MEASUREMENT OF GRAIN ENZYME DIFFUSION RATES AND GRAIN THRESHOLD PARTICLE SIZE CALCULATOR (4B-123)

Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

Rationale

The factors in grain-based diets that are responsible for pig production performance are incompletely understood. This project evaluated the potential of determining the enzyme (amylase) diffusion coefficient within grain samples as a fundamental characteristic of grains that is directly linked with their efficient use in production diets. The rationale is that feed efficiency is likely to be maximized when all grain digestion occurs by the end of the small intestine, as energy is less completely harvested from subsequent colonic fermentation. In order to achieve this, the digestion time needs to be shorter than the small intestinal passage time.

Outcomes

From analysis of in vitro digestion rates as a function of (separated) particle size for 16 diverse grains, it was found that all samples showed the same inverse square dependence of digestion rate coefficient against particle size. The slope of this relationship is linearly related to the enzyme diffusion coefficient, a size-independent measure of the intrinsic digestibility of each grain sample. Diffusion coefficients were similar for wheat and barley samples, and both were higher than those for sorghum samples. Damaged, sprouted or immature grain samples each had much faster diffusion coefficients. However, this value alone was not sufficient to explain previously measured ileal digestibility values, suggesting that passage rate (as influenced by fibre content) as well as intrinsic digestibility controls the extent of digestion in the small intestine.
A limitation of the approach is that measurement of amylase diffusion coefficient requires a lengthy high-skill method. The feasibility of developing a NIR calibration was evaluated and showed some promise, although many more samples would be needed to establish a robust calibration that could be used for selecting (milled) grains for optimal feed efficiency

Relevance

This study has shown that enzyme diffusion coefficients are a robust and meaningful characteristic of feed grains. In the sister project (4B-122), it is shown how the diffusion coefficients are directly related to FCR through the proportion of grain starch digested by the end of the small intestine.
One consequence of the large body of data collected is to reinforce the concept that it is the large particles in milled (and pelleted) grain that limit in vivo digestibility. If these can be avoided without generating fines (e.g. by two-pass milling), then efficient grain utilization by pigs would be more predictable. Size cut-offs (threshold particle size) for full digestion of wheat/barley and sorghum are estimated to be 0.8-1.0 mm and 0.6-0.8 mm respectively. Any milled grain particles larger than this (even though the average size may be lower than the threshold) run the risk of being used inefficiently in pig production.

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1. Introduction

Cereal grains play a vital role in nutrition for many animals including humans and pigs. The major component of cereal grains is starch, with its nutritional value being determined by its site of digestion (Black, 2016). Starch that is completely digested before the end of the small intestine (SI) provides the maximum amount of glucose from the grain to support growth in pigs. However, complete digestion of starch can have negative consequences for human health by providing excess energy and increasing risks of diabetes and obesity. Starch that is not digested by the ileum (end of the SI) passes to the large intestine where it is fermented by the resident microbial population, which is considered to be generally beneficial for human and pig health (Dhital et al, 2017). However, approximately 15% of energy digested in the large intestine is lost from the animal as heat of fermentation, methane and voided microbes, resulting in less energy being available for animal growth. An understanding of grain characteristics and processing conditions controlling starch digestion in the small intestine would assist the selection of processed grain for maximising pig growth and enhancing human health.

The general principle is well-established that smaller grain particle sizes result in faster digestion in vitro (Al-Rabadi et al., 2009; Heaton et al., 1988) and more efficient use of feed by pigs (De Jong et al., 2016; Saqui-Salces et al., 2017). Similarly, human ileostomates consuming coarse wheat (2mm) porridge showed significantly lower postprandial blood glucose and insulin levels than consumers of finer wheat (<0.2mm) porridge (Edwards et al., 2015). However, quantitative relationships between particle size and digestion both in vitro and in vivo have not been established across diverse grain types. Thus, there is no available in vitro method of determining ileal digestibility of cereal grain starch that can be associated directly with in vivo measurements of digestion in the SI of pigs.

Commercial pig feeds typically contain cereal grains as the main ingredient, and the digestibility of the grains can be estimated by different in vitro methods, including for example, by analogy with human digestion, in vitro prediction of glycaemic index (GI) of milled grains (Giuberti et al., 2012). However, such methods do not take into account effects of particle size distribution (PSD). Grain particle size is important because the rate of enzyme digestion depends on milled grain particle size, as does the efficiency of pig growth (Al-Rabadi et al., 2017; Paulk et al., 2015). From a study of amylase digestion of size-fractionated milled sorghum and barley grain particles, an inverse square dependence of (first order) starch digestion rate on particle size was found (Al-Rabadi et al., 2009). This inverse square relationship is consistent with a surface-controlled reaction, and can be considered to be equivalent to a diffusion-controlled process and formalised as an apparent diffusion coefficient (ADC) of amylase in milled grains (Al-Rabadi et al., 2009). However, limited knowledge exists on the association between predicted in vitro digestibility of grains and digestion of starch or energy in the SI of pigs. Moreover, the amount of starch digested in the SI depends on the combined effect of passage rate of the digesta through the SI and starch digestion rate (Lee et al., 2013). Therefore, a simple measurement such as the in vitro prediction of GI of ground grains without definition of PSD, is of limited use.

In commercial animal feeding practice, milling of grains generates a mixture of coarse and fine fractions, as characterised by the PSD. The overall digestion rate

can be hypothesised to be a function of the PSD and the intrinsic properties of the grain, which determines the enzyme diffusion rate (EDR), measured as the ADC. As ADC is independent of particle size, it is hypothesised that the overall digestion rate of a milled grain can be determined based on the ADC and the PSD of milled grains. The ADC is expected to depend on the surface and internal structure of grain fractions as these define the barriers to enzyme diffusion. These diffusion barriers will depend on the cereal cell wall structure, endosperm density and processing method (e.g. hammer- or disc-milling, heat or enzyme treatment) used to prepare the grain for use in feed. The maturity of grains at harvest and natural processes such as sprouting, can also affect the tissue structure of the grain and therefore EDR and digestibility. To test whether ADC/EDR values can be used to develop more robust in vitro predictions of in vivo digestion characteristics for milled grains, two main steps are required. First, the general nature of inverse square dependence of amylase digestion rate on milled grain particle size needs to be established for a range of grains. Secondly, the resulting ADC values need to be compared with in vivo ileal digestibility data to determine the potential for prediction of in vivo behaviour based on in vitro data. Ileal digestibility of grains in pigs can be measured from ileal cannulation experiments (Van Barneveld, 1993) comparing ileal digestible energy (IDE, MJ/kg, the fraction of total dietary energy that is utilised before the ileum) with faecal digestible energy (FDE, MJ/kg, the total dietary energy that is not excreted in faeces). Indices of effective digestibility can be obtained from e.g. the ratio of IDE:FDE, or an adjusted IDE value which takes into account the indigestible fibre that is present in the grain (Knudsen and Canibe, 2000; Knudsen et al., 1993).
The hypotheses tested in this work were that: 1) there is an inverse square dependence of rate coefficients for amylase-catalysed digestion of starch on particle size for diverse grains; 2) grain type and modified tissue structures affect ADC in a rational way; and 3) grain ADC values provide insights into feed factors affecting ileal digestibility in pigs. The objectives are therefore: 1) To determine the generality of the inverse square dependence of amylase digestion rate of starch for milled grains and compare the resulting ADC values for sixteen samples representing different cultivars and growing conditions of wheat, barley and sorghum, and 2) To investigate any associations between ADC and IDE:FDE or fibre-adjusted IDE of feeds primarily containing these grains.

2. Methodology

2.1 Grains, ileal and faecal digestible energy

Sixteen grain samples (Table 1), previously studied in the Premium Grains for Livestock Program (Black, 2008), were obtained from the University of Sydney grain archive, where the grains were stored at -20°C. The grains selected had been exposed to either natural or imposed growing conditions or post-harvest treatments to produce grains with a range of morphological, chemical and nutritional characteristics. The samples had previously been analysed (Table 1) for total starch (McCleary et al., 1997), crude protein (Method – 4.2.04) (AOAC, 1995), crude fat (Method – 4.5.01) (AOAC, 1995), acid detergent fibre (ADF) (Method – 4.6.03) (AOAC, 1995), neutral detergent fibre (NDF), IDE and FDE

Table 1. Grain samples with IDs from previously reported study (Black, 2008), used for determination of ADC. All grains were grown and treated under standard conditions apart from Wollaroi, Janz, Arapiles and Fitzroy. All % values based on dry matter.

Grain Type	Grain Variety	Grain ID	Starch (%)	Protein (%)	Fat (%)	NDF* (%)	ADF** (%)	HC*** (%)
Wheat	Wollaroi[a]	1772	64.0	18.5	2.3	9.3	2.3	-
	Janz[b]	1809	52.0	18.1	3.0	23.6	5.6	91.6
	H45	1841	65.9	16.7	2.0	21.8	4.1	52.8
	Red Wheat	1876	68.6	10.9	2.2	10.6	3.5	37.0
	Currawong	1906	64.9	14.7	2.0	13.2	3.9	55.5
Barley	Skiff	3814	58.3	13.6	2.2	17.8	5.2	-
	Arapiles[c]	3828	49.8	13.5	2.2	32.8	11.8	109.9
	Grout	3869	53.1	14.4	1.8	15.6	4.9	39.7
	Wyalong	3875	47.1	17.7	1.8	18.9	5.2	42.4
	Fitzroy[d]	3879	48.9	13.6	1.8	24.5	8.9	-
	Schooner	3904	58.3	11.3	2.3	20.7	5.4	49.0
Sorghum	Waxy Isoline	7710	73.8	13.4	3.8	16.4	2.8	40.2
	Normal Isoline	7711	73.0	13.5	3.7	16.8	3.8	32.2
	Boomer	7812	74.5	10.4	4.0	11.8	4.7	28.1
	MR Maxi	7855	68.5	11.4	2.7	7.2	4.0	35.2
	ICSV400	7870	72.4	12.3	3.2	7.3	3.5	30.4

[a] Normal growth, but naturally sprouted before harvest, [b] Dry prior to harvest and grains screened through 2.2 mm sieve, [c] Experienced a frost prior to harvest that reduced grain maturity, [d] Sprayed with glyphosate six weeks after flowering that reduced grain maturity

*Neutral detergent fibre, **Acid detergent fibre, ***Hydration Capacity

contents (Black, 2008). IDE and FDE were determined in diets where approximately 81% (Table A1) of the diet was from the cereal grain. A cannulation methodology (Van Barneveld, 1993) had been used to determine IDE and FDE. Briefly, T-piece cannulas were inserted at the terminal ileum in pigs (~45kg). Pigs were fed twice daily for six consecutive days followed by two days of continuous digesta collection for 2-8h period after the morning meal. Faecal samples were also collected during this time. Faecal and ileal samples were immediately frozen. IDE and FDE was determined by adiabatic bomb calorimetry of freeze dried digesta and faecal samples. Hydration capacity (%) was measured by determining the amount of water absorbed by 100 whole grains after 16 hours of soaking in excess water at ambient temperature.

2.2 Milling and sieving

Each of the grain samples (100g) were hammer-milled (PX-MFC 90 D, Culatti AG, Zurich) at 6000rpm with a 2mm sieve in place. The ground grains were then individually size-fractionated by sifting for 30 minutes through a vibrating automatic sieve-shaker (Minor-1807-07, Endecotts Ltd., England) using multiple sieves (1.4, 1.2, 1, 0.85, 0.71, 0.5, 0.25, 0.075mm and a pan). Size distributions for all fractions by percent weight were determined for all samples and the geometric mean diameter (d_{gw}) was determined for each sample using the standard method (ASABE, 2013). Of the nine fractions, those retained on five

sieves (1, 0.71, 0.5, 0.25 and .075mm) were obtained in sufficient quantities for starch digestion analysis to derive digestion rate coefficients based on first order kinetics, and to determine total starch content. The average particle size for each fraction was taken as the average of the adjacent sieve sizes that each fraction was retained within. The average particle sizes of the five fractions analysed were therefore 1.1, 0.78, 0.605, 0.375 and 0.163mm.

2.2 Total starch determination

Total starch in each size fraction of each grain sample was determined in duplicate using the Megazyme Total Starch Assay kit (K-TSTA, Megazyme International Ireland Ltd.) based on the dimethyl sulfoxide method (McCleary et al., 1997) with modifications. A control of standardised regular maize starch (Megazyme) and a reagent blank were analysed with each grain sample. Each grain sample (100 $\pm$ 5mg) was dispersed and vortexed with 0.4ml ethanol (80% v/v) in a 15ml tube. Then, 2ml dimethyl sulfoxide was added and further vortexed. Tubes were then placed for 10 – 20 minutes in a water-bath maintained at 100^0C and under vigorous stirring conditions. The 1.1mm size fraction from each of the grain samples took up to 20 minutes for starch to dissolve. The resulting digesta was mixed with 3ml thermostable *alpha* – amylase (Megazyme, 54U/mg) dissolved at a concentration of 3.23% in 50mM MOPS buffer (pH 7) and subsequently maintained at 100^0C under vigorous stirring conditions for 12 minutes. Tubes were then transferred to a water bath at 50^0C and 4ml 200mM sodium acetate buffer (pH 4.5) was added. The whole mixture was then incubated after addition of 0.1ml amyloglucosidase (Megazyme, E-AMGDF, 3260 U/ml) at 50^0C for 30 minutes with constant stirring. After incubation, 0.1ml of the mixture was diluted to 1ml for wheat and barley, and 1.6ml for sorghum and centrifuged at 4000g for 2 minutes. Then, 50µl of the supernatant was transferred to another microcentrifuge tube and 1ml GOPOD reagent (glucose oxidase) was added. After 30 minutes, absorbance was measured spectrophotometrically (UV-1700 Pharmaspec, Shimadzu, Japan) at 505nm to determine glucose concentration. A factor of 0.9 was used to convert glucose to starch.

2.3 In vitro starch digestion

A three step digestion model (Al-Rabadi et al., 2009) simulating digestion in the mouth, stomach and the SI was used with modifications for all the milled grain fractions in duplicate. A control of standardised regular maize starch (Megazyme) and a reagent blank were analysed with each grain sample. Firstly, 100 $\pm$ 5mg sample was mixed with 2.17mg of alpha-amylase (Megazyme, E-BLAAM, 54U/mg) dissolved in 1ml phosphate buffer (pH 7) to simulate oral digestion for 45 seconds followed by immediate addition of 2ml 0.02M HCl to simulate entrance into the stomach. This digesta was then incubated with constant stirring at 100rpm to simulate gastric digestion at 37 ^{0}C for 30 minutes with 1mg pepsin (Sigma, P-6887, 3200U/mg) dissolved in 1ml 0.02ml HCl. To stop the pepsin activity, 3ml of 0.02M NaOH was added to digesta to raise the pH to 11.0. The pH was then reduced to 6.7 with addition of 4ml 0.2 M sodium acetate (pH 6.0) buffer containing 0.2% sodium azide. Then, 8mg of pancreatin (Sigma, P-1750, 4 x U.S.P specification) and 38µL of amyloglucosidase (Megazyme, E-AMGDF, 3260 U/ml) dissolved in 1ml sodium acetate (0.2M) buffer were added to the digesta. The digesta was then incubated at 37 ^{0}C with constant stirring using a magnetic stirrer (100rpm) to simulate small intestinal digestion. Periodically, 50µl of digesta was taken after 5, 10, 20, 40, 60, 90, 120, 240, 360, and 480 minutes, and mixed with 300µl 0.3M

Na_2CO_3 to stop the enzymic activity for colorimetric assay. The relatively long digestion time of up to 480 minutes was chosen so that rate coefficients for slowly-digested larger fractions could be determined. 1ml GOPOD reagent (glucose oxidase / peroxidase) was added to a 50µl aliquot taken from the digesta- Na_2CO_3 mixture. After 30 minutes, absorbance was determined to measure glucose concentration spectrophotometrically at 505nm. A factor of 0.9 was used to convert glucose to starch, which was expressed as a percent fraction of total starch digested over time in hours (Fig.1A). All the results were reported on dry matter basis.

2.4 Determination of digestion rate- and diffusion- coefficients

The fractional digestion rate coefficients and ADC were calculated as described by Al-Rabadi et al. (Al-Rabadi et al., 2009). Starch digestion in milled grains typically exhibits first order kinetics

$$C_i = 1 - e^{-k_i t} \quad (1)$$

where Ci is starch in the grain fraction digested at time t in sieve with mesh size i; k_i is the fractional digestion rate coefficient (h^{-1}), t is the digestion time (h). This approach assumes that all starch is digested with a single rate coefficient, as would be predicted for diffusion through the grain particle being the rate-determining step.

For diffusion/surface-controlled reactions, there is a linear relationship between the reciprocal of the rate coefficient and particle size squared. Rate coefficients for each size fraction were determined in duplicate, and inverse of their means were plotted against the square of their corresponding average particle sizes. The slope of the plot provides a solution for the equation:

$$K_i = \frac{6D}{Si_{avg}{}^2} \quad (2)$$

Where D is the apparent diffusion coefficient (ADC) and Ki is the rate constant for digestion at a particle size average of Si_{avg}. ADC values for all the grain samples were calculated in units of mm^2/h and cm^2/s (Table 3).

2.5 Statistical Analysis

ADC for all grains grown under standard conditions were analysed using a one-way ANOVA, with grain type as a factor, followed by post-hoc Tukey HSD to determine significant differences in ADC values between wheat, barley and sorghum. A linear model was fitted between IDE adjusted for NDF and ADC, and between ADC and NDF. A multivariate (bivariate) approach was used to fit a model to IDE (not adjusted for fibre) as a function of ADC influenced by NDF. All the statistical analyses were undertaken using R (version 3.4.2, https://www.R-project.org).

3. Outcomes

3.1 Inverse square dependence of rate coefficient on particle size

An example of the dependence of amylase digestion of starch on particle size is shown for the waxy sorghum sample in Fig.1. The graph shows (a) progressive starch digestion over eight hours for different size fractions with smaller fractions digesting faster (Fig 1 A), (b) first order kinetics behaviour of starch-digestion (Fig

1 B), and (c) inverse square dependence of rate coefficients on average particle sizes (Fig 1 C; R2=0.97). The rate coefficients (Table 2) for the milled grain fractions from wheat, barley and sorghum all increased with decreasing particle size, which was consistent with the findings reported earlier for barley and sorghum (Al-Rabadi et al., 2009). All sixteen cultivars of wheat, barley, and sorghum showed strong inverse square dependence (R2>0.9) of rate coefficients on particle sizes (Table 3). Previously, such dependence has been reported for only single samples of barley (Al-Rabadi et al., 2009) and sorghum (Al-Rabadi et al., 2009; Mahasukhonthachat et al., 2010). This study shows the generality of the inverse square dependence of amylase digestion of starch on particle size for wheat, barley and sorghum grains based on a large number of grain cultivars including those with widely varying growing conditions (Table 1).

3.2 Enzyme diffusion rate or ADC of wheat, barley and sorghum samples

ADC provides a single in vitro measurement of the rate of amylase penetration into milled grains and should reflect different grain-specific factors which affect starch digestion. Having values for 16 grains now allows an analysis of the factors determining ADC values, with grain structure, composition, and processing methods used expected to be the main factors affecting grain starch digestion (Giuberti et al., 2014; Taylor et al., 2015).

There were similarities and differences in ADC between the three grains studied. The ADC values (**Error! Reference source not found.**) were within a relatively narrow range of 0.014 – 0.021 mm^2/h for all five cultivars of sorghum, and were significantly lower than wheat ($P<0.05$) and barley ($P<0.001$) samples (**Error! Reference source not found.**) (**Error! Reference source not found.**). Two samples of wheat (Wollaroi, Janz) and two samples of barley (Arapiles, Fitzroy) had ADC values more than twice the average of the other samples of the same grain type. These samples were either pre-harvest sprouted, immature or frost-damaged, as discussed in detail later. When these wheat and barley samples with the higher ADC values were excluded, the ADC values for wheat and barley were not significantly ($p>0.05$) different.

The peripheral endosperm region of sorghum is dense and high in protein, making it resistant to water absorption and digestion (Rooney and Pflugfelder, 1986). Waxy Isoline sorghum ($0.020 mm^2/h$) had a higher ADC than Normal Isoline sorghum ($0.014 mm^2/h$). The faster rate of amylase diffusion in the waxy isoline may be expected because of the higher proportion of amylopectin with its more open structure than amylose. A comparison of digestibility for different sorghum varieties analysed in various in vitro and in vivo studies found waxy sorghum to be more digestible than normal sorghum (Rooney and Pflugfelder, 1986). Previously, a slightly higher ADC of 0.027 mm^2/h (Al-Rabadi et al., 2009) was reported for sorghum (cultivar: Buster) than found in this study for the five sorghum cultivars. The wheat cultivars Wollaroi and Janz, and barley cultivars Arapiles and Fitzroy showed markedly higher ADC with values 0.069, 0.06, 0.074 and 0.07 mm2/h respectively, than the other wheat and barley samples. The Wollaroi sample was from a pre-harvest sprouted or partially germinated crop. Germination would be expected to disrupt the cellular structure of the grain and allow amylase to diffuse at a higher rate. Olaerts et al. (Olaerts et al., 2016) found that sprouting of wheat in the field in contrast to sprouting under laboratory conditions, caused

no initial starch hydrolysis from the action of amylolytic enzymes which developed during sprouting. This suggests that disruption to cellular structure was the cause of faster hydrolysis of starch in pre-harvest sprouted grains.

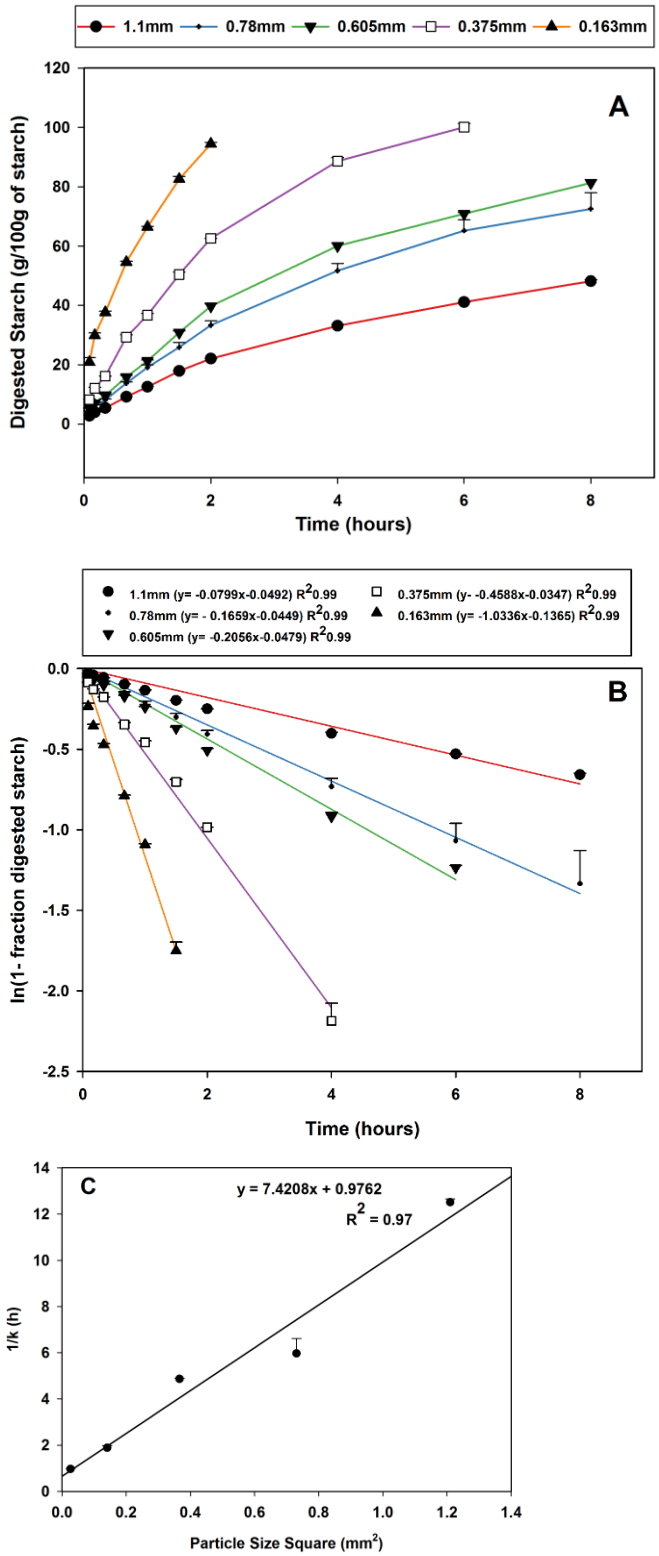

Figure 1. Starch digestion for a sorghum sample (Waxy Isoline) with SEM: A) digestograms as a function of particle size, B) first order kinetics fit – rate coefficient k (h^{-1}) for each fraction is given by the slope, C) Relationship between square of particle size (mm^2) and inverse of rate coefficient 1/k (h) – the slope gives the apparent diffusion coefficient (ADC).

Table 2. Geometric mean diameter (dgw) and rate coefficients k (h^{-1}) ± SEM (from Equation 1) for five particle size fractions from each of 16 grains.

Grain Type	Grain Variety	d_{gw} (mm)	Average Particle Size (mm)				
			1.1	0.78	0.605	0.375	0.163
			k (h^{-1})	k (h^{-1})	k (h^{-1})	k (h^{-1})	k (h^{-1})
			SEM	SEM	SEM	SEM	SEM
Wheat	Wollaroi	0.56	0.367	0.595	0.649	1.028	1.413
			0.012	0.015	0.012	0.08	0.044
	Janz	0.46	0.304	0.468	0.676	1.020	1.057
			0.008	0.036	0.045	0.058	0.053
	H45		0.159	0.206	0.221	0.233	0.278
			0.018	0.007	0.011	0.011	0.005
	Red Wheat	0.47	0.123	0.213	0.322	0.487	0.865
			0.000	0.005	0.021	0.017	0.011
	Currawong	0.54	0.128	0.163	0.193	0.299	0.413
			0.028	0.006	0.005	0.001	0.037
Barley	Skiff	0.49	0.164	0.285	0.336	0.556	0.847
			0.008	0.031	0.020	0.064	0.024
	Arapiles	0.39	0.400	0.443	0.742	0.853	0.964
			0.032	0.022	0.010	0.036	0.016
	Grout	0.49	0.145	0.292	0.463	0.673	1.582
			0.005	0.012	0.011	0.008	0.016
	Wyalong	0.48	0.149	0.302	0.396	0.612	0.738
			0.002	0.004	0.004	0.022	0.011
	Fitzroy	0.41	0.346	0.643	0.751	0.917	1.060
			0.030	0.003	0.001	0.035	0.028
	Schooner	0.47	0.133	0.223	0.346	0.645	0.818
			0.006	0.002	0.018	0.019	0.033
Sorghum	Waxy Isoline	0.51	0.097	0.184	0.228	0.459	1.125
			0.002	0.008	0.008	0.003	0.085
	Normal Isoline		0.074	0.109	0.152	0.217	0.321
			0.001	0.005	0.005	0.008	0.007
	Boomer	0.51	0.112	0.156	0.195	0.262	0.433
			0.001	0.015	0.011	0.012	0.004
	MR Maxi		0.081	0.136	0.171	0.207	0.326
			0.001	0.014	0.003	0.013	0.008
	ICSV400	0.52	0.101	0.166	0.189	0.282	0.582
			0.013	0.007	0.006	0.011	0.050

Table 3. Inverse square dependence (R^2>0.9) of rate coefficients on particle sizes for all grains and the corresponding apparent diffusion coefficients (ADC) from Equation 2. IDE:FDE is derived from IDE and FDE values obtained with feed containing the same grain samples (Black, 2008).

Grain Type	**Grain Variety**	**Dependence between particle size squared (mm^2) and inverse of rate coefficient (1/k)**			**ADC**		**IDE:FDE**
		Slope (m)	**Intercept (c)**	**R^2**	**mm^2/hr**	**cm^2/s ($x10^{-7}$)**	
Wheat	Wollaroi	1.5856	0.7458	0.96	0.069	1.92	0.900
	Janz	2.0213	0.7763	0.99	0.060	1.67	0.742
	H45	2.1192	3.7146	0.93	0.029	0.81	0.794
	Red Wheat	5.6406	1.0417	0.99	0.025	0.69	0.850
	Currawong	4.9869	2.6909	0.97	0.022	0.61	0.885
Barley	Skiff	3.9277	1.2096	0.97	0.032	0.89	0.866
	Arapiles	1.3672	0.9957	0.94	0.071	1.97	0.548
	Grout	5.0001	0.4485	0.97	0.031	0.86	0.769
	Wyalong	4.361	0.9527	0.95	0.031	0.86	0.828
	Fitzroy	1.5729	0.7926	0.91	0.070	1.94	0.827
	Schooner	5.3356	0.9049	0.99	0.027	0.75	0.882
Sorghum	Waxy Isoline	7.4208	0.9762	0.97	0.020	0.56	0.868
	Normal Isoline	8.5653	3.1936	0.99	0.014	0.39	0.853
	Boomer	5.1665	2.7878	0.97	0.021	0.58	0.909
	MR Maxi	7.1657	3.1713	0.97	0.016	0.44	0.841
	ICSV400	6.4806	2.1750	0.95	0.019	0.53	0.787

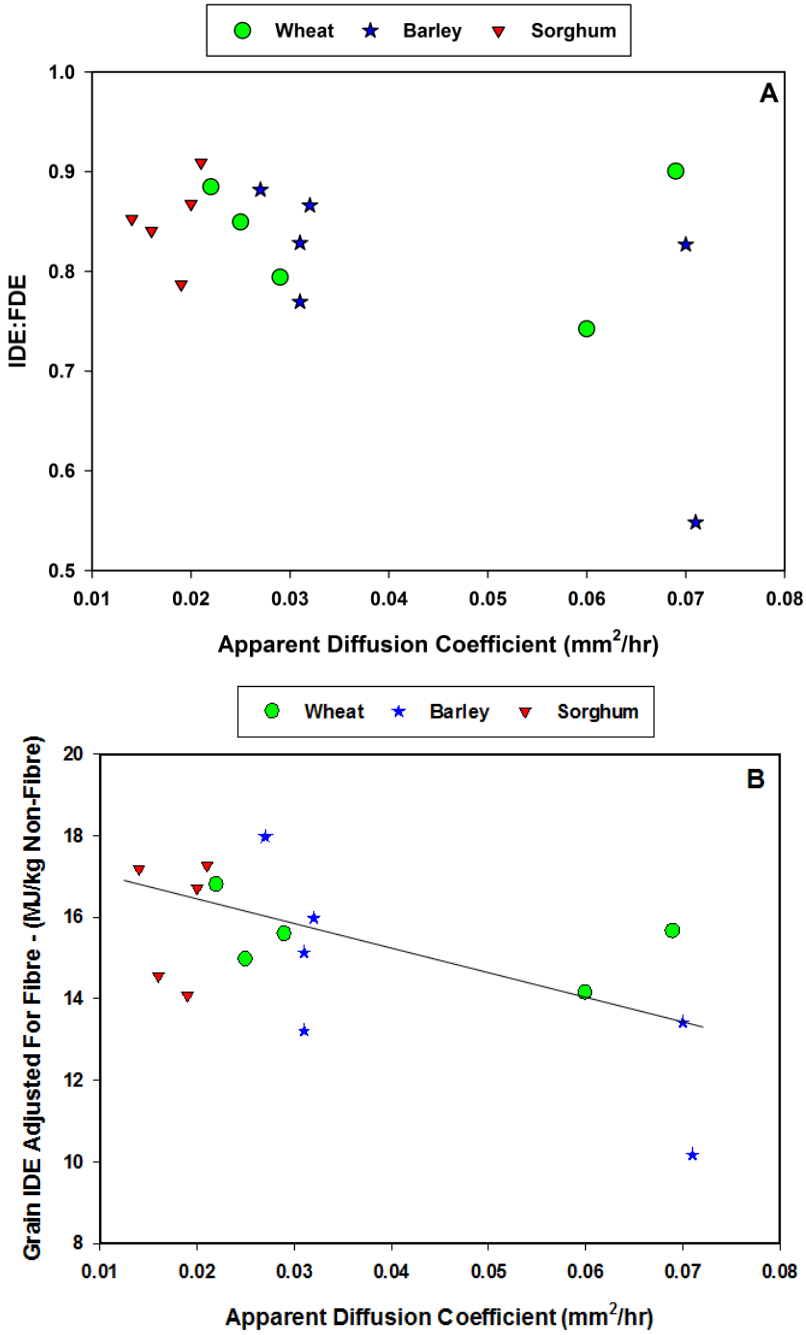

Figure 2 (a). Values of ADC for 16 grains compared with the ratio between ileal and faecal digestibility extents in pigs for feeds based on the same grains; (b). Relationship between ADC and IDE adjusted for NDF content (MJ/kg DM-NDF) for the sixteen grains fed to pigs. NDF adjusted IDE = 17.2 – 57.3 * ADC (R^2 = 0.36).

The sample of Janz was grown under dry conditions and the grains selected were screened to be less than 2 mm in size. This immature Janz wheat sample had a NDF value (Table 1) over 20% and a corresponding low starch content of 52%, whereas the three other wheat samples had NDF values of around 10% and starch contents of around 65%. These differences suggest that the Janz sample studied had a lower proportion of starchy endosperm to outer layers of grain than the three other wheat samples, consistent with it being less mature with less

developed cell walls allowing faster amylase penetration. During cultivation in the field, the sample of barley cultivar Fitzroy was sprayed with herbicide (Glyphosate), which prevented the grain from fully maturing. Similar to the immature Janz wheat, the Fitzroy sample would have less developed cell walls allowing faster diffusion rate of amylase. An in vitro investigation (Ahmed et al., 2014) on corn grain found that starch digestibility was significantly reduced with advancing maturity. Frosting of the barley crop during maturation reduced starch content in the Arapiles sample by approximately 20% compared to unfrosted Arapiles (Black, 2008). In a study (Craven et al., 2007) of the effect of cold temperatures during grain maturation stage on quality parameters of wheat, two out of three cultivars showed a significant decrease in Hagberg Falling Number, an indirect measure of starch digestion rate.
A sample of the barley cultivar, Binalong, grown under normal conditions was found to have a higher ADC value of $0.062mm^2/h$ (Al-Rabadi et al., 2009) than measured for normally grown barley samples in this study (average ADC $0.030mm^2/h$). This difference suggests that the sample analysed by Al-Rabadi et al., (2009) may have been immature or damaged as described above for the four examples in the current study, because ADC values were similar.

3.3 Implications of ADC in grain milling

The fractional digestion rate coefficient, k_i (h^{-1}) can be determined from the starch fraction digested (C_i) as a function of digestion time t (h) using **Error! Reference source not found.**. Then, **Error! Reference source not found.** can be solved with known values of k_i (h^{-1}) and ADC (mm^2/h) to determine the average particle size, Si_{avg} (mm), which will be digested to a specified extent in a specified time. A potentially important application would be if the residence time in the SI of pigs is known, then the particle size that would be e.g. 95% or 99% digested in the SI could be predicted. This approach needs to be validated based on measurements for a range of grain samples of known ileal digestibility and rate of passage of digesta through the SI in pigs. However, data on passage rates in the SI are very difficult to obtain, as they typically require a dual cannulation approach at the duodenum and terminal ileum, which is ethically and technically challenging. Measurement of mouth to ileum passage time is not sufficient, as this does not distinguish between residence times in the stomach and SI.

3.4 Association between in vitro ADC of grains and in vivo digestion in the SI

IDE: FDE is a ratio that provides a measurement of energy utilised before the end of ileum as a fraction of the total energy from the feed digested in the whole digestive tract. Digestion in the SI yields more energy from macronutrients than fermentation in the large intestine (Gerrits et al., 2012). An IDE:FDE of 1 means that all of the energy available from the feed is absorbed by the end of the SI. However, dietary fibre that is not digested by animal enzymes in the SI, but can be fermented by microbes in the large intestine (LI) will reduce IDE:FDE below 1. Interactions between rate of macronutrient digestion and rate of passage of digesta through the SI also affects IDE:FDE because digestible substrates may flow to the LI before there is sufficient time in the SI to be fully digested. Starch digestion can be reduced by 1. viscosity effects of soluble fibre reducing

accessibility of amylase to starch granules, although this can be overcome by efficient mixing (Dhital, Dolan, et al., 2014); 2. amylase binding to insoluble fibre such as cellulose or wheat bran (Dhital, Warren, et al., 2014) and; 3. increase in SI passage rate (if grains are slowly digestible), e.g. by wheat bran (Wilfart et al., 2007).

Fig. 2 shows that most grains have ADC values of around 0.014 – 0.031mm^2/h and IDE:FDE lying between 0.75 – 0.92. The four with high ADC values had variable IDE:FDE values. The Wollaroi grain sample from normal growing conditions but naturally germinated had a high IDE:FDE, suggesting that most starch digestion occurred before the end of the ileum owing to the higher enzyme diffusion. The small grained Janz sample, despite a higher ADC, had a lower IDE:FDE than grains with lower ADC. This may be because of the relatively high NDF value. (Table 1). Little NDF is digested in the SI of pigs. Consequently, an alternative method for assessing the impact of ADC on grain digestion in the SI of pigs is to investigate the relationship with IDE when NDF is removed; that is, IDE expressed as MJ/kg DM-NDF rather than MJ/kg DM (Figure 2b). There is a moderate relationship (R2=0.36, P<0.05) between NDF adjusted IDE and ADC, but adjusted IDE declined as ADC increased, rather than increase as would be expected if the rate and extent of starch digestion was increased with increasing ADC. However, the negative relationship is influenced strongly by the four samples with high ADC values. Based on all 16 grains, there is a moderate positive correlation (R2=0.33, P<0.05) between ADC and NDF, and this moderate collinearity might have affected the bivariate analysis described in the next section. However, based on 12 grains, considered representative of normal growth conditions, with lower ADC values there is no apparent relationship between NDF adjusted IDE and ADC (Fig 2b).

The bivariate model fitted to IDE (not adjusted for NDF) as a function of ADC influenced by NDF exhibited a negative correlation (R2=0.67, P<0.001). This implies that grains with high NDF content are likely to result in a more rapid transit though the gastrointestinal tract than grains with low NDF. Thus, even when the intrinsic digestibility of a grain is greater, increases in passage rate may reduce the time the grain is in contact with intestinal amylases and reduce digestion relative to low NDF, low ADC grains. Wilfart et al (Wilfart et al., 2007) found that increasing amounts of added wheat bran, which contains high amounts of NDF, in wheat- and barley- based diets, significantly decreased transit time in the SI. The Arapiles sample with an ADC even higher than the Janz sample, has a low IDE:FDE of only 0.55. Both Janz and Arapiles also had higher ADF of 5.6 and 11.8% and hydration capacities (related to swellable fibre) of 92 and 110% respectively (Table 1). This is a strong indication that grains with higher NDF may not achieve higher IDE:FDE or digestion of available substrates in the SI.

There is probably an optimum level of NDF at which higher ADC of grain digestion gives maximum NDF adjusted IDE or IDE:FDE. This optimum is suggested in Figure 3 where NDF adjusted IDE is maximised with grains when NDF is approximately 15%. The combination of Figures 2 and 3 suggest there are strong interactions between 1) increasing enzyme diffusion rate and rate of digestion and 2) NDF affecting rate of digesta passage in the SI and extent of grain digestion in the SI.

4. Application of Research

This research has demonstrated that amylase diffusion rates can be obtained for all of a wide range of grain samples, including damaged grains. The method therefore allows a robust single number assessment of the intrinsic starch digestibility of a grain sample. Project 4B-122 Final Report provides a number of examples from animal trials of how this impacts directly on feed efficiency and less directly on feed intake.

Knowing the amylase diffusion coefficient of a grain sample allows calculation of the extent of digestion of any given particle size from that sample (Equations 1 and 2). If it is assumed that secreted amylase levels are not limiting, and if a passage time in the small intestine is specified, the extent of digestion of any particular particle size for a grain sample of known amylase diffusion coefficient can be estimated. However, the asymptotic nature of digestion progress curves (Fig 1A) means that there can be a long (equivalent small intestine passage) time between 95% cf 99% or 99.9% digestion, limiting the usefulness of predictions. However, what is clear is that for the same particle size, grains with lower amylase diffusion coefficient will be less digested. This is indeed found for sorghum cf wheat (Project 4B 122 Final Report), leading to the conclusion that the particle size cut-off for sorghum needs to be lower than for wheat (or barley) to ensure essentially complete small intestinal digestion. From previous experiments (Al-Rabadi et al, 2017), results from Project 4B-122, and calculations based on equations 1 and 2, it is likely that these cut-offs are in the range 0.8-1.0 mm for wheat/barley and 0.6-0.8 mm for sorghum, for both mash and pelleted forms. Further studies with multi-pass milling of grains to isolate sufficient quantities of specific size fractions would be needed to test these predictions in animal trials.

Although amylase diffusion coefficient provides a very useful characterization of grain materials, its measurement involves a lengthy and demanding laboratory process (Fig 1). For further utilization, it would be a great advantage to be able to predict the diffusion coefficient directly, particularly if NIR technology could be used. We therefore evaluated the potential for this, as described below.

Feasibility of NIR (near infrared) technology for measuring amylase diffusion rates in field or mill samples.
Sixteen whole grain samples (100g each) (Table 1), in total, from wheat, barley and sorghum were scanned with an NIR scanner to obtain NIR spectra. The NIR scans were conducted using a Bruker MPA with a spectral range of 10000 cm to 4000 cm (750 nm to 2500 nm).
The grains were then hammer-milled using 2mm sieve and scanned again to obtain NIR spectra of the ground grains. Enzyme diffusion rates (Table 3) of the sixteen grain samples were measured as described above. Calibrations (Table 4) were developed using Partial least Square algorithms with the measured enzyme diffusion rates.
The NIR calibration results indicate that ground samples provide a better predictive model (with n=16 samples; Table 4) compared with whole grain samples. However, with grain samples with an ADC (apparent amylase diffusion

coefficient) of less than 0.4 mm^2/h, slightly better predictions were achieved (n=12 samples; Table 4). A statistic used by NIR users is the **R**atio of standard error of **P**rediction against the standard **D**eviation of the chemical assay (RPD), which indicates the possible application of the model. The standard error of prediction is the standard error for cross validation (SECV) (Table 4). Where the RPD is less than 2, the model would be best for predicting enzyme diffusion rates in grains with

Table 4. NIR calibration, with statistical indicators of reliability, derived from the NIR spectra of whole and ground grains.

Calibration	N	[1]ADC (Mean) mm2/h	Std dev (ADC)	RSQ (CAL)	[2]SEC	Factors	RSQ (CV)	SECV**	RPD
ground grain	16	0.035	0.02	0.7344	0.01	3	0.316	0.017	1.2
whole grain	16	0.035	0.02	0.9659	0.003	7	0.44	0.015	1.3
*ground grain <0.04	12	0.024	0.006	0.8107	0.003	2	0.689	0.004	1.5
*whole grain < 0.04	12	0.024	0.006	0.9236	0.002	4	0.544	0.004	1.5

[1]apparent amylase diffusion coefficient, a measure of enzyme diffusion rate (EDR); [2] standard error of calibration; [3]standard error for cross validation; *grains with ADC below 0.04 mm^2/h.

higher values compared with grains with low diffusion rates. An RPD greater than 3 allows the user to rank individual samples, and where the RPD is greater than 3 then the predicted values are more quantitative. Based on the calibration developed with a relatively small number of samples, the standard deviation remains reasonably high compared to the SEP (SECV) (Table 4), which is based on the standard error of the method.

Figure 3 and 4 show the scatter plots of reference values (X axis) compared to the predicted values (Y axis) for ground samples and whole grains samples respectively. The blue dots represent grain sample calibrations, and the red dots represent the internal cross validation predictions. The ground samples had closer predicted values to the matching calibration samples (Figure 3) compared to the whole grain predictions. Since ground grain provide larger surface area to volume ratio, the dispersion and penetration of the NIR radiation per unit weight of ground grain samples is greater compared with whole grain samples. In addition, lesser scattering of light through the tightly packed ground material than whole grains, provides more data on the absorbance of the NIR light. As a result, the ground sample calibrations require fewer factors in building the models (Table 4). Furthermore, the structural characteristics of grain-influencing parameters such as EDR/ADC are more exposed to the vibration effects near infrared light source. Therefore, ground grains are more suitable for NIR studies than whole grains, which allow limited penetration of the transmitted NIR light.

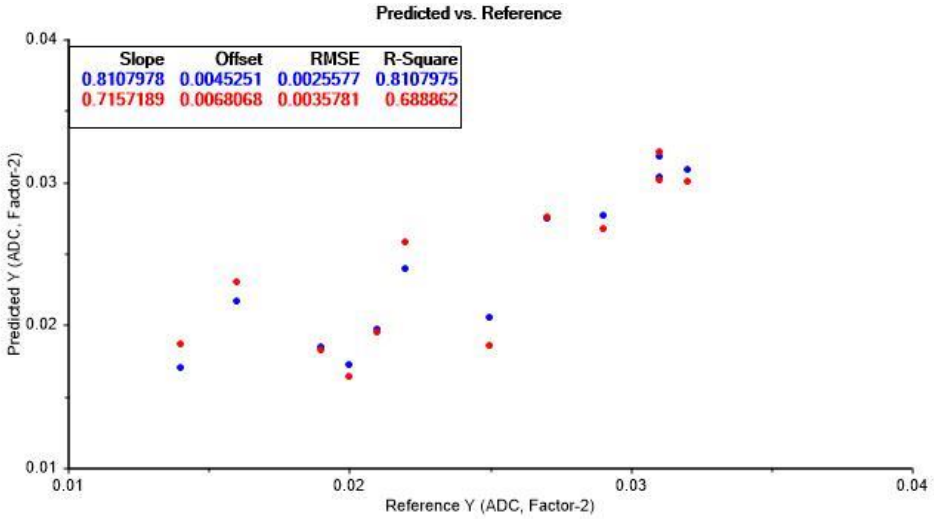

Figure 3. Scatter plot of reference vs predicted values for ground grain. Blue dots are grain sample calibrations, red dots are predicted ADC values from the best-fit calibration.

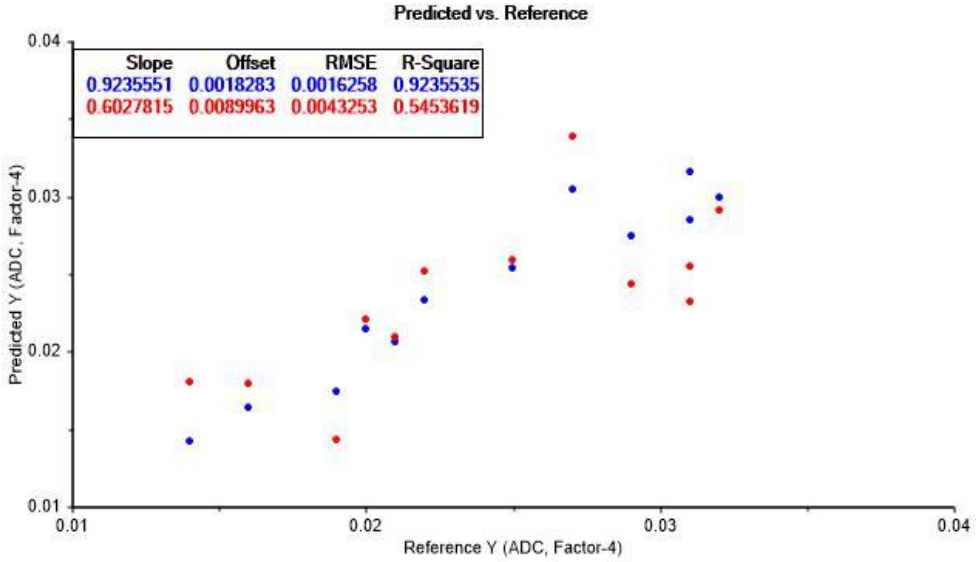

Figure 4. Scatter plot of reference vs predicted values for whole grain. Blue dots are grain sample calibrations, red dots are predicted ADC values from the best-fit calibration.

Interestingly, grain type had no effect on the predicted enzyme diffusion rates. This suggests that the calibration has a potential to be applied across at least these three grain types (wheat, barley and sorghum) without requiring separate calibrations for individual grain types. The whole grain calibration may have been more affected by the different grain types. The findings suggest that NIR holds a potential to accurately predict grain enzyme diffusion rates, provided that the calibrations are built based on a larger data set.

Currently (Black, 2016), NIR calibrations based on enzyme diffusion rate of grains are being considered to predict grain digestibility to improve feed efficiency in different animals.

5. Conclusion

In summary, it has been shown that (a) each of the three grains studied (wheat, barley, sorghum) exhibited a clear inverse square dependence of rate coefficients for amylase-catalysed digestion of starch on milled grain particle sizes, and that the determination of ADC is a robust method for assessing amylase diffusion rate for these three grains; (b) sorghum has a lower ADC compared to wheat and barley for samples grown under standard conditions; (c) major differences in grains (such as sprouting, germination and immaturity) result in different ADC values that can be related to in vivo digestibility, and (d) there is an optimum level of fibre (measured as NDF) of between 10 and 20% that results in maximal starch digestibility in the pig small intestine. Based on the findings that immature grains with high fibre have higher ADC and lower IDE:FDE, it can be concluded that the true potential for efficient feed utilisation depends not only on a fast grain starch digestion rate, but also the rate of passage of digesta, so that residence time in the SI is long enough for grain fractions to be digested completely.

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6. Limitations/Risks

A main limitation to the use of amylase diffusion coefficients as a predictive characteristic of feed grains is the non-routine nature of its measurement. Further work would be needed to integrate grain characterization with diffusion coefficient measurement and production animal performance. This is all possible, but will require more feeding trials to develop a NIR calibration with amylase diffusion coefficient that can be directly linked with feed performance.

A second limitation is that precise particle size cut-offs for complete small intestinal digestion of grains with defined diffusion coefficients are imprecise. This is because there is a slow (asymptotic) progression of digestion when it nears completion e.g. at greater than 95% digestion. For a given small intestine passage rate, it is possible to calculate the predicted particle size that results in e.g. 95, 99, or 99.9% digestion, but this varies widely depending on the target digestion level. Further trials with specific grain size fractions (e.g. from multi-pass milling) would be required to determine the relevant digestion extent for maximizing feed conversion efficiency.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

- The particle size distribution of milled grains should be routinely assessed
- For efficient use in feeds, it is predicted that milled wheat/barley should not contain particles greater than 0.8-1.0 mm. Multi-pass milling is one method of achieving this without generating fines (Al-Rabadi et al, 2017)
- For sorghum, the particle size cut-off is predicted to lower at about 0.6-0.8 mm
- Further work should be carried out to generate a NIR calibration for amylase diffusion coefficient and link this to particle size specifications for maximal feed efficiency from grains.

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Appendices

Appendix 1:

Table A1. Description of grains used in the experiment with growing conditions or post-harvest treatment.

Grain Type	Grain Variety	Grain ID	Growing condition or post-harvest treatment
Wheat	Wollaroi	1772	Normal growth, but naturally sprouted before harvest
	Janz	1809	Dry prior to harvest and grains screened through 2.2 mm sieve
	H45	1841	Normal growing season and grains screened to be > 2.2 mm
	Red Wheat	1876	A red wheat variety grown under good conditions
	Currawong	1906	Grown under normal conditions
Barley	Skiff	3814	Grown under normal conditions
	Arapiles	3828	Experienced a frost prior to harvest that reduced grain maturity
	Grout	3869	Irrigated during the growing season
	Wyalong	3875	Grown under normal conditions
	Fitzroy	3879	Sprayed with glyphosate six weeks after flowering that reduced grain maturity
	Schooner	3904	Grown under normal conditions
Sorghum	Waxy Isoline	7710	A breeding line of sorghum selected for high amylopectin:amylose ratio – waxy gene
	Normal Isoline	7711	The same breeding line as 7710, but not selected for the waxy gene
	Boomer	7812	Grown under normal conditions
	MR Maxi	7855	A midge-resistant cultivar grown under normal conditions
	ICSV400	7870	A breeding line grown under normal conditions