide and total AX contents, as well as the A:X ratios

for maize were all similar to the other 3 cereals studied (Tables 1 and 2). The total AX content of maize determined in this study is similar to that described previously (Choct, 1997); (Malathi and Devegowda, 2001). Despite the AX contents and A:X ratios found in maize in this study, these findings suggest that the structure and or accessibility of the AX in maize makes it less susceptible to degradation into lower molecular weight material observed in the present study (degree of polymerisation (DP) < 5). It could have been the case that higher molecular weight material (e.g. DP > 50) was released, but not processed down into oligosaccharides or monosaccharides. This higher DP material could still be fermented as a substrate by the gut microbiome in the caeca.

4.3 Oats

Of the 4 cereals studied, oats contained the lowest total AX content, but the most galactose. In a previous study, Westerlund et al. (1993) showed the total AX content of flaked oats to be 7.4% of total dry mass, whereas the AX content in the present study was 4.71%. This difference could be because the previous study used the dry weight, whereas we determined values as percentages of total biomass (on a wet weight basis).

Oats generated much smaller amounts of the XOS than either barley or wheat, and there were clear differential effects of the 3 enzymes. Only Barley P700 induced any generation of xylotetraose (Figure 1); while only the combination of Econase MP1000 and oats induced an appreciable generation of xylotriose (Figure 2) and there was virtually no xylobiose released from Barley (Figure 3), either in the absence or presence of any enzyme. Perhaps the addition of a mannanase enzyme in the oats sample gave a unique additive effect to the enzyme hydrolysis, bringing about a spike in xylotriose production. Similarly, only Econase XT and Econase MP1000 induced the release of xylose, arabinose and galactose (Figures 4-6), with no effects of Barley P700 over the release observed in the absence of any enzyme. It is therefore unclear which enzyme might be best in oats-based feeds, but Barley P700 is unlikely to have much effect, since it only increased the generation of xylotetraose and glucose from oats. Once again this will need to be confirmed by *in vivo* studies.

4.4 Wheat

Of all the cereals studied, wheat contained the most arabinose and had the highest A:X ratio but was intermediate in terms of the other monosaccharide contents following TFA hydrolysis. These levels are in line with those published for a variety of wheat cultivars. For example, Pritchard et al. (2011) quantified 211 varieties of wheat and showed that the total AX contents and A:X ratios ranged from 2.37-10.75% and 0.4-1.3 respectively, which agrees with the 7.57% and 1.02 in the present study. Similarly, Lafond et al. (2015) characterised the total carbohydrate composition of 6 wheat cultivars

and showed average contents of 2.65%, 4.57%, 0.59% and 7.22% for arabinose, xylose, galactose and total AX contents respectively, which are all similar to the levels observed in the present study. Importantly, Lafond et al. (2015) showed that the wheat cultivar with the highest AX content delivered significantly more arabinose and xylose into the ileum compared to the cultivar with the lowest AX content. They also showed that the AX was not degraded in the absence of xylanase. This implies that high AX contents may result in higher digestion potential *in vivo* when a xylanase is fed.

In the present study, wheat samples generated all 3 XOS (xylotetraose, xylotriose and xylobiose), but in quantitively smaller amounts than the amount of xylose released through enzyme hydrolysis. Wheat was also the most responsive to enzyme supplementation in terms of release of both xylose (Figure 4) and arabinose (Figure 5), with Econase XT releasing slightly more than Econase MP1000, and Barley P700 only releasing slightly more than the no enzyme controls. Wheat was the only cereal to release noticably more xylotetraose in combination with Econase XT supplementation, suggesting the enzyme activity here is leading to greater production of DP4 XOS *in vitro.* In contrast, wheat didn't respond as much as barley or oats in terms of enzyme-induced release of glucose, probably due to the greater number of β-glucans present for hydrolysis into glucose in barley and oats (Figure 7). These results again indicate the importance of enzyme-cereal combinations in terms of the magnitude of the response seen, but it is important that these differences are confirmed *in vivo* before any recommendations are possible.

5. Conclusions

There were clear differences between cereals in terms of the effects of the 3 commercially available (AB Vista, UK) exogenous enzyme preparations on the sugars generated and released *in vitro*. It is surprising that all of the enzyme mixes contain endo-xylanase activity yet gave differing results when other enzymes were added to the mix. For example, the generation of xylobiose was generally greatest with Econase XT, but the addition of other enzymes in Econase MP1000 and Barley P700 mixes reduced xylobiose generation. This suggests that it might be possible to optimise the enzyme used for a diet containing a particular cereal, but also that the addition of enzymes to some diets may be ineffective due to the cereals used.

This study suggests improvements in current enzyme technologies may be achieved by targeting which xylooligosaccharides are produced as end products and ensuring the products do not further degrade these beneficial xylooligosaccharides to lower the degree of polymerisation or even to monosaccharides which may reduce the bioefficacy of the enzyme.

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CRediT authorship contribution statement

Tom Dale: Investigation, Methodology, Formal analysis, Visualisation, Writing – Original draft. Michael Bedford: Conceptualisation, Resources, Writing – review and editing. Gregory Tucker: Visualisation, Supervision, Writing – review and editing. John Brameld: Conceptualisation, Resources, Visualisation, Supervision, Writing – review and editing. Tim Parr: Conceptualisation, Resources, Visualisation, Supervision, Writing – review and editing.

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Table 1

Monosaccharide composition of various cereals after Trifluroacetic acid hydrolysis.

Cereal	**Barley**		**Maize**		**Oats**		**Wheat**		**P-Value[2]**
Sugar (g/100g)[1]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	
Arabinose	3.15 [c]	0.08	2.63 [b]	0.10	1.17 [a]	0.02	3.82 [d]	0.18	**<0.001**
Galactose	0.00 [a]	0.00	0.64 [c]	0.03	1.03 [d]	0.02	0.40 [b]	0.01	**<0.001**
Glucose	43.40 [a]	1.53	55.58 [c]	1.81	49.34 [b]	0.92	50.68 [b]	2.63	**<0.001**
Xylose	5.22 [c]	0.17	4.46 [b]	0.14	3.54 [a]	0.06	3.75 [a]	0.22	**<0.001**
Total	51.76 [a]	1.78	63.31 [c]	2.08	55.08 [a]	1.02	58.64[b]	3.05	**<0.001**

[1] Values are expressed as grams of each monosaccharide per 100g of cereal ± SD, standard deviation for biological triplicates.

[2] One-way ANOVA indicated significant differences between cereals for each sugar.

[a,b,c,d] Mean values with different superscript letters within a row were significantly different ($P<0.05$, Tukey's post hoc test).

Table 2

Total Arabinoxylan (AX) contents and Arabinose:Xylose (A:X) ratios of various cereals.

Cereal	**Barley**		**Maize**		**Oats**		**Wheat**		**P-Value[3]**
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	
Total AX (g/100g) [1]	8.36 [c]	0.25	7.08 [b]	0.24	4.71 [a]	0.08	7.57 [b]	0.40	**<0.001**
A:X ratio [2]	0.60 [b]	0.01	0.59 [b]	0.01	0.33 [a]	0.01	1.02 [c]	0.01	**<0.001**

[1]Total AX values are expressed as grams of arabinoxylan per 100g cereal ± SD, standard deviation for biological triplicates.

[2]A:X values represent grams of arabinose per 100g cereal divided by grams of xylose per 100g cereal ± SD, standard deviation for biological triplicates.

[3] One way ANOVA indicated significant differences between cereals for Total AX and A:X ratio.

[a,b,c] Mean values with different superscript letters within a row were significantly different ($P<0.05$, Tukey's post hoc test).

Figures and Figure Legends

Figure 1

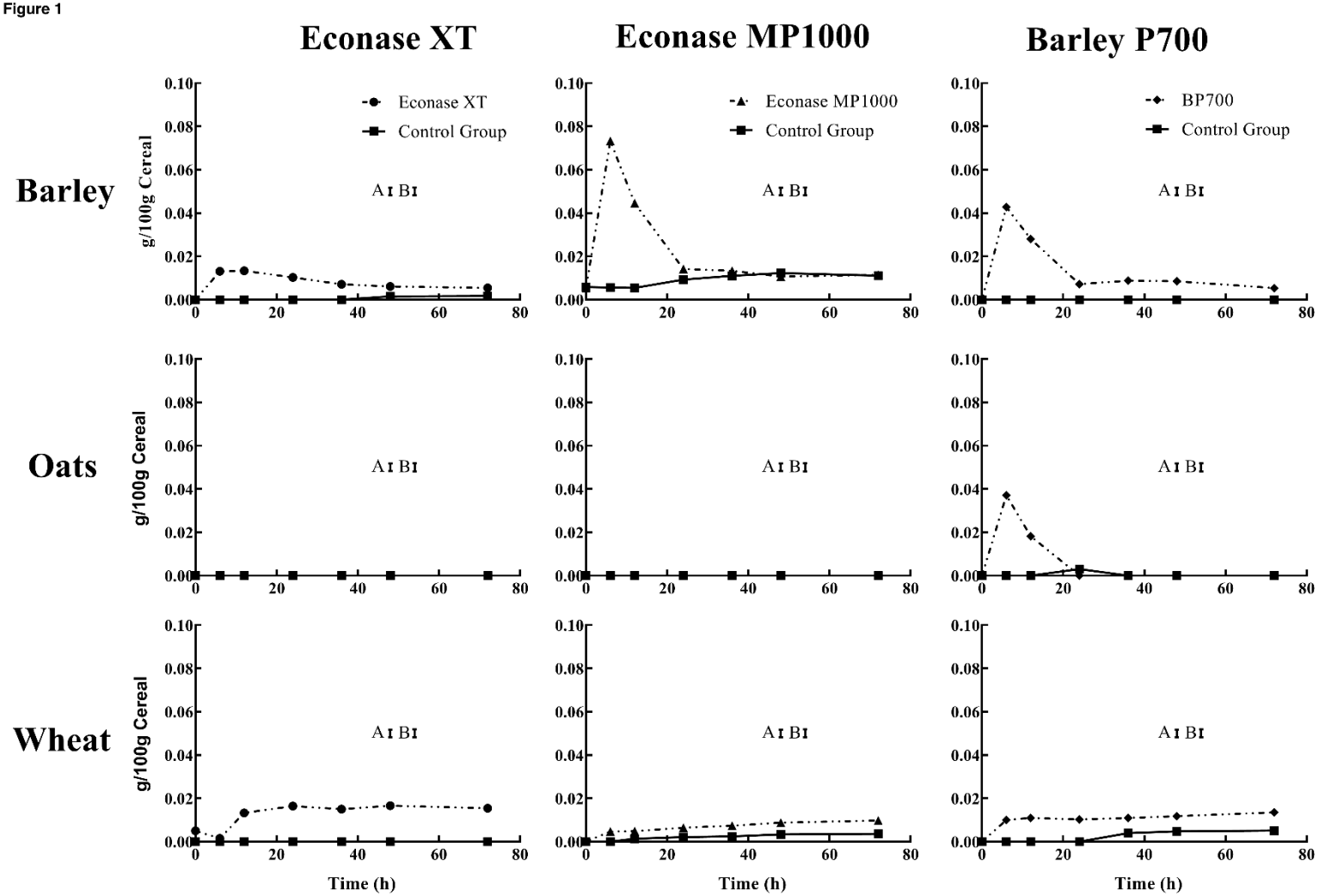

Fig. 1. Comparison of the effectiveness of different enzymes on the release of xylotetraose from different cereals. Mean values expressed as total xylotetraose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No xylotetraose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.002).

Figure 2

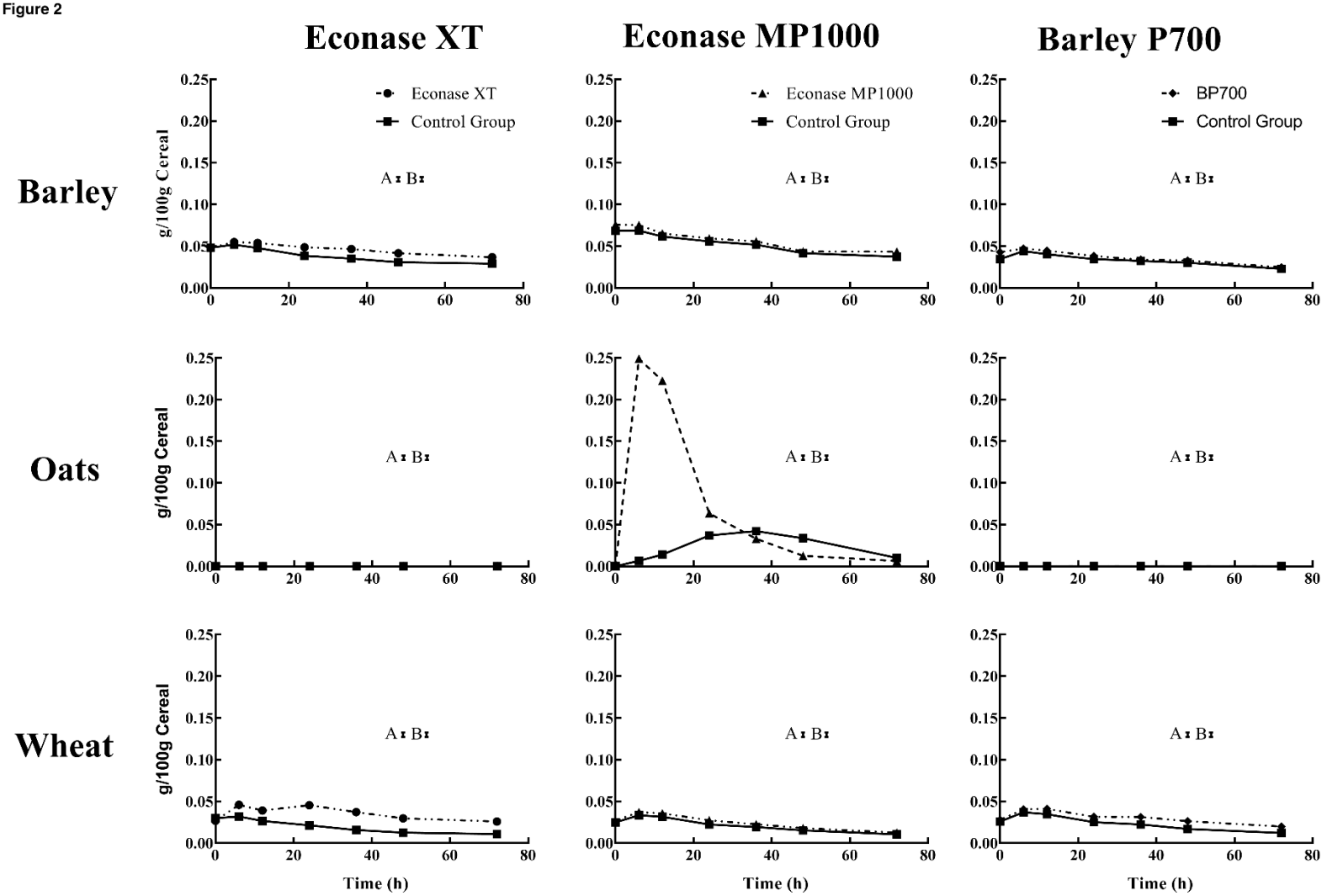

Fig. 2. Comparison of the effectiveness of different enzymes on the release of xylotriose from different cereals. Mean values expressed as total xylotriose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No xylotriose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.003).

Figure 3

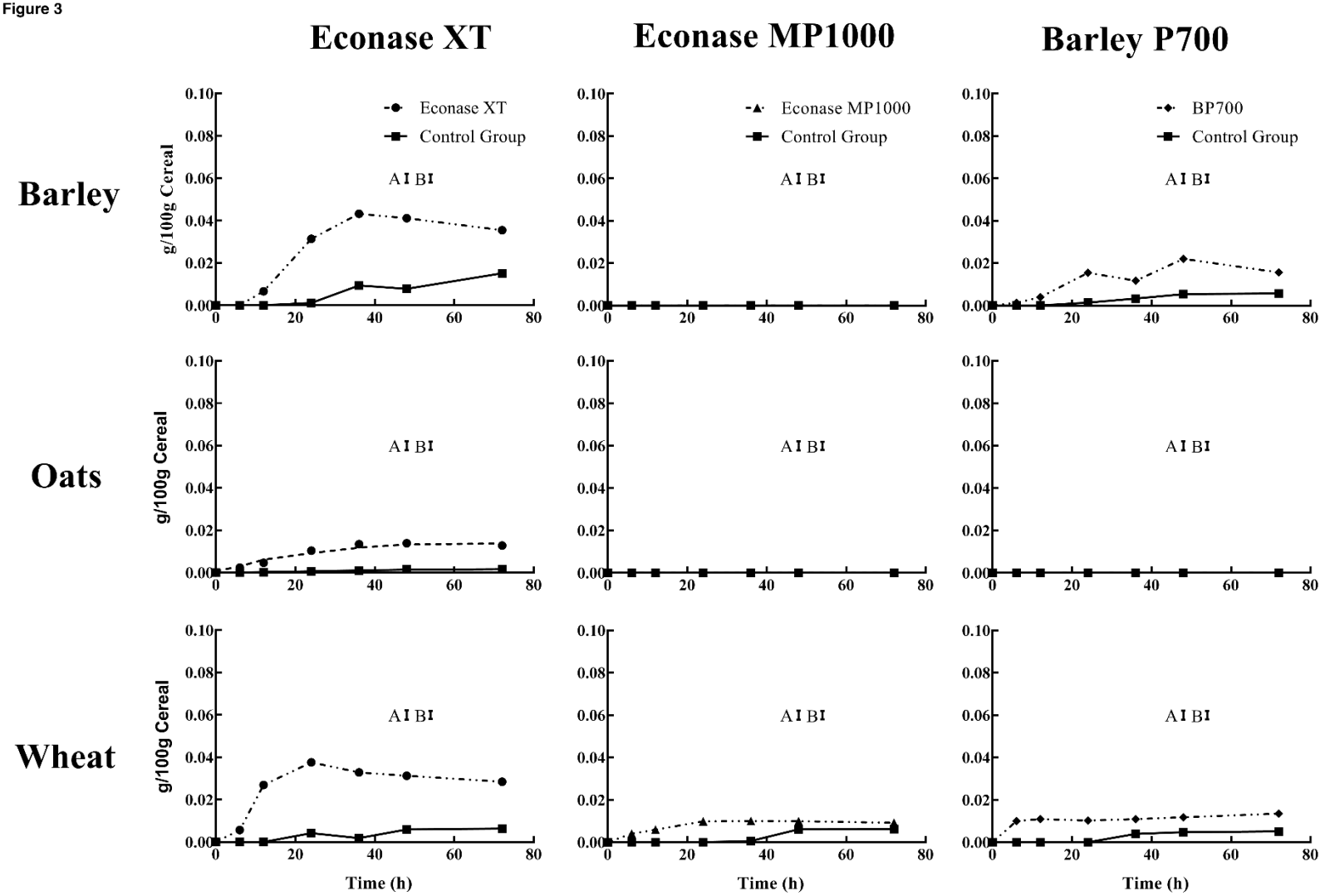

Fig. 3. Comparison of the effectiveness of different enzymes on the release of xylobiose from different cereals. Mean values expressed as total xylobiose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No xylobiose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.002).

Figure 4

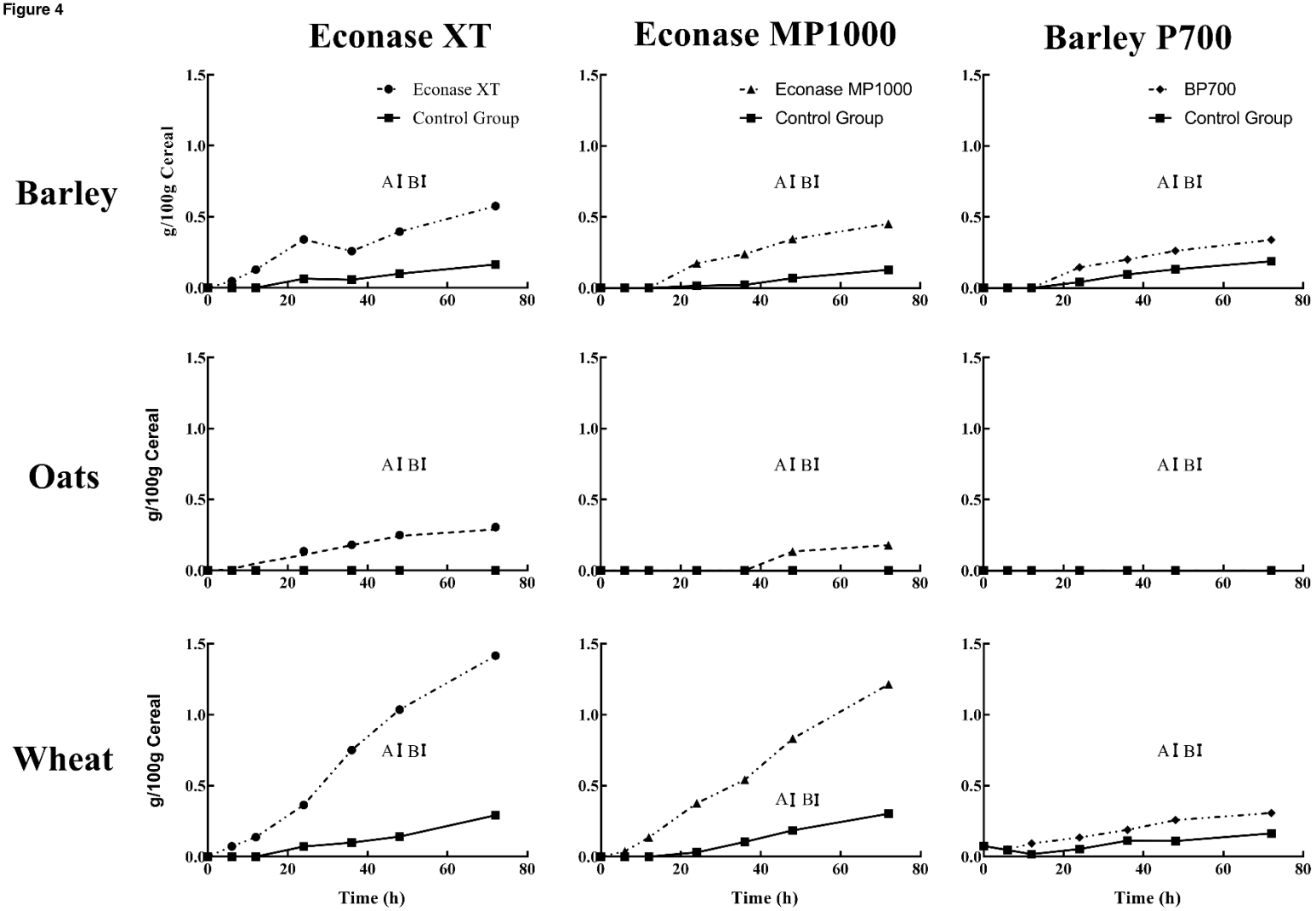

Fig. 4. Comparison of the effectiveness of different enzymes on the release of xylose from different cereals. Mean values expressed as total xylose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No xylose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.042).

Figure 5

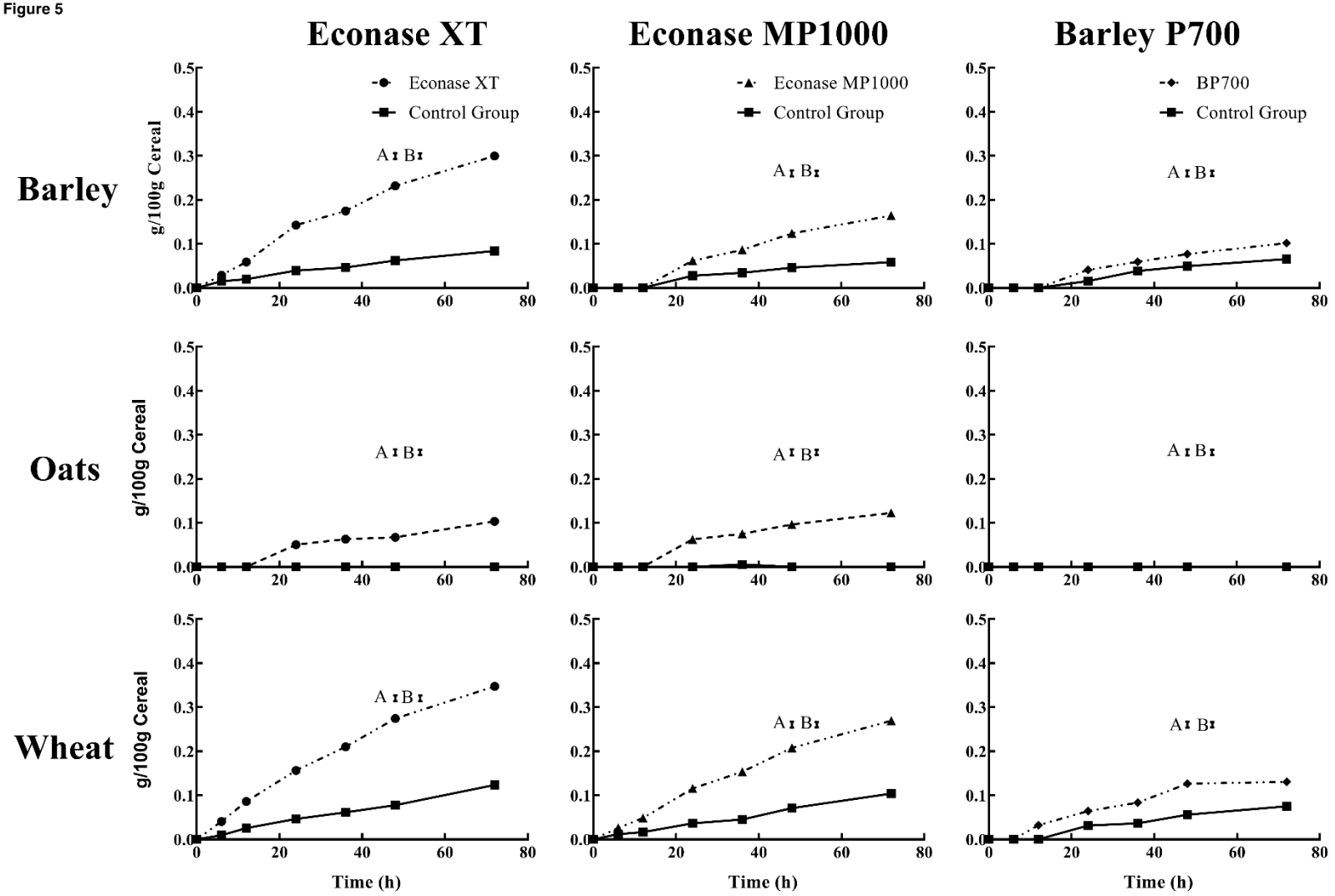

Fig. 5. Comparison of the effectiveness of different enzymes on the release of arabinose from different cereals. Mean values expressed as total arabinose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No arabinose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a significant enzyme x cereal x time interaction (P=0.007).

Figure 6

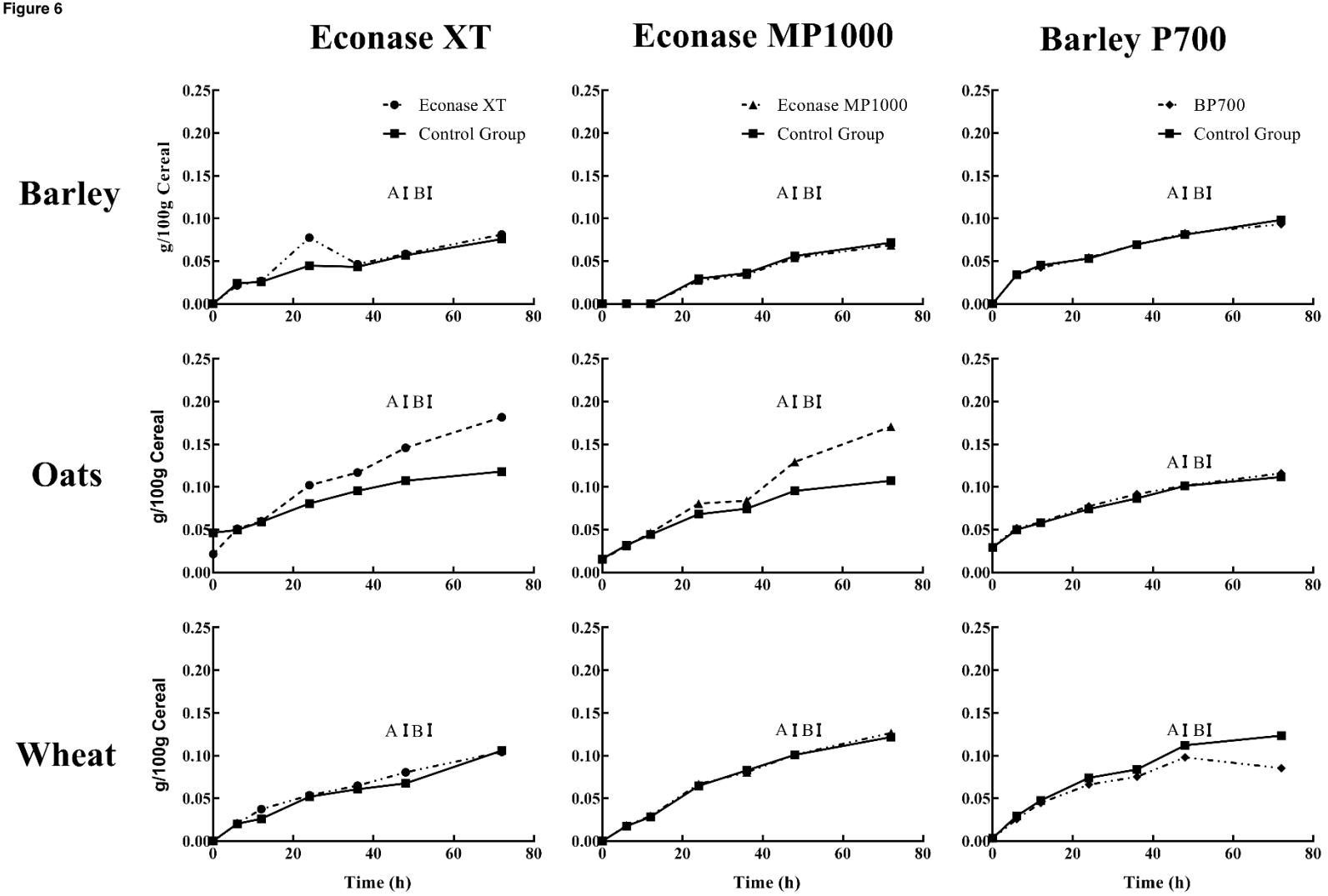

Fig. 6. Comparison of the effectiveness of different enzymes on the release of galactose from different cereals. Mean values expressed as total galactose g/100g biomass released during in-vitro digestion over 72 h at 41°C. No galactose was detected for maize in any of the control or enzyme-treated digestions. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated significant enzyme x cereal x time interaction (P=0.007).

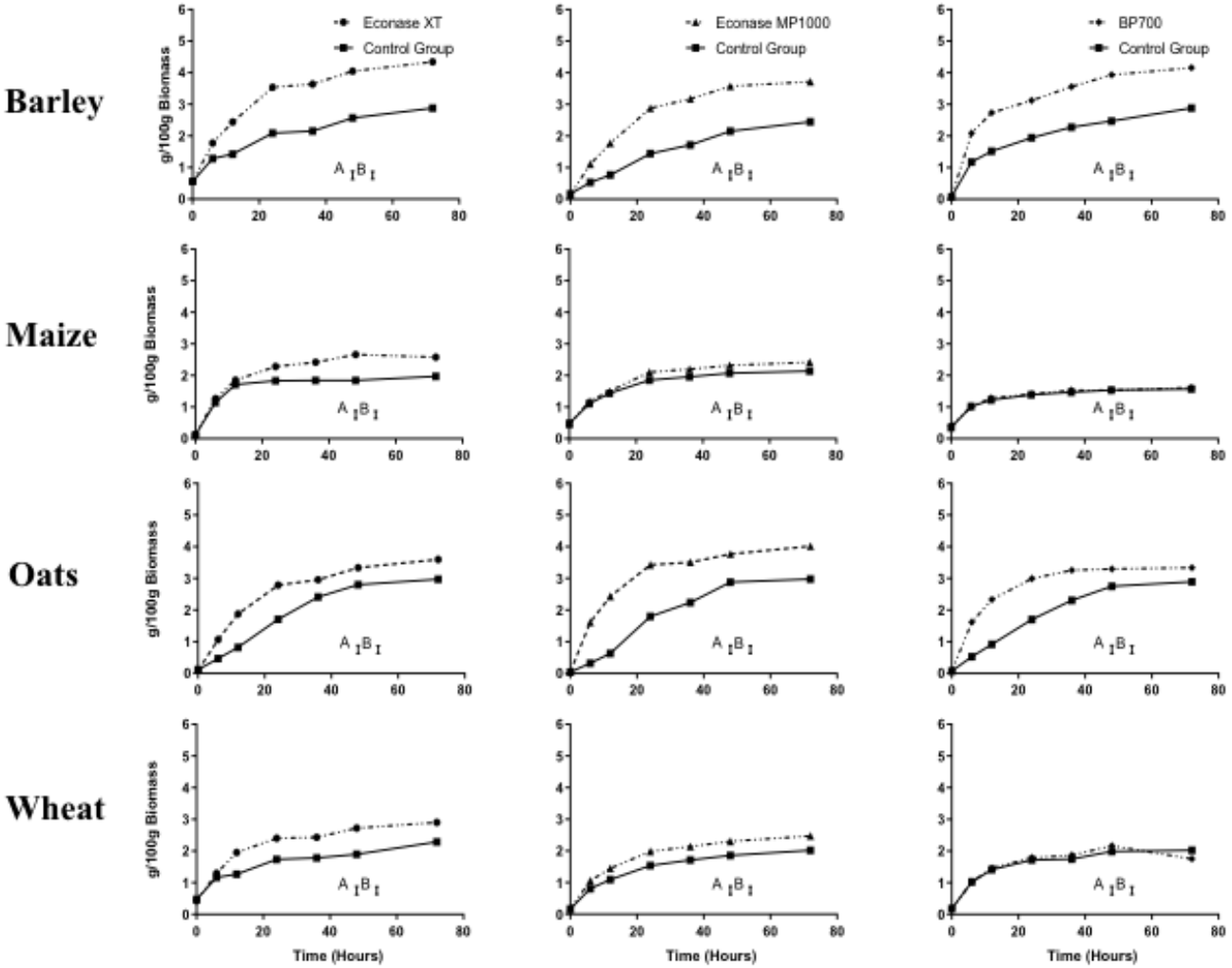

Fig. 7. Comparison of the effectiveness of different enzymes on the release of glucose from different cereals. Mean values expressed as total glucose g/100g biomass released during in-vitro digestion over 72 h at 41°C. The error bars are the standard error of the difference of the means for comparison in the same graph (see Materials and Methods), 'A' for comparing control and enzyme, 'B' for comparing within the same treatment (control or enzyme). Three-way ANOVA indicated a trend for enzyme x time (P= 0.090) and cereal x enzyme (P= 0.090) interactions, but significant effects of time (P= 0.036) and cereal (P=0.047) individually.