Subtilisin protease increases digestible energy in Sorghum and wheat based diets

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By

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Executive Summary

The majority of Australian vegetable protein meals and meat meals are significantly less digestible than imported soybean protein. The international demand and supply of American Soybean meal is increasingly volatile, hence the need for quality locally produced protein sources for pig diets. Specific exogenous proteases have the potential to significantly increase the amino acid availability of lower quality protein meals like meat meal and legumes such as peas and lupins.

Two experiments were designed to evaluate whether a specific Subtilisin protease was able to significantly increase the ileal digestibility of vegetable and animal proteins. Experiment 1 used an *in vitro* technique to measure the effects of the Subtilisin protease on the digestibility and rate of digestion of 15 samples of legumes, oilseed meals, meat meal, cereals and cereal by-products. The experiment showed that the canola and soybean meals exhibited the highest protein digestibility and sorghum protein had the lowest digestibility. There was a significant effect of protease on protein digestibility and rate of digestion, although the response was the higher with proteins of lower digestibility. There was no effect of protease on soybean and canola meals (eg. protein of high digestibility). The increased rate of protein digestibility was also greater on protein meals with low digestibility, and in fact there was almost a significant interaction (P=0.0524) as the protease tended to reduce the rate of digestion in ingredients with higher protein digestibility (particularly canola and soybean). It was hypothesised that the effects of protease would be the lowest in diets containing soybean, canola and the cereal wheat, and that sorghum based diets with peas and meat meal would be the most responsive to exogenous protease.

The second experiment was designed to test this hypothesis. A 2 x 2 x 2 factorial array was designed to measure the ileal digestibility of amino acids and energy of experimental diets in digestibility crates using male pigs between 25 and 45 kg live weight. The factors were cereal type (wheat or sorghum), protein source (soybean/canola meal or peas/meat meal), and protease (0 and 4000 units/g). The protein digestibilities were significantly higher in soybean and canola protein sources and in the wheat based diets. The difference in protein digestibilities also coincided with substantial differences in ileal and faecal energy, with again the soybean and canola meal protein producing higher ileal and faecal digestible energy (DE) and the wheat based diets exhibited superior faecal DE compared to sorghum.

The protease had no direct effect on increasing protein digestibility in the protein sources or the two cereals, however there were tends towards the protease having a greater effect on the pea and meat meal based diets and the diets containing wheat. Therefore, part of the hypothesis was not supported, there being no effect on amino acid and total protein digestibility in sorghum diets. The protease did, however, significantly increase the faecal DE across all treatments with the largest effect in diets containing soybean and canola protein and sorghum, which is opposite to the protein digestibility effects. The protease increased the ileal DE of the sorghum diets containing soybean and canola by 1.05 MJ/kg (P=0.025). The corresponding increase in faecal DE was 0.61 MJ/kg.

The protease was much more consistent at increasing the energy of all the diets, compared to the inconsistency of the enzyme on protein digestibility. The average increase in faecal DE was 0.35 MJ/kg which equates to $8.75/tonne if the cost of 1 MJ of DE is $25/tonne. At a cost of 2.50/tonne, the enzyme provides a payback of 3:1 to 4:1. And although the amino acid or protein uplift was not consistent, further gains would be made on diets containing inferior protein digestibility.

Finally, an adjunct experiment was run in combination with the second study 2 and was designed to compare Titanium Oxide against Celite (acid insoluble ash) as digestibility markers. The two markers were found to produce similar standard errors, with the

Titanium Oxide producing slightly lower standard errors for the ileal digestibility coefficients. Celite produced numerically better standard errors in the faecal digestibility coefficients. The advantage of Titanium Oxide is that it is cheaper to add, costs less to analyse and the procedure is considerably more rapid.

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1. Introduction

The majority of Australian vegetable protein meals and meat meal are less digestible than imported soybean protein. The international demand and supply of American Soybean meal is becoming increasingly volatile, hence the need for quality locally produced protein sources for pig diets. In past research, the Subtilisin protease has had no significant influence on the digestibility of soybean meal, however research at Auburn University by Professor Ed Moran has demonstrated Substilisin to significantly improve protein digestibility of meat meal in broilers. The protease has ability to digest insoluble protein by hydrolysing hydrophobic bonds. The digestibility of cysteine is substantially lower than other amino acids, due to the amino acid being part of such hydrophobic proteins. Threonine digestibility is also low, and lysine digestibility can vary considerably.

Recent evidence also shows, even if the protein quality is high in imported soybean, the non-starch carbohydrate or fibre fraction is fermented very quickly in the pig hindgut, and high levels cause scours and increase the proliferation of enteric pathogens, especially *Lawsonia* spp.

The main objective was to investigate if the Subtilisin protease can significantly improve Australian grown or produced protein meals. The same protease has been shown to hydrolyse the main indigestible protein in sorghum (Kafirin). Protein meals such as meat meal, canola (solvent and expeller), sunflower and cottonseed meal, and the legumes field peas and lupins generally have inferior essential amino acid availability compared to soybean. If effective the protease would reduce the reliance off imported soybean, and address the outputs for sub-program 4B of Pork CRC.

The project used *in vitro* protein digestibility analysis developed by the University of Queensland and as positive responses were observed, further work was conducted using the Melbourne using digestibility crates. Other objectives within this project were to produce more recent ileal digestibility values for protein and essential amino acids of locally produced protein meals and to assess if there is a difference in diet digestibility between expeller and solvent process canola meal.

This project continues work commenced under Project 2H-103 of the CRC for an Internationally Competitive Pork Industry ("CRC1") and reflects the Project Details as originally presented in that project, amended to remove completed Milestones and Budget items already paid/contributed or no longer required under Project 2H-103.

Australia currently imports in excess of 700,000 tonnes of soybean meal annually for animal feeding. The availability of canola meal is increasing as Australian crushing plants expand and new plants come into production, this being driven by increasing canola oil consumption for both food and biofuels. Other vegetable and animal protein meals are not as cost effective due to their lower essential amino acid digestibility, compared to soybean. Other locally produced protein meals such as field peas and meat meal could be value added by enzymes like protease to increase their value and take the emphasis off imported soybean meal.

This project has the potential to significantly reduce the cost of protein supplements for Australian pig producers.

2. Methodology

Introduction (Experiment 1)

Protein is a key component of feed, and when digested, it contributes to nutrients and energy. Protein digestibility varies with source, cultivar or variety and processing. In view of the different types of ingredients used in feed manufacture, it is important to quantify and understand protein digestibility of common feed ingredients. Protein digestibility can be assessed by *in-vivo* and *in-vitro* techniques. *In-vitro* protein digestion is fast and more commonly used, particularly to screen a large number of samples.
In this report, samples with a wide range of composition, obtained from a commercial partner, were analysed, and their in-vitro protein digestibility is discussed. Moreover, the effects of treating the samples with a commercial protease enzyme on in-vitro protein digestibility of the samples were investigated.

2.1. Materials (Part 1)

Fifteen samples, described in Table 1, were received and were stored refrigerated until analysed.

Table 1 - Information on the samples invstigated

Date	Product	Supplier	No.
--	Canola Meal	Riverland oil seed	SS0012467
--	Field Peas	--	12489
2009-2-1	Lupins	--	84717
2010-9-3	Meat Meal	John Dee (Warwick)	890010339
2010-8-31	Meat Meal 4749	THR	4749
--	Meat Meal Pakenham	--	SS0012507
--	Millrun	Manildra	SS0012493
2010-9-3	Mung Bean	Champion Seeds	890010330
2010-8-30	Sorghum	River City Contract	890010286
2010-8-30	Soya Meal 4733	AWH	4733
--	Soya bean meal 48%	Viterra	SS0012470
2010-8-30	Sunflower Meal	Cargill Narrabri	890010287
--	White Sorghum	--	--
--	Canola meal Numurkah	Riverland expeller	--
--	Canola meal Millicent	Riverland expeller	--

The information on the commercial protease enzyme received is given below:
Avizyme 1510 Protease, 4881422173, 1 kg, Finland, 40,000 units/g, Genencor International, B.V. Archimedesweg 30, 2333 CN Leiden, Netherlands

2.2. Methods

2.2.1. *Sample preparation*

Upon receipt, the samples were ground with a coffee grinder (Sunbeam, EM0400, Lord Street, Botany NSW 2019), and sieved using a 1 mm sieve, prior to analysis.

2.2.2. *Moisture content analysis*

Moisture content was determined after drying overnight in a vacuum oven (<100mHg, 13.3kPa) at 70^{o}C. The moisture content was used to calculate all the reported parameters on dry basis.

2.2.3. *Protein content analysis*

The total nitrogen content was determined in a LECO CNS 2000 combustion analyser, where a known quantity (about 150mg) of representative sample was burnt with pure

oxygen at 1100°C which volatilizes all forms of nitrogen. The various nitrogen oxides produced (NO, NO_2) were reduced to nitrogen (N_2) gas by passing a small aliquot of the collected gas through a copper catalyst column heated to 700°C. All other gases except the carrier helium gas were scrubbed from the aliquot prior to passage through a thermal conductivity cell, producing a change in the output voltage of the cell which was related back to the response from the Ethylenediaminetetraacetic acid (EDTA) standard used to calibrate the instrument (Press, 1992). A factor of 6.25 was used to compute the protein value.

Protease treatment

According to the client, the commercial protease enzyme was applied at a concentration of 250 ppm per sample. Because of the density and viscosity of the enzyme, a stock solution was prepared at a concentration of150 µg/mL, from which appropriate volumes were mixed with the samples. The weights and volumes of the samples, stock solution and MilliQ water used are summarised in Table 2. The samples were rehydrated for about 1 hr in a reciprocating (85 rpm) water bath at 37°C in the protease enzyme solution, which was adjusted to pH ≈ 8.0 with HCl and/or NaOH before in-vitro protein digestion.

***In-vitro* protein digestion**

An appropriate weight of each sample, calculated using Eqn. [1], was dispersed in 10 mL of either distilled water (No Protease) or protease enzyme solution (Protease) to give 6.25 mg protein/mL (Table 2). A multi-enzyme solution (10mL) was prepared, containing 16mg trypsin (Type IX, bovine pancreas), 31 mg chymotrypsin (type II, bovine pancreas) and 13 mg bacterial protease (Type XIV, *Streptomyces griseus*); the enzymes were obtained from Sigma-Aldrich (Castle Hill NSW 1765). The pH of the multi-enzyme solution was also adjusted to ≈ 8.0 with HCl and/or NaOH. One milliliter (1 mL) of the multi-enzyme solution was added to each sample suspension after rehydration, and the pH was recorded automatically every 5 s, over 15 min. This procedure is essential for the pH drop method of Hsu *et al.* (1977) for determining protein degradation. Casein (Sigma-Aldrich, C7078) was run as the standard, and a conversion factor was calculated assuming complete (100%) digestion of casein. *In-vitro* protein digestibility was defined relative to casein digestion.

Weight of sample = (6.25 x 10 x 100)/P, where P = protein content (g/100 g solids)

Table 2 - Weights and volumes of samples and protease enzyme solution

Sample	Protein (g/100g Solids)		Weight of sample (mg)[1]	Weight of protease (µg)[2]	Volume of stock solution* (µL)[3]	Volume of water (mL)[4]	Equivalent ppm (enzyme:sample)[5]
Canola Meal	42.3	C[#]	147.75	36.9	246	9.754	250
Canola Millicent	36.0	D	173.38	43.3	289	9.711	250
Canola Numurkah	37.3	D	167.75	41.9	280	9.720	250
Field Peas	28.7	E	217.50	54.4	363	9.637	250
Lupins	35.2	D	177.64	44.4	296	9.704	250
Meat Meal	54.8	B	101.72	25.4	170	9.830	250
Meat Meal 4749	61.4	A	106.91	26.7	178	9.822	250
Meat Meal Pakenham	58.5	A B	114.00	28.5	190	9.810	250
Millrun	22.5	F	277.94	69.5	463	9.537	250
Mung Bean	29.3	E	213.54	53.4	356	9.644	250
Sorghum	14.5	G	431.46	107.9	719	9.281	250
Soya Meal 4733	55.9	B	111.76	27.9	186	9.814	250
Soya bean meal 48%	55.6	B	112.47	28.1	187	9.813	250
Sunflower Meal	37.0	D	168.70	42.2	281	9.719	250
White Sorghum	12.9	G	484.94	121.2	808	9.192	250

1 = (6.25 x 10 x 100)/Protein

2 = 250 x 10-6 x Weight of sample x 1000

*Stock enzyme solution = 150 µg/mL

3 = 1000 x Weight of protease/150

4 = 10 - (Volume of stock enzyme solution/1000)

5 = 1000 x Weight of protease /Weight of sample

[#]Values with the same letters are not significantly ($p>0.05$) different. This applies to all tables and figures where they appear

Hsu *et al.* (1977) proposed the equivalent *in-vitro* protein digestibility (IVPD) as:

$\mathrm{IVPD} = 210.46 - 18.10\ \mathrm{pH}_{10\ \mathrm{min.}}$, where $\mathrm{pH}_{10\ \mathrm{min.}}$ = pH at the 10th minute. Eqn.[2] can be rearranged as in Eqn.[3] using the change in pH within 10 min.

$\mathrm{IVPD} = 65.66 + 18.10\ \Delta\mathrm{pH}$, where ΔpH = $\mathrm{pH}_{10\ \mathrm{min.}}$ - pH_o, pH_o = the starting pH, which is about 8.0.

2.2.4. *Statistical analysis*

Preliminary studies in our laboratory revealed that the plot of pH against time (protein digestograms) can be described using a first-order kinetic. A modified first-kinetic model was developed in terms of pH as shown in Eqn[4]:

$\mathrm{pH_t} = \mathrm{pH_0} + \mathrm{pH_{\infty-0}}\left(1 - \exp(-\mathrm{Kt})\right)$, where $\mathrm{pH_t}$= pH at time t, $\mathrm{pH_0}$= pH at time t = 0, $\mathrm{pH_\infty}$= ($\mathrm{pH_o}$ + $\mathrm{pH_{\infty-o}}$) pH at time = ∞, the minimum pH; K = rate constant ($\mathrm{min^{-1}}$).

Using Eqn.[4], $\mathrm{pH_o}$ and $\mathrm{pH_{10\ min.}}$ were obtained, from which ΔpH was calculated and used in Eqn.[3] to calculate IVPD.

Wherever possible, the samples were randomized before analysis, which was done in duplicate. Statistical analysis of the data was performed by the General Linear Model (GLM) in Minitab(R) version16 using a significance level of 5% ($p<0.05$).

The Microsoft Excel Solver® was used to compute the parameters of the modified first-order kinetic model (Eq. [4]) by minimising the sums of squares of residuals (SSQ) with constraints that $\mathrm{pH_0} \leq$ 8 and $\mathrm{pH_\infty} \geq 0$ following the procedures in Mahasukhonthachat et al. (2010).

Materials and methods (Experiment 2)

The hypothesis was that protease enzyme will have a greater beneficial effect for sorghum than wheat based diets and also for diets with meat meal and field peas compared to soybean and canola meal.

The sub-aim of this experiment was to determine if titanium oxide can be used instead of acid insoluble ash as a marker. All diets contained both markers.

Treatments:

The experiment consisted of 4 diets with and without subtilism (added protease enzymes).

Diet Number	Main Ingredients	Protease Enzyme
1	sorghum, meat meal, field peas	no
2	sorghum, soybean, canola meal	no
3	wheat, meat meal, field peas	no
4	wheat, soybean, canola meal	no
5	sorghum, meat meal, field peas	yes
6	sorghum, soybean, canola meal	yes
7	wheat, meat meal, field peas	yes
8	wheat, soybean, canola meal	yes

The grains/ingredients were pelleted by Donald Nicholson (mill manager) and staff at the University of Sydney, Camden campus. The diets were pelleted in accordance with the partially replicated pelleting design below, thus the 8 diets were labelled and fed to pigs as 12 batches of pelleted feed.

Where a diet was pelleted in two batches, each batch was fed to half the number of pigs compared to diets with only one pellet batch. In this way the number of times each "diet" was fed was approximately equal.

Number of replicates required:

At least 5 replicates per diet

Physical restriction to number of replicates:

The amount of feed produced, the number of cages/pigs

Physical Dimensions: (description)

Number of cages: 15

Number of rows: 4

Number of columns: 2

Protocol

Animal Protocol:

A total of 15 pigs were used in this experiment. Within the run there were three feeding/sampling periods where, 14 Pure bred Large White male pigs (approx. 35kgs) were fed the 8 different diets for a period of 7 days. One pig was always on hand as a "spare" pig and fed a standard grower diet unless needed

Diet Composition:

The diets consisted of different combinations of wheat, sorghum, soybean, canola meal, meat meal and field peas with or without the protease enzyme. All diets contained 2% Celite and 0.3% titanium oxide.

Diets were prepared with the following percentage of ingredients

Table 3 - Percentage of ingredients in each diet.

ingredient	DIET 1	DIET 2	DIET 3	DIET 4	DIET 5	DIET 6	DIET 7	DIET 8
SORGHUM 9.0%	67.7	67.7			67.67	67.67		
WHEAT 11%			68.7	68.7			68.67	68.67
PEAS	10		10.09		10		10.09	
CANOLA MEAL 34%		7		8.3		7		8.3
SOYABEANMEAL-48%		12		9		12		9
MEATMEAL 52%	7.5		7.3		7.5		7.3	
MILLMIX	3.75	6.61	3	6.46	3.75	6.61	3	6.46
SUNFLOWERMEAL-31%	7.5		6.5		7.5		6.5	
SUNFLOWER OIL		0.6	1	1.7		0.6	1	1.7
SALT	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
LYSINE-HCL	0.52	0.44	0.45	0.42	0.52	0.44	0.45	0.42
LIMESTONE		1		0.9		1		0.9
DL-METHIONINE	0.12	0.09	0.07	0.06	0.12	0.09	0.07	0.06
DICALPHOS		1.7		1.6		1.7		1.6
TRYPTOPHAN H/A	0.02		0.01		0.02		0.01	
THREONINE	0.19	0.16	0.18	0.16	0.19	0.16	0.18	0.16
GROWER PMX	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
CELITE	2	2	2	2	2	2	2	2
TITANIUM OXIDE	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
PROTEASE ENZYME					0.03	0.03	0.03	0.03

Diets were steam pelleted at 85ºC using a 4mm die in batches according to the pelleting design above.

Feeding Protocol:

Diets were fed at 2.5 × the maintenance level.

Calculation: (2.5 × (0.5 MJ DE kg^{-1} body weight$^{0.75}$)/diet DE)

Note: Digestible energy was formulated to contain 14MJ DE/Kg.

Daily rations were halved and fed at 8 h intervals and water provided *ad libitum* via nipple drinkers.

Period/Run Protocol

- Fourteen pure bred large white male pigs (approximately 35 kgs) are selected from the commercial herd.
- Pigs are housed in purpose built individual pens, in an environmentally controlled room at 24 ºC.
- The room also has bar heaters for providing warmth to pigs immediately after surgery in the cooler/cold periods.
- Pigs receive water *ad libitum* and are fed a commercial diet.
- Pigs have a settling in period of 7 days where they acclimatise and become accustomed to being handled and are monitored for signs of distress or disease before being chosen to participate in the trial.
- Of the 14 pigs, 11 are selected for surgery (insertion of an ileal cannula), this allows for 10 cannulated pigs for the trial with 1 reserve (as a contingency for cannula breakages or any mortalities).
- Pigs have 7 – 8 days post operative care, after which the trial and reserve pigs are selected and feeding of the trial diets will commence. Selection is generally based on temperament, well being and feeding behaviour.
- Trial Diets will be fed for 7 days, (commencing on a Friday) with a 5 day period of diet adaptation, prior to animals being transferred into metabolic crates for digesta and faecal sample collection on days 6 and 7 (Wednesday and Thursday).
- Pigs continue to be fed trial diets whilst in the crates.

- Pigs stay in the metabolic crates overnight.
- Pigs are then weighed and returned to individual pens and started on new diets.
- Weighing each pig at the completion of their time in the crates allows for the calculation of the amount that each animal will receive for the following week. (2.5 times maintenance is used).
- The process is repeated over five weeks (i.e. collections 1 – 5).

Metabolic room

10 metabolic crates are housed in an environmentally controlled room.

Once the pig is in the crate the bung is removed from the cannular.

A length of clear plastic tubing is attached to the cannular to facilitate the collection of ileal digesta samples.

The tubing with an attached bulldog clip at the end is placed in a bucket with ice to keep the digest cool before samples are removed hourly and stored frozen.

A bulldog clip is attached to the end of the tubing so the digest does not leak.

Measurements recorded for database:
The chemical analysis measurements made on the freeze dried faecal samples, pelleted diet and grain samples were as follows:

- *Grain samples* – dry matter (DM), acid insoluble ash (A), nitrogen (N), starch (S), gross energy (GE), calcium (Ca), phosphorus (p), non-starch polysaccharides (NSP).
- *Pelleted diet samples* – dry matter (DM), acid insoluble ash (AIA), gross energy (GE).
- *Faecal & ileal samples (freeze dried)* - dry matter (DM), acid insoluble ash (AIA), gross energy (GE).
- *Note: AIA of the freeze dried faecal samples was conducted in accordance with a replicated experimental design to produce statistically adjusted values for use in the determination of DE below.*
- A physical analysis was also be conducted on the raw grain samples including: 1000 grain weight, specific weight, screening %

Formula for derived responses:

Faecal DE – All diets included about 2% Acid insoluble ash (AIA) marker, thus as the diet was digested the concentration of the AIA marker increases. The AIA marker was used to determine digestibility with the following measurements needed to calculate faecal digestibility. All values are initially calculated on an as fed basis and then converted to a dry matter basis.

Digestible energy (DE) values were calculated using the following equations:

$$\text{faecal DE of the diet (ar)} = \left(\text{pellet GE (dm)} - \frac{\text{(faecal GE(dm) x pellet AIA(dm)}}{\text{faecal AIA(dm)}}\right) \times \frac{\text{pellet dm\%}}{100}$$

$$\text{ileal DE of the diet (ar)} = \left(\text{pellet GE (dm)} - \frac{\text{(ileal GE(dm) x pellet AIA(dm)}}{\text{ileal AIA(dm)}}\right) \times \frac{\text{pellet dm\%}}{100}$$

$$\text{ingredient (grain) faecal DE (ar)} = \frac{\text{(diet faecal DE(ar) - DE other diet components)}}{\text{\% grain in diet}}$$

$$\text{ingredient (grain) ileal DE (ar)} = \frac{\text{(diet faecal DE(ar) - DE other diet components)}}{\text{\% grain in diet}}$$

$$\text{ingredient (grain) faecal DE (dm)} = \frac{\text{ingredient (grain) faecal DE (ar)}}{\text{pellet dry matter}}$$

$$\text{ingredient (grain) ileal DE (dm)} = \frac{\text{ingredient (grain) ileal DE (ar)}}{\text{pellet dry matter}}$$

ar = as received basis
dm = dry matter basis
DE of the other diet components = 0.387
% grain in diet = 0.94505

3. Results

Experiment 1

Figure 1 shows the protein digestograms of typical samples. It can be observed that the pH reduced with time of digestion as amino acids were produced during proteolysis. With or without the protease treatment, the modified first-order kinetic model was suitable in describing the protein digestograms of the samples (Fig. 1) with r^2 = 0.882 – 0.990 (n = 180).

Table 4 shows the *in-vitro* protein digestion parameters of the samples with and without the protease. Irrespective of the samples, protein digestion was more than 80%. As expected, the protein contents of the samples were significantly ($p<0.05$) different (Table 2), so also was the protein digestibility of the samples (Table 4). The canola and soybean meals were generally the most digestible, while the sorghums were the least digestible (Fig. 2A). It appears that samples with high protein content were generally more digestible. Irrespective of the source, the samples were less digestible than casein.

Generally, treating the samples with the commercial protease significantly ($p<0.05$) enhanced *in-vitro* protein digestibility (Fig. 2A-2B and Table 5). This shows the suitability of the protease for a wide range of samples. Although there was no significant interaction between the protease and type of ingredient, the protease generally increased the protein digestibility of most ingredients, expect for the canola and soybean meals.

There was a significant difference (P=0.011) in the rate of protein digestion (K) between different raw ingredients, the meat meals generally had a high rate of digestion compared to the other tested ingredients, while the white sorghum exhibited a higher rate than the red sorghum (Fig. 2C). The commercial protease significantly ($p<0.05$) enhanced the rate of protein digestion in the samples (Fig. 2C-2D). There was almost a significant interactions between the protease and the tested ingredients (P=0.0524), where the protease increased the rate of protein digestion of all ingredients expect for the canola meals, soybean meals and one sample of meat meal.

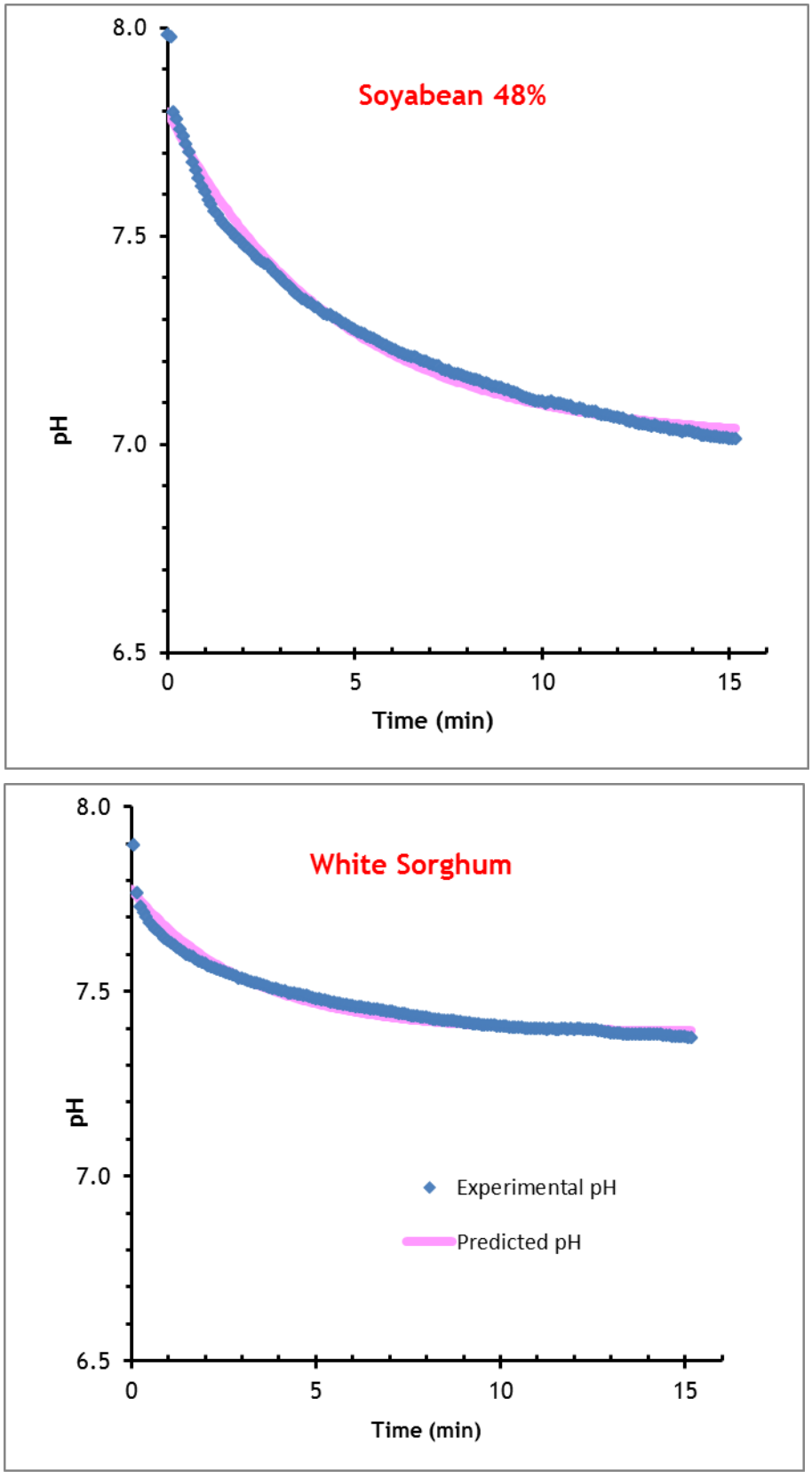

Figure 1 - Typical protein digestograms of the samples

Table 4 - *In-vitro* protein digestion parameters of the samples with (+) and without (-) the protease treatment*

Sample	*Protease**	*IVPD#*	*K x 10^{-3}*
Canola Meal	+	92.8 A B C D	329.4
Canola Meal	-	92.4 A B C D	260.1
Canola Millicent	+	95.5 A B	306.7
Canola Millicent	-	96.1 A	386.2
Canola Numurkah	+	95.9 A	341.8
Canola Numurkah	-	95.4 A B	371.8
Field Peas	+	90.4 A B C D	377.6
Field Peas	-	89.4 A B C D	243.3
Lupins	+	92.9 A B C D	297.4
Lupins	-	91.5 A B C D	280.3
Meat Meal	+	93.4 A B C D	436.2
Meat Meal	-	91.1 A B C D	287.8
Meat Meal Pakenham	+	91.4 A B C D	352
Meat Meal Pakenham	-	86.6 C D	275.4
Meat Meal4749	+	93.3 A B C D	366.8
Meat Meal4749	-	91.1 A B C D	399.2
Millrun	+	90.8 A B C D	341
Millrun	-	89.8 A B C D	293.2
Mung Bean	+	90.7 A B C D	400.1
Mung Bean	-	86.6 C D	253.7
Sorghum	+	87.5 A B C D	323.9
Sorghum	-	84.8 D	242.1
Soya Meal4733	+	93.3 A B C D	246.3
Soya Meal4733	-	94.1 A B C	249.5
Soyabean meal 48%	+	94.1 A B C	289.4
Soyabean meal 48%	-	94.4 A B C	302.1
Sunflower Meal	+	89.6 A B C D	320.9
Sunflower Meal	-	87.2 B C D	277.6
White Sorghum	+	87.5 A B C D	403.1
White Sorghum	-	85.0 D	328.9
SEM		0.460	9.67
Mean		91.2	319.5

*Protease (+) = Treated with the commercial protease, Protease (-) = Not treated with the commercial protease

#In-vitro Protein Digestibility; Casein was used as the reference and =100. Rows with the same letter are not significantly different (P>0.05)

Table 5 - *In-vitro* protein digestion parameters of the combined samples with and without the protease treatment*

Protease	IVPD#	K x 10^{-3}
-	90.3	342.2

+	92.0	296.8
P Values		
Ingredient type (I)	<0.001	0.494
Protease (P)	0.011	0.010
I x P	0.576	.0524

*Protease (+) = Treated with the commercial protease, Protease (-) = Not treated with the commercial protease
#In-vitro Protein Digestibility; Casein was used as the reference and =100.

Conclusion (experiment 1)

Protein digestion is dependent on materials. Within a material, the amount of protein, the manufacturing process and variety/cultivar can affect protein digestibility. The materials investigated have different amino acid profiles, and knowing their protein digestibility potential can guide the choice of feed formulations involving them for specific amino acid digestibility. Importantly, treating the samples with the commercial protease enzyme (Avizyme 1510) was found beneficial, and could lead to better utilisation of the protein component for maximum delivery to animals (e.g. pigs).

Experiment 2: The ileal digestibility experiment

Results

Comparison of Titanium Oxide and Acid insoluble ash as digestibility markers

The ileal and faecal gross energy digestibilities determined using Titanium Oxide and acid insoluble ash are shown, in Tables 6 and 7 respectively. Overall the standard errors for the ileal and faecal gross energy digestibilities were similar for both markers with no significant difference between the means. The Titanium Oxide produced a numerically lower standard error for the ileal digestibility and the acid insoluble ash produced a numerically lower variation in faecal digestibility.

Although the standard errors were similar between the two markers, some of the P values for treatment effects and their interactions were different, particularly for the faecal digestibility results. The acid insoluble ash produced considerably more P values below the 5% probability level in the faecal digestibility figures between the treatments and their interactions.

For this reason, the gross energy ileal digestibility results were analysed and discussed further based on Titanium Oxide.

Influence of grain, protein source and exogenous protease on ileal DE and Faecal DE

There were no differences in ileal DE between the two cereal grains, however the wheat based diets did exhibit a higher faecal DE (P=0.001) than sorghum.

Even though all diets were formulated to 14.0 MJ/kg DE, the soybean and canola protein source produced a superior ileal DE of between 0.82 and 0.88 MJ/kg higher than the Pea and meat meal protein mix. The faecal DE was 0.41 MJ/kg higher for the Soybean-Canola protein (P=0.001).

There was no significant influence of protease on ileal DE. The enzyme, however, improved (P=0.002) faecal DE, and the effect was more pronounced in the sorghum based diets (P=0.049), and when the soybean/canola protein source was present (P=0.037). Other significant interactions were between grain and protein source, where the addition of peas and meat meal proteins produced an inferior faecal DE in sorghum diets, but there was little influence in the wheat based diets (P=0.002).

There was also a three way interaction on ileal DE (P=0.025; Table 6 AIA marker) where protease produced a large improvement in ileal DE on the sorghum diet, containing soybean/canola meal proteins, but had no effect on the wheat plus soybean/canola diets. However, the protease increased ileal DE by 0.42 MJ/kg in the wheat plus pea/meat meal diets.

Ileal digestibility of protein and amino acids

Table 8 shows the wheat based diets and the soybean/canola protein sources produced the highest protein digestibilities by 5.2% (P=0.004) and 3.2% (P=0.033), respectively. A little over half of the individual amino acids were significantly influenced by either grain or protein source. The amino acids that exhibited higher digestibilities in wheat, compared to sorghum, were leucine (P=0.023), serine (P=0.006), glycine (P=0.001), glutamic acid (P=0.001) and proline (0.001).

It is noteworthy that proline digestibility in sorghum diets was 22% lower than in wheat diets. Glycine digestibility was also 22% lower in sorghum compared to wheat (Table 9).

The amino acids that were found to be higher in ileal digestibility in the soybean and canola diets were the branch chain amino acids (valine, isoleucine and leucine), serine (P=0.007), aspartic acid (P=0.001), glutamic acid (P=0.002) and alanine (P=0.002).

Protease supplementation did not have a direct influence on ileal digestibility of individual amino acids or protein.

Discussion

Comparison of Titanium Oxide and Acid insoluble ash as digestibility markers

Generally, both the Celite acid insoluble ash and Titanium Oxide markers produced very similar digestibility values for ileal and faecal DE (P>0.10). Interestingly the standard errors were lower for the Titanium Oxide maker for the ileal DE means (0.47 versus 0.51) and the Celite produced a lower standard error for the faecal DE means (0.17 versus 0.21), which in turn produced lower P values.

Titanium Oxide has a significantly more rapid analysis time frame, and generally the analytical errors are lower. The other advantage for using the Titanium Oxide marker is only 0.3% is required, compared to 2% for Celite, which means much less dilution of dietary nutrients and less potential confounding effects as no extra oil or synthetic amino acids are required to balance for the dilution effect.

The Celite acid insoluble ash marker did produce considerably lower P values for the Faecal DE, and therefore may be a preference if only faecal collection and subsequent digestibilities are required, and it seems to reduce the variation more than using Titanium Oxide. Titanium Oxide, on the other hand, seems to be the preference if only analysing for ileal digestibility of nutrients due to the lower variation and the ease (and cheaper) of analysis.

The magnitude of enzyme response was greater for ileal digestibility and faecal digestibility for the Celite marker, almost by two fold. The reason for the larger enzyme response when the acid insoluble ash marker is used is difficult to explain and further work is required to see whether different digestibility markers are better than others to measure the influence of enzymes on nutrient/energy digestion, particularly on faecal digestibilities.

Influence of protein source, grain and protease supplementation on the digestibility of protein and energy.

The diets containing soybean and canola meal exhibited significantly higher protein digestibility than the pea and meat meal diets, and although there was no significant interaction (P=0.075), the protease had a greater numerical improvement in the pea/meat meal diets compared to the soybean/canola diets, which supported the vitro-results in

experiment 1. The present study again supports past experiments where the protease had little to no effect on the soybean and canola diets due to the digestibility already being very high. In contrary to the in-vitro studies in by Finn et al. (2005) the protease had no effect on increasing the amino acid digestibility of sorghum, and in fact the enzyme had a greater numerical effect on the wheat based diets. Moreover, there was a trend for an interaction between protein type, grain and protease (P=0.091), where the protease was more effective on wheat based diets, containing peas and meat meal, which is in contrary to the hypothesis, where it was thought that the protease would be more effective on Sorghum diets containing peas and meat meal.

The combination of the soybean and canola protein source was superior in both energy and protein digestibility compared to the diets with the pea and meat meal protein sources, even though the diets were formulated to the same digestible amino acid levels. The 3% lower faecal DE in the peas/meat meal diets was similar to the 3.2% lower ileal digestibility of protein. The superior digestibility of soybean and canola meal were shown in experiment 1, whereas the protein digestibility of meat meal varied depending on source, all meat meal protein digestibilities were well below that of the oilseed meals.

The ileal digestibility of protein in the sorghum based diets was inferior to that of wheat. There was some variation between different amino acids. All but lysine had a lower digestibility value in sorghum based diets. The biggest standout and concern is the very low digestibility of proline and glycine in the sorghum diets. In low protein diets both of these amino acids can be classified as semi-essential, particularly when Arginine and Glutamine are marginal and gut health is compromised (Ball, unpublished). Proline digestibility was only 53.2% in the sorghum/pea-meat meal diet, and averaged 59.6% across all diets, whereas proline digestibility was 76.9% across all wheat diets. Glycine digestibility averaged 57.1% across all sorghum diets as opposed to 68% across all wheat diets. The Kafirin storage proteins are high in proline and glutamic acid, and the Kafirin protein is generally poorly digested in sorghum whereas wheat proteins are relatively soluble and more available for enzymatic breakdown. Another reason for the very low Proline digestibility is the poly-phenols in sorghum preferentially bind to proline and proteins rich in proline. Wheat is very low in poly-phenols. This may also be the reason for the inability of the protease to significantly increase ileal amino acid and protein digestibility, due to the poly-phenol bound proteins being somewhat protected from the Substilisin protease which preferentially hydrolyses proteins rich in proline.

All the experimental diets were formulated to 14 MJ/kg DE (faecal) and to 0.70 grams of available lysine per MJ DE (0.98% available lysine). The measured DE was found to be very similar at 14.1 MJ/kg. The sorghum diets were analysed to be 2% lower or 0.26 MJ/kg in faecal DE (P=0.001), even though the ileal DE was similar to that of the wheat diets. This suggests large intestinal fermentation was able to extract more energy out of the wheat diets. The protease was more effective on sorghum based diets, increasing the faecal DE by 0.64 MJ/kg compared to 0.20 MJ/kg in wheat. So the unexpected result was a significant increase in energy gained by protease supplementation, rather than ileal amino acid or protein digestibility. Although there was no effect of protease on ileal DE, most of the energy gain was produced by more efficient energy digestion/fermentation in the large intestine. This suggests the protease was providing better assess to nutritional substrates for colon microbes to ferment and produce more volatile fatty acids.

Table 6 - Effects of grain, protein source and exogenous protease on Faecal DE using Acid Insoluble Ash as the digestibility marker

Grain	Protein source	Protease	Ileal DE (MJ/kg)	SE	Faecal DE (MJ/kg)	SE
Sorghum	SOY + Canola Meal	-	11.53	0.59	13.96	0.20
Sorghum	SOY + Canola Meal	+	12.68	0.43	14.57	0.15
Sorghum	Peas + Meat Meal	-	11.00	0.43	13.54	0.15
Sorghum	Peas + Meat Meal	+	11.20	0.59	13.81	0.20
Wheat	SOY + Canola Meal	-	11.80	0.59	14.20	0.20

Grain	Protein source	Protease	Ileal DE (MJ/kg)	SE	Faecal DE (MJ/kg)	SE
Wheat	SOY + Canola Meal	+	11.72	0.43	14.46	0.15
Wheat	Peas + Meat Meal	-	10.94	0.43	14.07	0.15
Wheat	Peas + Meat Meal	+	11.36	0.59	14.20	0.20
Mean			11.51	0.51	14.10	0.17
Main Effects						
Grain						
Sorghum			11.59		13.97	
Wheat			11.44		14.23	
Protein						
Soy+CSM			11.93		14.31	
Peas and MM			11.11		13.90	
Protease						
No enzyme			11.30		13.93	
Enzyme			11.74		14.28	
P values						
Grain (G)			0.416		0.001	
Protein (P)			0.001		0.001	
Enzyme (E)			0.225		0.002	
G x P			0.218		0.002	
G x E			0.148		0.049	
P x E			0.344		0.037	
G x P x E			0.025		0.355	

Table 7 - Effects of grain, protein source and exogenous protease on Ileal and faecal DE using Titanium Oxide as the digestibility marker

Grain	Protein source	Protease	Ileal DE (MJ/kg)	SE	Faecal DE (MJ/kg)	SE
Sorghum	SOY + Canola Meal	-	11.87	0.54	14.23	0.21
Sorghum	SOY + Canola Meal	+	12.18	0.41	14.40	0.20
Sorghum	Peas + Meat Meal	-	10.97	0.41	13.97	0.20
Sorghum	Peas + Meat Meal	+	11.19	0.54	13.60	0.21
Wheat	SOY + Canola Meal	-	12.32	0.54	14.49	0.21
Wheat	SOY + Canola Meal	+	12.25	0.41	14.65	0.20
Wheat	Peas + Meat Meal	-	10.90	0.41	14.07	0.20
Wheat	Peas + Meat Meal	+	11.21	0.54	13.83	0.21
Mean			11.74	0.47	14.15	0.21
Main Effects						
Grain						
Sorghum			11.73		14.04	
Wheat			11.67		14.27	
Protein						
Soy+CSM			12.14		14.36	
Peas and MM			11.26		13.95	
Protease						
No enzyme			11.60		14.10	
Enzyme			11.80		14.21	
P values						
Grain (G)			0.736		0.009	
Protein (P)			0.001		0.001	
Enzyme (E)			0.343		0.265	
G x P			0.040		0.164	
G x E			0.320		0.771	
P x E			0.287		0.001	
G x P x E			0.060		0.547	

Table 8 - The effects protease supplementation and diet grain type and protein sources on the apparent ileal digestibility coefficients of the essential amino acids and protein (using the Titanium marker)

Grain	Protein source	Protease	Lysine	Threonine	Methionine	Valine	Isoleucine	Leucine	Phenyl	Tyrosine	Protein
Sorghum	SOY + CSM	-	87.5	78.8	90.8	78.3	79.6	81.5	0.809	73.6	79.7
Sorghum	SOY + CSM	+	86.8	78.1	91.2	77.6	79.3	80.6	0.814	75.8	77.9
Sorghum	Peas + MM	-	85.0	76.1	90.5	74.0	75.9	77.7	0.784	74.4	74.1
Sorghum	Peas + MM	+	85.6	76.3	90.2	74.2	76.6	78.5	0.792	76.0	75.6
Wheat	SOY + CSM	-	82.2	75.7	88.3	78.2	80.3	82.7	0.818	77.8	80.6
Wheat	SOY + CSM	+	84.3	79.4	91.5	81.1	83.3	84.6	0.847	81.4	82.5
Wheat	Peas + MM	-	82.8	75.2	89.5	74.7	77.5	79.8	0.806	73.6	78.7
Wheat	Peas + MM	+	86.0	80.0	90.2	79.1	82.1	82.8	0.844	75.4	82.1
Main Effects											
Grain											
Sorghum			86.2	77.3	90.7	0.760	77.8	79.6[a]	80.0	75.0	76.8[a]
Wheat			83.8	77.6	89.9	0.783	80.8	82.5[b]	82.9	77.0	81.0[b]
Protein											
Soy+CSM			85.2	78.0	90.5	78.8	80.6	82.4[a]	82.2	77.1	80.2[a]
Peas and MM			84.9	76.9	90.1	75.5	78.0	79.7[b]	80.7	74.9	77.6[b]
Protease											
No enzyme			84.4	76.5	89.8	76.3	78.3	80.4	80.4	74.9	78.3
Enzyme			85.7	78.4	90.8	78.0	80.3	81.6	82.4	77.2	79.5
P values											
Grain (G)			NS	NS	NS	NS	0.086	0.023	NS	NS	0.004
Protein (P)			NS	NS	NS	0.022	0.059	0.018	NS	NS	0.033
Enzyme (E)			NS	NS	NS	NS	NS	NS	NS	NS	NS
G x P			NS	NS	NS	NS	NS	NS	NS	NS	NS
G x E			NS	NS	NS	NS	NS	NS	NS	NS	NS
P x E			NS	NS	NS	NS	NS	NS	NS	NS	NS
G x P x E			NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 9 - Effects of diet grain and protein sources on the apparent ileal digestibility coefficients of the non- essential amino acids (using the Titanium marker)

Grain	Protein source	Protease	Histidine	Serine	Arginine	Glycine	Aspartic acid	Glutamic acid	Alanine	Proline
Sorghum	SOY + CSM	-	80.0	76.4	85.0	61.3	78.3	85.2	78.3	66.5
Sorghum	SOY + CSM	+	80.1	75.7	84.6	53.5	76.9	83.5	76.4	57.1
Sorghum	Peas + MM	-	75.2	69.9	84.2	55.4	70.4	78.3	73.7	53.2
Sorghum	Peas + MM	+	75.7	70.8	84.5	58.3	71.3	79.3	75.3	61.6
Wheat	SOY + CSM	-	79.9	78.3	83.0	68.3	77.6	86.5	76.5	75.6
Wheat	SOY + CSM	+	83.3	81.2	85.2	68.0	79.2	87.3	78.6	76.5
Wheat	Peas + MM	-	77.7	74.4	82.9	66.3	70.4	86.9	73.5	74.4
Wheat	Peas + MM	+	82.5	78.1	86.1	69.4	75.2	88.4	76.8	81.1
Main Effects										
Grain										
Sorghum			77.7	73.2[a]	84.6	57.1[a]	74.3	81.6[a]	75.9	59.6[a]
Wheat			80.9	78.0[b]	84.3	68.0[b]	75.6	87.3[b]	76.4	76.9[b]
Protein										
Soy+CSM			80.8	77.9[a]	84.4	62.8	78.0[a]	85.6[b]	77.4[a]	68.9
Peas and MM			77.8	73.3[b]	84.4	62.3	71.8[b]	83.2[a]	74.8[b]	67.6
Protease										
No enzyme			78.2	74.8	83.7	62.8	74.2	84.2	75.5	67.4
Enzyme			80.4	76.4	85.1	62.3	75.6	84.6	76.8	69.1
P values										
Grain (G)			0.115	0.006	NS	0.001	NS	0.001	NS	0.001
Protein (P)			0.071	0.007	NS	NS	0.001	0.003	0.002	NS
Enzyme (E)			NS	NS	NS	NS	NS	NS	NS	NS
G x P			NS	NS	NS	NS	NS	NS	NS	NS
G x E			NS	NS	NS	NS	NS	0.001	0.087	NS
P x E			NS	NS	NS	NS	NS	NS	NS	NS
G x P x E			NS	NS	NS	0.09	NS	NS	NS	0.065

4. Application of Research

The *in vivo* benefits of protease supplementation were to increase the faecal energy digestibility rather than ileal protein digestibility initially shown in the in-vitro study. The protease may reduce the energy required to digest protein, as well as maximize the ability for microbes in the large intestine to digest or ferment nutrients that were previously unavailable.

The present experiment again highlights that sorghum digestibility is less consistent in pigs, and generally lower compared to wheat. The sorghum based diets were significantly lower in protein and faecal gross energy digestibility, even though they were formulated to contain similar DE and essential amino acid levels. Enzymes and heat processing techniques are essential to maximize sorghum digestibility, as shown in the current study as well as previous Pork CRC experiments.

Protease supplementation significantly improved the energy digestibility in sorghum diets, but had no effect on protein digestibility, which was unexpected. The benefits of added protease are mostly likely due to improving starch digestibility, as well as maximizing energy digestibility (fermentation) in the large intestine.

Due to the lower protein digestibility of sorghum, it is suggested that an emphasis be placed on higher quality protein sources to be used in combination with sorghum. The very low proline and glycine digestibilities are unusual and support the need for higher quality proteins to be used in sorghum diets.

The Titanium Oxide marker is equal to or better than Celite when measuring ileal digestibility and should be considered to replace the acid insoluble ash measurement due to less variation, lower cost and more rapid analysis. The Celite marker, however, looks to be the preferred option when analyzing for fecal Digestibility of nutrient.

5. Conclusion

The protease had no direct effect on increasing protein digestibility in the protein sources or the two cereals however, there were trends towards the protease having a greater effect on the pea and meat meal based diets and the diets containing wheat. Therefore part of the hypothesis was no supported, with no effect of amino acid and total protein digestibility in sorghum diets. The protease did, however significantly increase the faecal DE across all treatments, although the largest effect was on diets containing soybean and canola protein and sorghum, which is the opposite effect of protein digestibility effects. The protease increased the ileal DE of the sorghum diets containing soybean and canola by 1.05 MJ/kg. The corresponding increase in faecal DE was 0.61 MJ/kg.

The protease was much more consistent at increasing the energy of all the dietary treatments, compared to the inconsistency of the enzyme on protein digestibility.

The two markers, Celite and Titanium Oxide were found to produce similar standard errors, with the Titanium Oxide producing slightly lower standard errors in the ileal digestibility coefficients and the Celite produced numerically better standard errors in the faecal digestibility coefficients. The advantage of Titanium

Oxide is it is cheaper to add, a lower cost to analyse for and a more rapid procedure.

6. Limitations/Risks

The protease supplementation costs between $2.20 and $2.50 per tonne of feed. The average 0.35 MJ/kg increase in DE of the experimental diets (average of 0.44 for the sorghum diets), is worth approximately $8.75 per tonne if the cost of 1 MJ/kg DE is $25. Therefore the protease supplementation to improve DE only, is a 4:1 payback, and there maybe some small increases in protein digestibility as observed in experiment 1 (*in vitro*), although not significant in experiment 2.

There is very little risk of using Titanium Oxide instead of Celite, and the use of Titanium Oxide as a marker is well documented in scientific literature.

7. Recommendations

The use of protease in sorghum based diets to improve energy and protein digestibility is recommended, and will produce at least a 4:1 payback in energy (DE) yield, with some extra benefits of improved protein digestibility (supported by the in-vitro results in experiment 1).

The protein digestibility of sorghum based diets was observed to be inferior compared to wheat, and it is highly recommended that superior protein sources such as canola and soybean meal are used. The poor proline and glycine digestibility may impair young pig growth (higher requirement and may cause Arginine deficiency) and supports the past recommendations of restricting the use in diets for pigs under 25 kg.

The digestibility markers Celite and Titanium Oxide are similar, although Titanium Oxide maybe better when measuring ileal digestibility of nutrients, and Celite/acid insoluble ash markers better when measuring faecal digestibilities

8. References

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