MECHANISMS OF ACTION OF XYLANASE AND ITS COMMERCIAL APPLICATION IN PIG DIETS.

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ABSTRACT

Xylanase is an exogenous enzyme that is added to non-ruminants diets in order to break down and ameliorate the effects that non-starch polysaccharides (NSPs) have on feed digestibility. Dietary supplementary xylanase reduces viscosity of the digesta to improve feed digestibility and growth performance. Recently a study in broiler chickens (Singh et al., 2012) showed a significant increase in circulating serum peptide-YY (PYY) concentration ($p<0.001$) in response to xylanase supplementation. PYY is a satiety hormone that inhibits gastric motility, increasing digesta retention times in non-ruminants which has also been identified to occur in studies where xylanase has been supplemented. It is thought this could potentially be another mechanism by which xylanase exerts its beneficial effects on growth outcomes. Therefore the aim of the current study was to explore the effects that xylanase (16,000 BXU/kg diet, Econase XT, AB Vista, UK) supplementation, of a wheat-based feed, has on growth performance parameters and gut/metabolic hormones that are produced from the gastrointestinal tract in pigs, and if age at supplementation affects the efficacy of the enzyme.

In a preliminary trial a human multiplex assay (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA) was successfully used to measure porcine gut/metabolic hormones simultaneously, and the assay was hence used in subsequent trials. In the same trial it was determined that animals subjected to commercial

slaughter (S), which had an increased time to tissue sample collection (approximately 18 hours) and exposes carcasses to a 60°C water bath, did not affect the quality of RNA extracted from gut, pancreatic and liver tissue and subsequent relative abundance of gene transcripts over animals not subjected to this slaughter method (NS).

In two trials on weaned piglets, it was found that dietary xylanase supplementation at 0-2 weeks post-wean decreased glucose-dependent insulinotrophic peptide (GIP), glucagon like peptide-1 (GLP-1) and pancreatic polypeptide (PP) plasma concentrations ($p<0.05$) which resulted in a tendency for insulin and C-peptide ($p<0.08$) to be decreased in these animals when bloods were analysed at 6 weeks post-wean. Also at this time point PYY concentrations were found to be decreased ($p<0.01$) in animals fed diets supplemented with xylanase between 2 and 6 weeks post-wean. In a third trial, when blood parameters were measured at 13 weeks post-wean, leptin and ghrelin levels were higher in animals provided with the exogenous enzyme, supplemented feed at 0-2 weeks post-wean.

In conclusion, it was found growth performance in pigs fed a wheat-based diet supplemented with xylanase was not significantly improved at any age. However timing and/or duration of supplementation affected production of certain gut/metabolic hormones, in particular those produced in the pancreas or those that influence production of these

hormones, suggesting dietary xylanase supplementation fed to weaned pigs may affect pancreas function and development.

CONFERENCE PRESENTATIONS

May K, Wiseman J, Brameld JM, Masey O'Neill HV, Parr T (2013) RNA degradation in tissues collected from pigs subjected to two different slaughtering methods. Advances in Animal Biosciences. 5:139.

May K, Brameld JM, Masey O'Neill HV, Wiseman J, Parr T, O'Sullivan SE (2015) Measuring metabolic hormones in pig blood using a human bead-based assay. Advances in Animal Biosciences. 6: 245.

May K, Brameld JM, Masey O'Neill HV, Wiseman J, Parr T, O'Sullivan SE (2015) Xylanase supplementation in feed reduced incretin and PYY levels in piglets. In 2015 Conference, Joint Annual Meeting of ADSA and ASAS, Florida.

May K (2016) Exogenous enzyme use in the UK pig industry. In 2016 workshop, Sustainable Pig Production: the knowledge and technology exchange between UK and Thailand. Thailand

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LIST OF ABBREVIATIONS

ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
AgRP	Agouti-related Peptide
ARC	Arcuate Nucleus
CCK	Cholecystokinin
cDNA	Complementary Deoxyribonucleic Acid
Co	Control diet
CV	Coefficient of Variation
DPPIV	Dipeptidyl Peptidase-IV
Fa	Fasted
FCR	Feed Conversion Ratio (feed intake/weight gain)
Fe	Fed
FI	Feed Intake
g	G force
GHRL	Ghrelin
GIP	Glucose-dependent insulinotrophic peptide
GIT	Gastrointestinal Tract
GLP-1	Glucose like peptide-1
Nm	Nanometer
NPY	Neuropeptide Y
NS	Non-commercial Slaughter Method
NSP	Non Starch Polysaccharide
NTS	Neurotensin

RIN	RNA Integrity Number
RNA	Ribonucleic Acid
rpm	Revolutions per minute
S	Commercial Slaughter Method
SD	Standard Deviation
SEM	Standard Error of the Mean
sNSP	Soluble Non Starch Polysaccharide
P1	Period 1
P2	Period 2
POMC	Pro-opimelanocortin
PP	Pancreatic Polypeptide
PVN	Paraventricular Nucleus
PYY	Peptide YY
TMB	3,3’-5,5’-tetramethylbenzidine
V	Volts
XS	Xylanase supplemented diet

1. INTRODUCTION

In the current climate it is essential to direct research in the area of global food security, due to the enormity of providing food in a sustainable and environmentally friendly manner for a forecasted world population of 9 billion people in 2050. One area of interest that has the potential to help alleviate this issue is to improve the production efficiency of livestock. Exogenous enzymes added to compound diets have been suggested as one way of achieving this prospect.

Xylanase is one such example of a commercially used exogenous enzyme that is supplemented into the feed of non-ruminants to break down the non-starch polysaccharide (NSP) fraction of cereals commonly used in commercial compound feeds. Research in this area has mainly been directed into exploring the benefits, in terms of growth and other performance parameters, of supplementing this enzyme into pig and broiler chicken feeds. Studies have established that the addition of this enzyme (alone or as part of a cocktail) has the potential to increase average daily weight gain (ADWG) and improve feed conversion ratios (FCR) in both species.

The mechanisms by which xylanase facilitates these improvements have been suggested as; cell wall dissolution and a reduction in digesta viscosity. However it has been hypothesised that the enzyme has other mechanisms of action, such as acting as a prebiotic and influencing the

secretion of gut hormones involved in regulating the motility of the gastrointestinal tract (GIT). This thesis will mainly investigate the impact of xylanase on porcine gut hormones. Therefore the following literature review will succinctly address global food security and how it is impacted by livestock production. Also our current knowledge of the effect of xylanase on gut hormones that could potentially be affected when supplementing with this enzyme.

1.1. GLOBAL FOOD SECURITY

In 1996, at the World Food Summit, food security was defined as a situation where:

'…at the individual, household, national, regional and global levels [is achieved] when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life.' (FAO, 2016)

Currently global food security is a commonly discussed topic. This is due to the many stakeholders involved in the activity of providing food for the projected global population of 9 billion people in 2050 by essentially increasing our food production by 70-100% (Tomlinson, 2013). Increasing the production of food (cereal and animal based) creates many issues of its own including Land Use and Environmental Impact that are discussed below.

1.1.1. Land Use

Producing more food inevitably means either using more land for agriculture and production facilities, or increasing the yield from existing land used. This needs to be done without encroaching on land that has other uses and changing the ecosystem or decreasing biodiversity. The scenario that is most likely to occur is that higher yields of crops for food will need to be obtained from the same or less land (Godfray et al., 2010). Currently 38% of the Earth's surface is used for agriculture and recent expansion has mostly been in the tropics (Foley et al., 2011). This expansion has meant the loss of parts of rainforest which impacts the global climate and results in the loss of many species of plants and animals (Smith, 2013; Martinelli et al., 2010).

Of the land used for agriculture approximately 75% is solely used for raising animals (Foley et al., 2011). There is the option to expand into non-arable land (land deemed unsuitable for growing crops), however this is met with its own set of challenges as typically livestock that can be reared on this land are less productive (for instance this is the case for 'hill farming' in Scotland; Scottish National Heritage, 2017), hence it may not be a sustainable option in terms of increasing production and again could also impact on biodiversity. Therefore to increase the yield from agriculture to provide food for the growing population we need to reduce the amount of land used for raising animals by making production more efficient. This is paramount as there is also competition from other

industries to use land which makes ownership more costly (Godfray et al., 2010).

1.1.2. Environmental Impact

There is pressure to improve livestock's impact on the environment, in terms of nutrient excretion and methane production. This pressure is increasing due to the increasing number of livestock being produced, as seen in figure 1.1. In 2009 it was estimated that annually around 200-230 million tonnes of meat was being produced, and that by 2020 this needs to increase by 100 million tonnes with pork production needing to be doubled (Martinez et al., 2009).

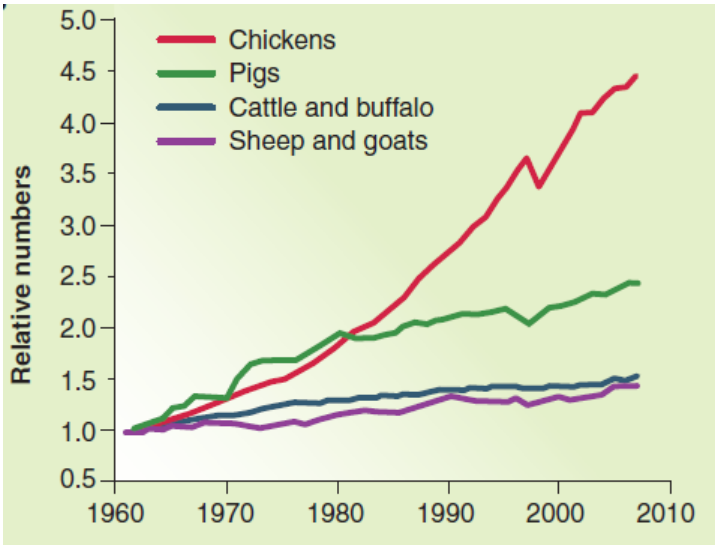


Figure 1.1 Global production of major livestock between 1960 and 2010. Adapted from Godfray et al., 2010.

One environmental issue raised from the intensification of animal production is nitrogen excretion in faeces and urine, which causes nitrification and acidification of water and soil. This in turn has effects on

the ecosystems of the area affected, resulting in a loss of fish and plant species (Milne, 2005). One study showed that in Europe pork production produces the second highest greenhouse gas emissions per kg of meat, which includes nitrous oxide and methane gases (Lesschen et al., 2011). Ammonia is released into the atmosphere via manure storage, spreading of manure over land and animal housing. Methane is also released from manure storage but also enteric production in ruminants. Ammonia is both a risk to the environment and the health of the animals and humans exposed to it due to its detrimental effects on lung health (Martinez et al., 2009). With the expected increases in animal production these environmental impacts, which are currently already trying to be reduced, will have a larger impact and methods to reduce them will need to implemented with greater force than being suggested currently.

1.2. EXOGENOUS ENZYMES

Exogenous enzymes (NSPases and phytases) are currently added to non-ruminant diets as both pigs and chickens are not as well adapted as ruminants in digesting the complex structural carbohydrates found in cereals, which is a problem as these make up the majority of the feed they are provided. The particular enzymes that are discussed here are used in production animal feeds as they have been found to be able to improve output parameters (e.g. FCR, weight gain and hence meat production) over animals not supplemented with the enzyme. Enzymes have been developed and used in non-ruminant diets since the 1980's (Choct, 2006) to target the NSP fraction of the cereals used in the feed and other anti-nutritional factors (e.g. phytate) that affect nutrient absorption. As well as to improve the digestibility of the feed, Bedford (2000) outlined three main reasons for the use of exogenous enzymes:

- Reduction in incidence of wet litter (chickens in particular).
- Decrease in the variation of nutrient quality of the feed.
- Diet costs are reduced.

There are also other benefits from using supplemental enzymes in poultry and pig diets, which will be discussed subsequently.

1.2.1. Exogenous enzymes and the environment

As well as reducing the incidence of wet litter, the addition of exogenous enzymes to feed can reduce the detrimental environmental impacts of animal waste. Atakora et al. (2011) reported that supplementation of phytase and xylanase into a wheat-based diet in growing finisher pigs

decreased the excretion of phosphorus (P), however they did not affect methane emissions. A study by Leek et al. (2007) showed that different diet preparations supplemented with a NSP degrading enzyme mixture have different effects on ammonia emissions in finisher pigs. When supplemented with a xylanase- β-glucanase combination pigs fed a wheat-based diet had decreased ammonia emissions from faecal samples, however in barley-based diets it increased the ammonia emissions. These results were also seen in a report by Garry et al. (2007a) in growing finisher pigs, however they saw no effect of enzyme supplementation in the barley-based diet on ammonia emissions. This result was substantiated by Garry et al. (2007b) in a similar study with only the wheat-based diet showing a decrease in ammonia emission. Ammonia emissions are potentially decreased when xylanase is included in feed as it has been shown to increase amino acid absorption (Cowieson & Ravindran, 2008), hence decreasing the amount of amino acid derived nitrogen available for conversion to ammonia and subsequently excreted.

1.2.2. Production efficiency effects when using exogenous enzymes

Another reason to use NSP-degrading enzymes is to increase the growth performance of the animal, this is achieved by the enzyme releasing extra nutrients to be utilised that would otherwise be inaccessible from the existing amount of feed. Bedford & Classen (1992) reported that, in broiler chicks fed a rye-based diet, inclusion of pentosanase (preparation

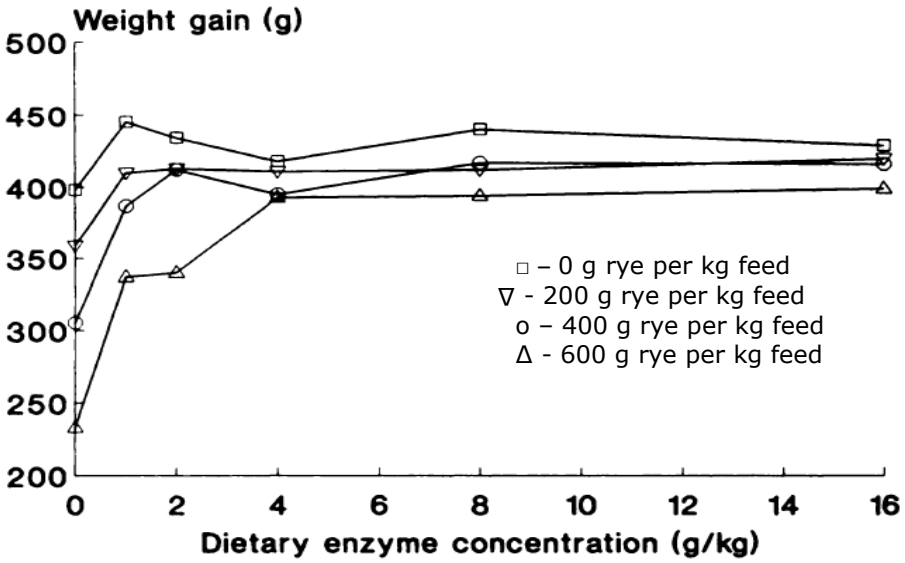


Figure 1.2 Weight gain of 19-day old broilers when fed diets with varying levels of rye (0 (□), 200 (▽), 400 (o) and 600 (Δ) g per kg total feed, each of these diets is depicted on the graph) and enzyme (0, 1, 2, 4, 8 and 16 g per kg of feed, supplemented in each diet containing varying rye levels). The increasing addition of pentosanase decreases the variability of weight gain between the groups containing different amounts of rye in their diets. Adapted from Bedford & Classen (1992).

of β-glucanase and xylanase) significantly improved weight gain and feed conversion efficiency (FCE). Their results on weight gain can be seen in figure 1.2. Since this early study there have been many other studies reporting on growth performance in response to exogenous enzyme supplementation. Barrera et al. (2004) reported that xylanase supplementation in wheat-based diets of growing pigs did improve weight gain but this was only a small increase. More significant improvements were seen in broilers on a wheat-based diet by Gao et al. (2008) where xylanase inclusion significantly increased weight gain and improved the FCR, $p<0.05$ and $p<0.01$ respectively. This was also previously found by Lázaro et al. (2003) in broilers fed a rye-based diet, weight gain, when supplemented with a combination of xylanase and β-glucanase,

increased significant to $p<0.001$, the FCR also improved ($p<0.001$). Cowieson & Ravindran (2008) also saw similar results when supplementing a maize-based diet in broilers with an enzyme mixture of xylanase, amylase and protease at a significance of $p<0.001$ for weight gain and $p<0.01$ for FCR. They also found that addition of this enzyme mixture increased the availability of amino acids and energy from the diet provided. Omogbenigun et al. (2004) found that in weaned pigs, fed a standard starter diet supplemented with an endogenous enzyme mixture, that compared to the control weight gain and FCR improved ($p=0.02$ and $p=0.01$ respectively). Furthermore, it was found that phosphorus digestibility in pigs fed with the enzyme supplemented diet was higher ($p=0.001$) and that they excreted less phosphorus ($p=0.04$) compared to the control.

Woyengo et al. (2008b) used a wheat-based diet in growing phase pigs to test performance response to supplementation with phytase and xylanase. However their findings contradicted those of the studies mentioned previously and reported that there were no significant improvements in growth performance when enzymes were included in the diet, but phosphorus and amino acid (AA) digestibilities did increase in response to phytase and xylanase supplementation, respectively. This is supported by Selle et al. (2009) who found inclusion of xylanase and phytase into a broiler wheat-based diet did not improve weight gain but improved AA digestibility. They also found that feed efficiency was significantly improved with exogenous enzyme inclusion, and the

responses they reported in conjunction with enzyme supplementation were greater when both enzymes were present than when individually supplemented with either one or the other suggesting a synergistic relationship. Another study by Woyengo et al. (2008a) does not support this synergistic interaction of the enzymes and did not report any positive response in performance to supplementation in broilers fed a wheat-based diet. Owusu-Asiedu et al. (2010) found no difference in weight gain in weanling pigs fed a wheat-barley-based diet supplemented with a xylanase and β-glucanase enzyme blend but did see improvements in feed efficiency when the dose of enzyme was at its highest in the study.

Finally, it has been suggested by studies into enzyme supplementation that by implementing these techniques (both phytases and NSPases) into feeding strategies it will enable reductions to be made in the production costs of rearing animals as lower quality and subsequently cheaper feedstuffs could be utilised in place of the current ingredients (Omogbenigun et al., 2004; Cowieson & Ravindran, 2008). In 2010 it was estimated that, in the global animal feed market, the use of these enzymes saves $3-5 billion annually (Cowieson et al. 2010). In pig production feed costs represent approximately 65-75% of total costs of production and the majority of this is on the grower-finisher feed, and so taking advantage of this technology is hugely beneficial to the industry (Mullan et al., 2011, as cited by Morel et al., 2012).

1.2.3. Variability of enzyme inclusion levels and performance outcomes

As discussed, the use of enzymes has been suggested to improve growth performance in non-ruminants. However the studies that have been presented here show a high variability in this response. This is detrimental to the producers of such enzymes as one of the selling points for using exogenous enzymes are that they decrease the variability of animal performance. Tables 1.1 and 1.2 summarise each study previously discussed detailing chicken and pig studies, respectively. It can clearly be discerned from comparing these studies that the level of inclusion of the enzyme does vary between each study and that the combination of enzymes varied as well; these are the two main factors that were consistently different between studies. This may provide reasoning for why the outcomes of the studies differed in regards to growth performance results. Some of the studies presented have both weight gain and feed efficiency improvements, however some have improvements in either one or the other or see no improvements at all. From these studies it is not discernible as to what the optimum level of inclusion is to see feed efficiency or weight gain consistently.

Table 1.1 Studies detailing the age, diet, level of enzyme inclusion and whether or not growth was increased in broilers under the dietary conditions outlined.

Study	Age/ d	Diet	Level of enzyme inclusion	Improved weight gain/feed efficiency?
Bedford & Classen (1992)	1-19	Rye-based	Pentosanase (xylanase 2150 U and β-glucanase 530 U) (g/kg): 0, 1, 2, 4, 8, 16	Yes/Yes
Gao et al. (2008)	7-49	Wheat-based	Xylanase, activity 1218 U of xylanase/g (g/kg): 0, 1	Yes/Yes
Selle et al. (2009)	1-21	Wheat-based	Xylanase (XU/kg): 0, 2000 Phytase (FTY/kg): 0, 500	No/Yes
Woyengo et al. (2008a)	1-23	Wheat-based	Xylanase, activity 4,000 XU/g (XU/kg): 0, 1,250, 2500 Phytase, activity 5,000 FTU/g (FTU/kg): 0, 250, 500	No/No
Lázaro et al. (2003)	4-25	Rye-based	Xylanase and β-glucanase combination, 864 IU/g and 858 IU/g respectively (ppm): 0, 500	Yes/Yes
Cowieson & Ravindran (2008)	1-21	Maize-based	Xylanase, amylase and protease combination, (U/kg): 0,0,0 & 300,400,4000 respectively	Yes/Yes

Table 1.2 Studies detailing the age, diet, level of enzyme inclusion and whether or not growth was increased in pigs under the dietary conditions outlined.

Study	Age	Diet	Level of enzyme inclusion	Improved weight gain/feed efficiency?
Barrera et al. (2004)	Grower (fed for 28 days)	Wheat-based	Xylanase, activity 0, 5,500, 11,000, 16,000 xylanase U/kg (XU/kg): 0, 5,500, 11,000, 16,000	Yes/No
Woyengo et al. (2008b)	Grower	Wheat-based	Xylanase (XU/kg): 0, 2,000, 4,000 Phytase (FTU/kg): 0, 250, 500	No/No
Omogbenigun et al. (2004)	25-53 days	Varying	Xylanase, glucanase, amylase, protease, invertase and phytase included at 250 U, 150 U, 0.001%, 0.0003%, 0.002% and 400 U /kg diet, respectively.	Yes/Yes
Owusu-Asiedu et al. (2010)	Piglets (fed for 6 weeks)	Wheat-barley-based	Xylanase and β-glucanase (g/MT): 0, 50, 100, 200	No/Yes

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1.2.4. Summary

Ensuring global food security presents many issues that need to be overcome in order to provide food for our ever growing population in a sustainable and environmentally friendly manner. Increasing food production means current issues are escalated, for example land use and pollution. It has been suggested here that introducing exogenous enzymes in non-ruminant diets can help alleviate the extent of these problems from these particular animal production systems. Ammonia production from manure has been found to be decreased when supplemented with NSPases, xylanase and β-glucanase, this may be due to the increase in amino acid digestibility when these enzymes are present in the diet.

Furthermore phytate digestibility increases with the addition of phytase to the feed, hence more phosphorus is available to the animal, meaning less is released into the environment increasing soil and water content of phosphorus, which is beneficial if the area is already being fertilised with phosphorus. By increasing the performance output of the animal by means which involve not increasing feed, land does not need to be acquired in order to provide more feed to increase animal production and hence can be utilised for other things, such as crops for human consumption. This may also mean that fewer animals have to be produced in order to meet demand for animal products as more output can be obtained, although numbers will still need to increase but hopefully not as drastically. Additionally costs of production will be

decreased in terms of feed costs as more nutrients can be utilised from the feed meaning less investment is needed in high energy feedstuffs and potentially supplements.

1.3. XYLANASE

Having detailed general benefits of using enzymes in non-ruminant diets and linked this to a current global issue this literature review will now focus on one particular exogenous enzyme, xylanase, and the research that has been undertaken in poultry and swine to detail its mechanisms of action.

Xylanase is an exogenous enzyme added to feeds containing cereal grains to improve the digestibility of the feed in non-ruminant production systems (Bedford, 2000) but is also used in other industries for different purposes (e.g. bleaching paper pulp, one process in the production of paper; Kamble and Jadhav, 2012). It is naturally produced by many species of microorganisms including bacteria, fungi and actinomycetes. For commercial purposes it is manufactured on large scale from microorganism strains that have a high capacity for producing the enzyme (Kamble and Jadhav, 2012).

Xylanase targets structural sugars called xylan (Collins et al., 2005) and arabinoxylan (Santana et al., 2011), these are hemicelluloses which make up a large proportion of the plant cell wall. Xylanase (E.C 3.2.1.8) endohydrolyses the β-1,4 glycosidic links of these pentose sugar-based polysaccharides (Collins et al., 2005).

1.3.1. Actions of xylanase

By breaking down the specified polysaccharides, xylanase improves the digestibility of feed. It is thought that this is achieved by cell wall dissolution and also in certain cereal based feeds (e.g. wheat) a reduction of digesta viscosity. There has also been evidence suggesting that prebiotic action occurs through the xylooligosaccharides produced from xylanases activity and that this has other benefits.

1.3.1.1. Viscosity of digesta

The hemicelluloses discussed, and in particular soluble arabinoxylan, create viscous solutions when consumed following partial dissolving in digesta, this causes some nutrients to become less available for digestion/absorption by the animal. It has been proposed xylanase activity reduces the viscosity of the feed in the GIT, this is supported firstly by Bedford & Classen (1992) who tested this in broilers fed a rye-based diet. They found that in the proximal and distal intestine viscosity of the digesta decreased with increasing inclusion of the enzyme supplementation, their results are shown in figure 1.3 which also includes the effects of viscosity on FCE (kg of weight gain per kg of feed consumed) and weight gain.

Lázaro et al. (2003) supported this conclusion, in broilers fed a rye-based diet, finding that in both 11- and 22-day old broilers intestinal viscosity was reduced when a xylanase and β-glucanase mixture was included in the diet ($p<0.001$), viscosity was measured from samples collected

between the end of the duodenum and the Meckel's diverticulum (in the distal ileum). Gao et al. (2008) also found that supplementation of xylanase into a wheat-based broiler diet decreased the viscosity of the digesta. This was significant in 21-day old birds in the proventriculus and jejunum ($p<0.05$) and in 49-day old birds in the colon ($p<0.05$) compared to broilers not supplemented with the enzyme. Woyengo et al. (2008a) saw decreases in digesta viscosity in broilers fed a wheat-based diet supplemented with phytase and xylanase also.

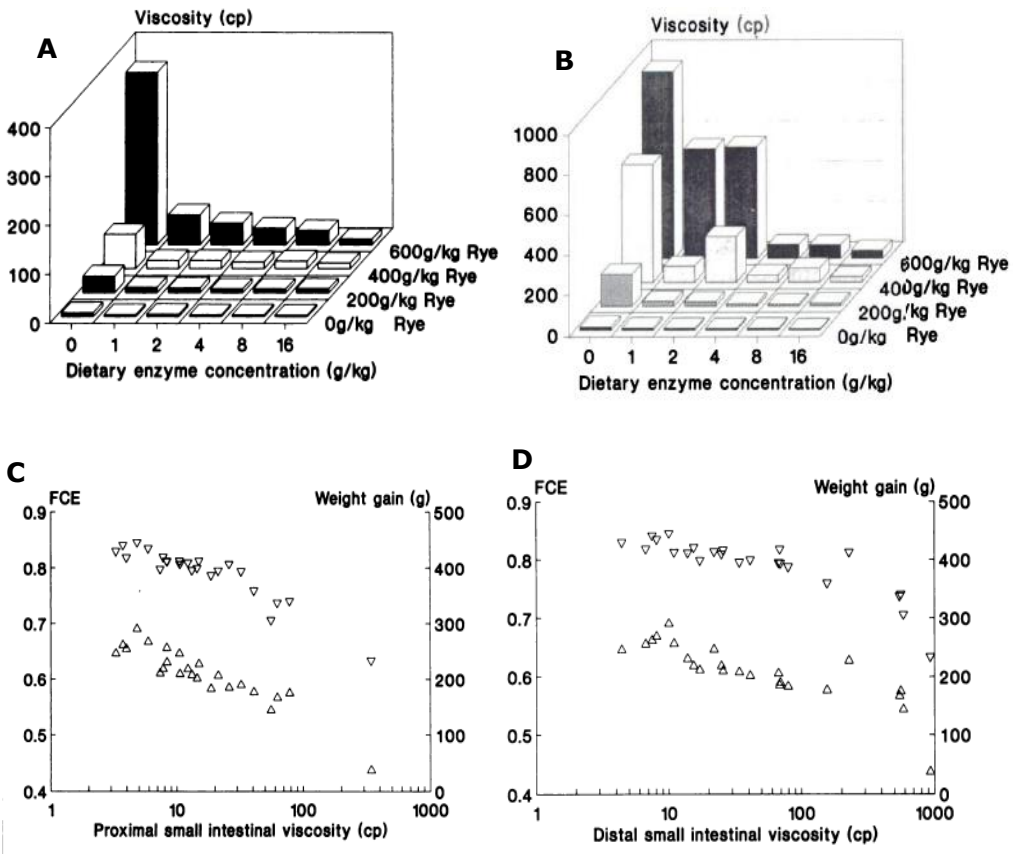


Figure 1.3 A and C show results obtained from the proximal intestine, and B and D show results from the distal intestine. A and B show how enzyme inclusion affected the viscosity (measured in centipoise (cp) where cp = 1/100 dyne second.cm^{-2}, using a digital viscometer) of the digesta in diets containing differing amounts of rye. In both parts of the intestine, even when small quantities of the enzyme were included, the viscosity decreased in each rye diet. C and D show how viscosity effects weight gain and FCE. In both parts of the intestine, but to a lesser extent in the distal small intestine, increased viscosity detrimentally effected weight gain and FCE. These results are from 19-day old broilers having

1.3.1.2. Cell wall dissolution

As improvements in growth and feed efficiency have been seen in both maize- and rye/wheat-based diets when supplemented with exogenous enzymes it is fair to assume that xylanase has more than one mode of action, as xylanase added to maize diets does not improve the viscosity of the digesta (Singh et al., 2012). This is due to the fact that maize contains lower concentrations of NSP's than wheat and rye. There have been a few suggestions as to how xylanase exerts its improved

performance effects. It was proposed that cell wall dissolution occurred, where the breakdown of the cell wall of the endosperm by exogenous enzymes releases nutrients such as protein and carbohydrates for digestion (Bedford & Schulze, 1998; Meng & Slominski, 2005). However the proficiency of this hypothesis has been debated as it has been seen that addition of enzymes into feed of non-ruminants improves the digestibility of fat more so than other components (due to digesta viscosity being reduced by inclusion of the exogenous enzyme, which in turn increases conjugated bile acid which can emulsify the dietary fat and ultimately increase its digestibility (Langhout et al., 1997 as cited by Tellez et al., 2014)) and, in the case of broilers, transit time is too short for this action to come into effect early enough for digestion/absorption of the trapped nutrients to occur, so it is not accepted that this action is fully explanatory of xylanases effects on digestibility (Bedford & Schulze, 1998; Singh et al., 2012).

1.3.1.3. Xylooligosaccharides as prebiotics

Another action that has been suggested is that the xylooligosaccharides produced from xylanases activity act as prebiotics. This entails them increasing the population of bacterium that are believed to have beneficial effects on the host, such as increasing the effectiveness of the host's immune system. The process involves the xylooligosaccharides being fermented in the large intestine by the bacterial colonies present producing short chain fatty acids (SCFA), which increases the acidity of the large intestine creating an environment where bacteria beneficial to

the host can thrive and growth of detrimental species is repressed (Blaut, 2002). Effects on the population size of certain species and total bacteria have been studied in both pigs and broilers.

Courtin et al. (2008) demonstrated the prebiotic effects of different preparations of oligosaccharides produced from the breakdown of xylan and arabinoxylan (xylooligosaccharides, XOS;, AXOS; soluble arabinoxylans, S-AX) in broilers. They examined the population of three types of bacteria (E*nterobacteriaceae*, *Lactobacilli*, and *Bifidobacteria*) from the caecum when the chicks were at one and two weeks of age. *Enterobacteriaceae* are considered to be harmful to the host, however *Lactobacilli* and *Bifidobacteria* are considered to be beneficial. Table 1.3 shows the results from each dietary trial on the population of each bacterium. No effects of dietary treatment were seen for *Enterobacteriaceae* or *Lactobacilli*, however the oligosaccharides did increase the population of *Bifidobacteria* compared to the control; XOS in the first week and AXOS and S-AX in the second week.

Xylanase supplementation was seen to decrease *Enterobacteriaceae* populations ($p<0.001$) though by Danicke et al. (1999) and also decrease total anaerobe population ($p<0.001$) in broilers at 16 days old. However although they took measurements of the microflora from each part of the GIT they stopped at the ileum and so did not include the colon or the caecum in their experiment. As most fermentation of oligosaccharides and hence prebiotic effects would be exerted in the last sections of the GIT their data may not be useful in commenting on the effects of xylanase

supplementation or its products on the different populations of microflora. In a study by Kiarie et al. (2007) addition of a multicarbohydrase enzyme, including xylanase, increased the population of lactobacilli significantly in the ileum of early-weaned pigs ($p=0.009$) fed a wheat/barley-based diet.

This being said there have also been studies where significant effects on the microbial population of the GIT have not been seen. Gao et al. (2008) demonstrated that in broilers chickens of 21 days of age there was no statistical difference in measured bacterial counts in the caecum when a multicarbohydrase enzyme mix was included in a wheat-based diet. Similarly, Singh et al. (2012) found that in broilers fed a maize-soy bean based diet that harmful bacteria to the host were not affected by xylanase supplementation when measured at 42 days of age. Finally, a study into the intestinal microflora in finisher pigs found that supplementing a multicarbohydrase enzyme into a barley-based diet increased the total count of *Lactobacilli* and *Bifidobacteria* ($p<0.01$ and $p<0.05$ respectively) in the caecum when the enzyme was present however it did not have the same effect when supplemented into an oat-base diet.

Table 1.3 Mean number of *Enterobacteriaceae*, *Lactobacilli* and *Bifidobacteria* (log CFU g-1) in the caecum of chicks fed three diets supplemented with oligosaccharides derived from arabinoxylan. Means in the same row not sharing a capital letter differ at p<0.05. For *Bifidobacteria*, means in the same column not sharing a lower case letter differ at p<0.05 (Adapted from Courtin et al., 2008). XOS= xylooligosaccharides; AXOS= arabinoxylooligosaccharides; S-AX= soluble arabinoxylans.

	Control		XOS		AXOS		S-AX	
Day	**n**	**Mean±SD**	**n**	**Mean±SD**	**n**	**Mean±SD**	**n**	**Mean±SD**
Enterobacteriaceae								
7	2	8.67±0.28 A	2	8.93±0.22 A	2	8.39±0.5 A	2	9.28±0.62 A
14	3	7.86±0.72 A	3	7.01±0.99 A	3	8.16±0.41 A	3	7.83±0.6 A
Lactobacilli								
7	2	8.11±0.04 A	2	7.83±0.27 A	2	7.90±0.25 A	2	8.39±0.2 A
14	3	7.26±0.44 A	3	7.84±0.06 A	3	7.46±0.7 A	3	8.05±0.35 A
Bifidobacteria								
7	2	3.29±0.18 Ba	2	8.50±0.02 Aa	1	2.05 Bb	2	3.05±1.08 Bb
14	3	2.71±0.43 Ba	3	6.60±0.56 Aa	3	7.63±1.34 Aa	3	7.62±0.96 Aa

1.3.2. Xylanase, fat and age of inclusion

Fat is viewed as the most potent macronutrient that affects the transit time of digesta in the GIT, increasing the contents rate of passage of digesta through the tract (van Citters & Lin, 2006), hence the more fat in the diet the longer the retention time. This has been linked to the stimulation of gut hormones, as fat has been found to stimulate the production of certain gut hormones which affect GIT motility (see section 1.4.2. Gut hormones), in turn affecting retention time. However there is conflicting evidence as Andreotti et al. (2004) found that incorporating varying levels of soybean oil into broilers feed linearly decreased ($p<0.05$) the retention times in these birds as the percentage included into the diet increased.

It has also been found that the type of fat incorporated into the feed effects the transit and retention of the digesta, for instance in broiler chickens who were supplemented either crude glycerol or tallow in their feed, the birds supplemented with tallow had a significantly ($p<0.01$) longer transit time than the control whereas the crude glycerol group was not significantly different from the control (Kim et al., 2013). This has also been met with conflicting evidence as previously Danicke et al. (1999) supplemented broiler diets with soybean oil or tallow and found no significant difference in overall tract retention time or retention time when recorded in each section of the tract. However the study reported that the retention time decreased in several sections of the tract and that the

decrease in the small intestine was most pronounced, although this was not significant.

It has been commented on by Singh et al. (2012) that due to xylanases effects on transit time the inclusion level of fat needs to be carefully considered when supplementing an exogenous enzyme into the diet. Transit time of digesta is an important variable to consider as it essentially determines the amount of time the feed is in the tract hence how long it is exposed to endogenous enzymes for digestion and subsequently the length of time nutrients are available to be absorbed. Cowieson et al. (2010) found that in a trial involving broilers on a maize-soy-based diet supplemented with xylanase and glucanase that at 21-days of age ileal amino acid digestibility was impeded by a reduction in fat content however this effect was not seen at 42-days of age and the enzyme activity was seen to be more effective in the later time-point. This could potentially be due to a reduction in retention time. They concluded from this study that when altering the fat contents of young broiler diets that have been supplemented with exogenous enzymes, care should be taken to ensure the fat reductions are not too extreme to impede performance, in agreement with Singh et al. (2012).

This shows a link between age and diet in the supplementation of xylanase and other exogenous enzymes. This is supported by Masey O'Neill & Cowieson (2013) who found that in broilers fed a wheat-based diet xylanase supplementation improved performance the most when it

was introduced from 1-day old rather than at 28-days old and when there was no reduction in dietary fat, although they did not report on retention time. Furthermore Masey O'Neill & Cowieson (2013) made reference to a holo-analysis performed by Rosen (2002) that showed when analysing 1322 studies xylanases effect was most prominent when supplemented immediately post-hatch, supporting the association between supplementation and the importance of age. Therefore when supplementing xylanase, or any exogenous enzyme, the underlying diet must be carefully formulated in relation to fat so as not to impede the enzymes efficacy, also the age of the animal at supplementation must be considered. As with fat, xylanase may exert its effects on transit time through hormones produced in the GIT.

1.3.3. Xylanase and gut hormones

It was suggested by Goodlad et al. (1987) that nutrients in the digestive tract could interact with intermediates to influence hormone production, which has since been confirmed (Anini et al., 1999; Strader & Woods, 2005; Voortman et al., 2012). In regards to research into xylanases mechanisms in improving feed digestibility, hormones that are of particular interest are ones linked with gut motility, influencing transit time of digesta within the tract as studies where supplementation with xylanase has been implemented have shown that this treatment affects the time it takes digesta to move through the digestive tract.

One particular hormone that has been investigated recently is PYY a gut hormone produced in the ileum and colon that slows down the gastric emptying of digesta but is classically known as being a satiety hormone (Sheikh et al., 1989). Singh et al. (2012) investigated how xylanase addition into a maize-soybean broiler diet effected the concentration of PYY in serum, amongst other parameters. They found that supplementation of xylanase into the diet significantly increased the levels of circulating PYY in broilers (see table 1.4). Currently there has been no further research into xylanases effects on the production of PYY

Table 1.4 Levels of gut hormone peptide YY (pg/ml) in broiler serum when maize-soybean diet was and was not supplemented with xylanase. Adapted from Singh et al. (2012).

Dietary Treatment	Peptide YY (pg/ml)
Without Xylanase	13.7
Supplemented with 16,000 U/kg Xylanase	34.8
P-value	<0.001

or any other hormones making this area of research quite novel. It has been reported in a review that SCFA increase the production of PYY as they act as ligands for receptors responsible for the release of some gut hormones including PYY and also have been known to cause contractions in the colon (Karaki & Kuwahara, 2010). This observation links into the suggestion that products from the enzymatic action of

xylanase act as prebiotics, increasing SCFA production and in turn influencing the production of certain gut hormones.

1.3.4. Summary

Xylanase is an enzyme that is supplemented into non-ruminant diets in order to increase performance response by making nutrients that were previously inaccessible available for digestion/absorption. It achieves this by targeting xylan and arabinoxylan, found in the plant cell walls, and producing fermentable xylooligosaccharides, this has been shown to decrease the viscosity of the digesta and increase the absorption of amino acids. Furthermore it has been suggested that the xylooligosaccharides produced act as prebiotics, increasing the populations of beneficial bacteria such as *Bifidobacteria*, and that the fermentation of these produces SCFA which create an environment in the distal digestive tract which is detrimental to the growth of harmful bacteria. These SCFA have also been linked to the production of PYY, a gut hormone involved in slowing down the transit time of digesta through the GIT. Furthermore, the success of xylanase supplementation has seen to be affected by the age of the animal that it is supplied to, and this has mostly been demonstrated in studies with broilers.

1.4. GASTROINTESTINAL TRACT

1.4.1. Anatomy and function of the digestive tract

Figure 1.4 shows the basic anatomy of the digestive tract of the pig, from stomach to colon including the pancreas. The main function of the digestive tract is to breakdown and absorb nutrients and water from the feed that has been consumed. It breaks down food through mechanical action (chewing and peristaltic movement of the stomach) and through acidic and enzymatic digestion of the nutrients, which mainly takes place in the stomach and the proximal section of the small intestine. Whilst digestion is occurring gut hormones are released in response to the ingestion of food and aid in the movement of the digesta through the digestive tract, the release of enzymes and other hormones to aid digestion as well as absorption and also relaying information to the brain about when to stop eating (and eventually start again).

The hormones relay these signals mainly through the vagal nerve however some are known to act directly upon the hypothalamus where the main centre for regulating energy homeostasis (appetite) is. The arcuate nucleus (ARC) within the hypothalamus contains neurons which either promote the release of orexigenic (appetite stimulating) peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), or anorectic (appetite suppressing) peptide pro-opimelanocortin (POMC) which mobilise to the paraventricular nucleus (PVN) and other nuclei within the hypothalamus to make either of these actions come about. This process

is demonstrated in figure 1.5 with some of the hormones that are discussed in the next section.

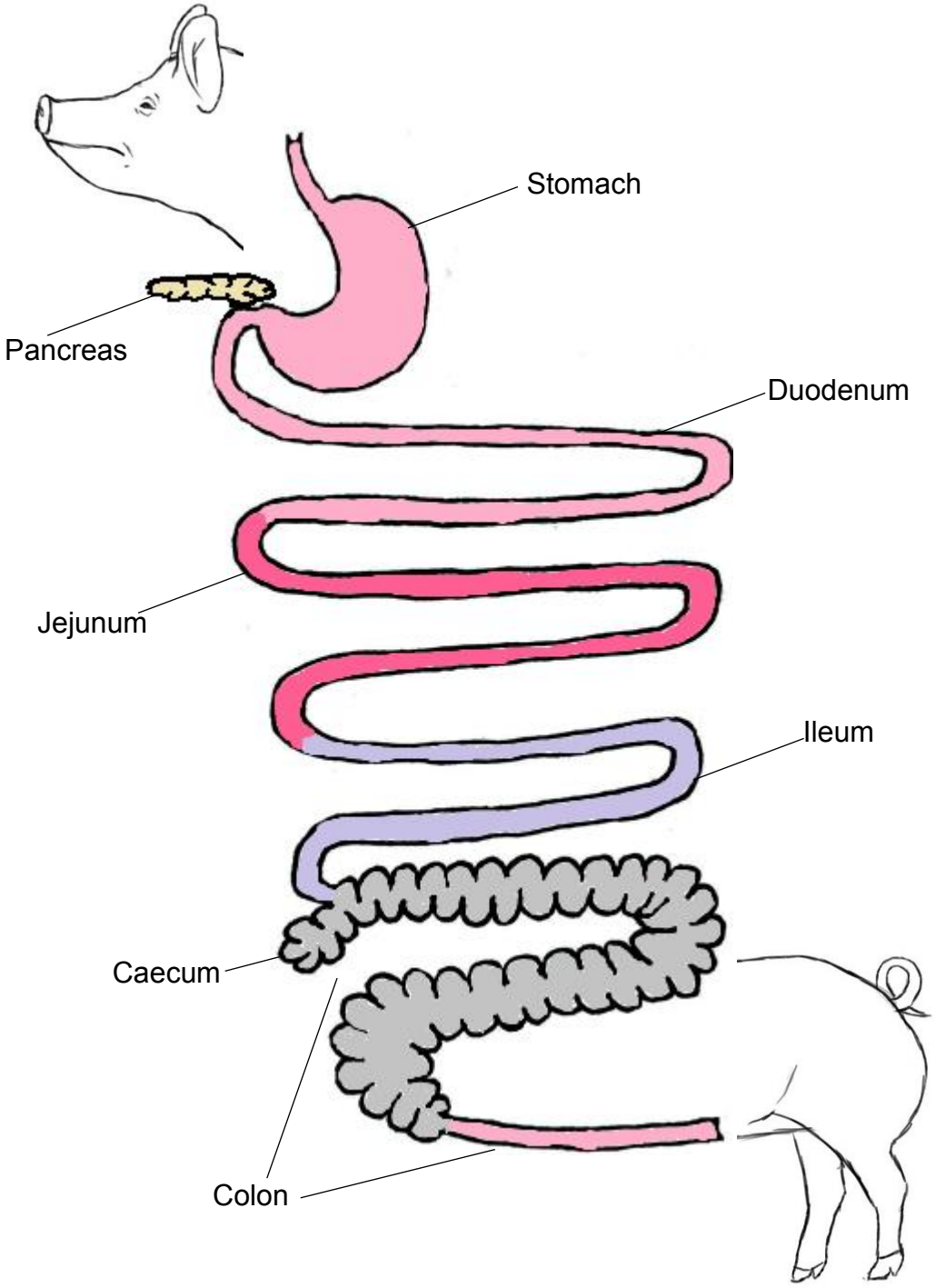


Figure 1.4 Basic depiction of the porcine digestive tract from the stomach to the colon.

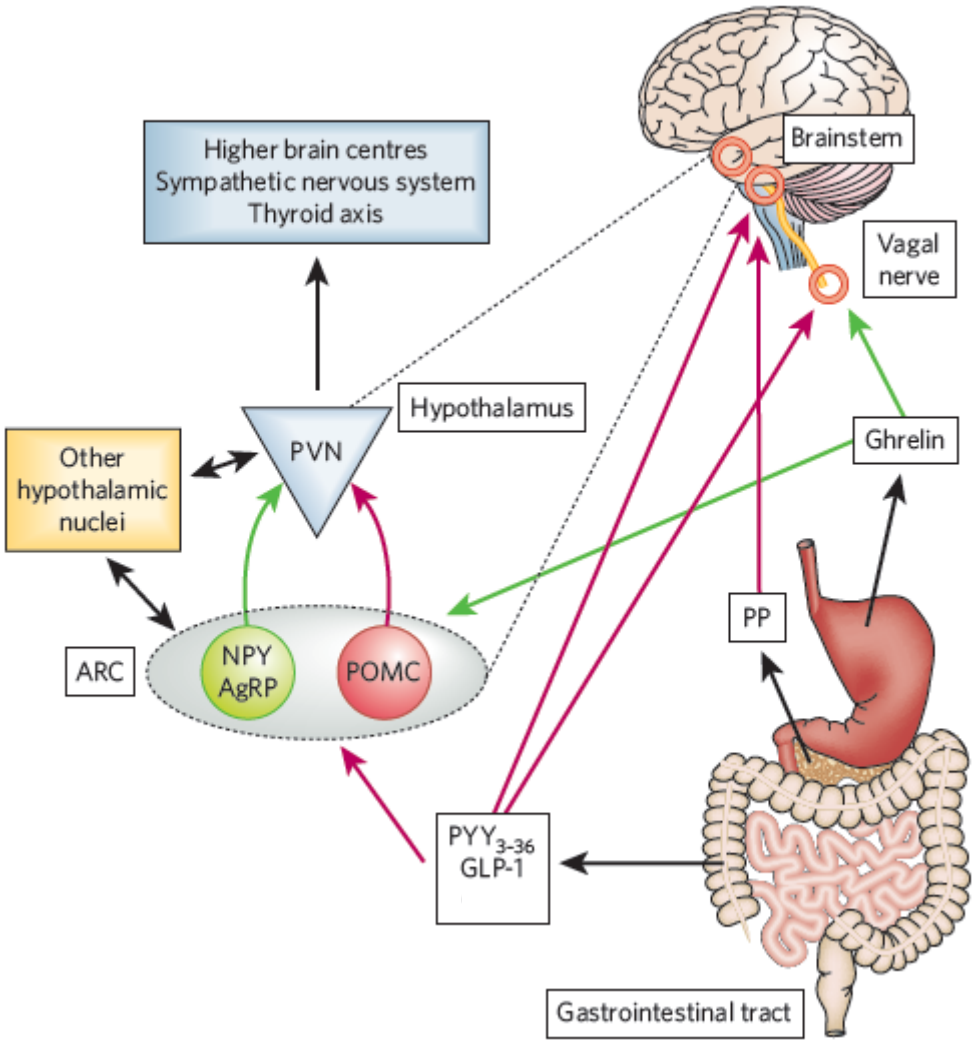


Figure 1.5 The process by which some gut and pancreatic hormones relay information to the hypothalamus. The green arrows represent appetite promoting signals whereas the red arrows show appetite supressing signals.
AgRP: agouti-related peptide. ARC: arcuate nucleus. GLP-1: glucagon like peptide-1. NPY: neuropeptide Y. POMC: pro-opiomelanocortin. PP: pancreatic polypeptide. PVN: paraventricular nucleus. PYY: peptide YY. Adapted from Murphy & Bloom, 2008.

1.4.2. Gut hormones

There are over 25 hormones produced in the gut (stomach to colon) and pancreas (Murphy and Bloom, 2008). The reason why they are so important to the animal feed industry and should be monitored are because they regulate gut motility/feed retention and appetite hence feed

intake, which can impact on feed efficiency, a key measurement in animal production. Most of the gut/metabolic hormones respond to the presence of food in the GIT and are either upregulated (e.g. insulin) or downregulated (e.g. ghrelin) in response to this stimuli, and even to specific nutrients being present. The hormones that are discussed in the current section were selected to be studied either because of this and/or they have an effect on gut motility when produced.

1.4.2.1. Amylin

Amylin (also referred to as islet amyloid polypeptide) is a 37 amino-acid peptide and is produced from the β-cells in the pancreas along with insulin, which are released together at a molar ratio of 1 amylin to 20 insulin (Martin, 2006), along with C-peptide. It is released in response to nutrient stimuli entering the digestive tract and is known to delay emptying of digesta from the stomach as well as supressing feed intake (Zhang et al., 2016).

1.4.2.2. Cholecystokinin

Cholecystokinin (CCK) is produced from I-cells which are located in the proximal part of the small intestine (duodenum and jejunum) (Little et al., 2005). It is released when food enters the digestive tract, meals high in fat and protein being the most potent stimuli to induce this response (Rehfeld, 2004). CCK is present in circulation in different forms varying in amino acid length (e.g. CCK-8, CCK-33, CCK-58 etc.) and the processing into these different forms is cell-specific with medium length

analogues being present the most in the gut (Little et al., 2006; Rehfeld, 2004). The main functions of CCK include slowing gastric emptying, stimulating pancreatic and gallbladder secretions, and appetite suppression (Wren & Bloom, 2007; Grider, 1994).

1.4.2.3. C-peptide

C-peptide is produced from the β-cells in the pancreas along with insulin and amylin. It is released in response to elevated blood levels of glucose and is also known as the connecting peptide as it joins the A and B chains of insulin of the insulin prohormone as seen in figure 1.6 (Yosten & Kolar, 2015). It is often measured to elucidate the functional efficacy of the β-cells (Yosten & Kolar, 2015).

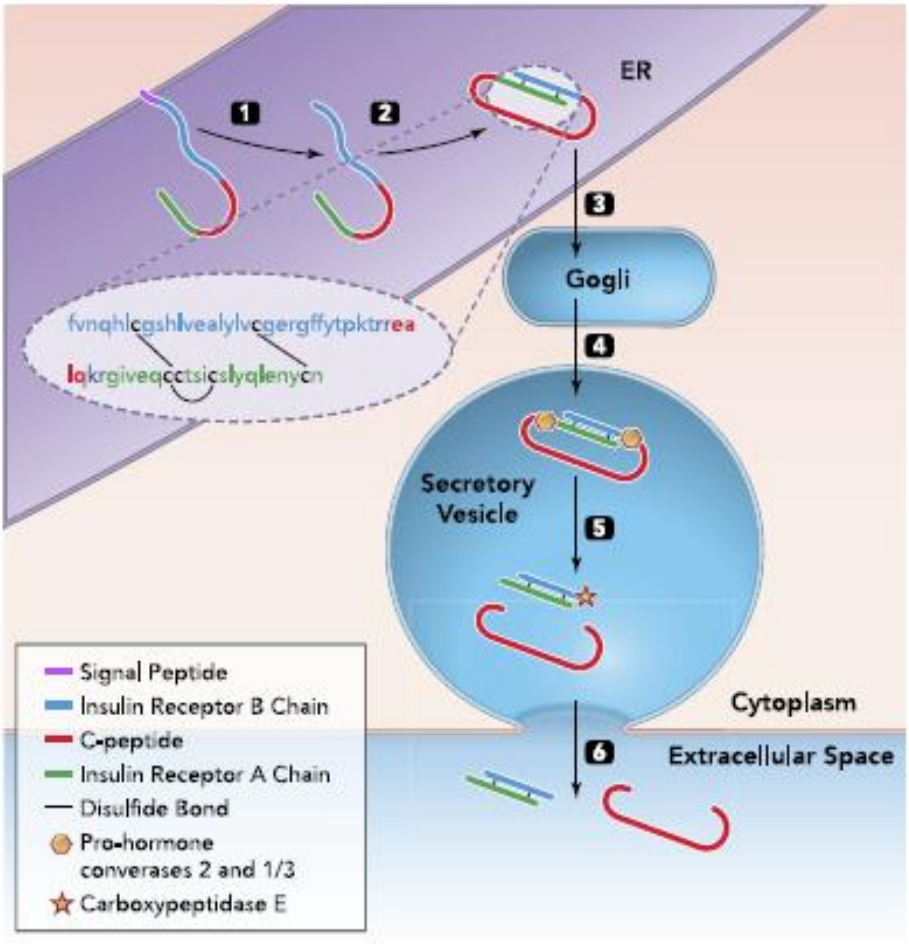


Figure 1.6 Processing of C-peptide and insulin from the proinsulin prohormone in the β-cells. Steps 1 to 3 show the signal peptide being cleaved, folding of the prohormone and the A and B chains being linked by two disulphide bonds and finally the prohormone moving into the Golgi. In steps 4 and 5 the prohormone is packaged into a secretory vesicle and the insulin and C-peptide peptides are separated enzymatically. In step 6 both hormones are released in response to stimuli, i.e. increase in blood glucose levels. Adapted from Yosten and Kolar, 2015.

1.4.2.4. Ghrelin

Ghrelin, known as the 'hunger hormone', is produced in the stomach although it has also been isolated in other peripheral tissues. On release it promotes feed intake and the feeling of hunger (Dong et al., 2009). Other actions include stimulating the release of growth hormone, increasing the rate of gastric emptying and gastric acid secretions (Dong et al., 2009).

1.4.2.5. Glucose-dependent insulinotrophic peptide

Glucose-dependent insulinotrophic peptide (GIP), is produced from K-cells in the proximal part of the small intestines (Baggio & Drucker, 2007). In figure 1.7 the biological actions that GIP has on the various parts of the anatomy are shown.

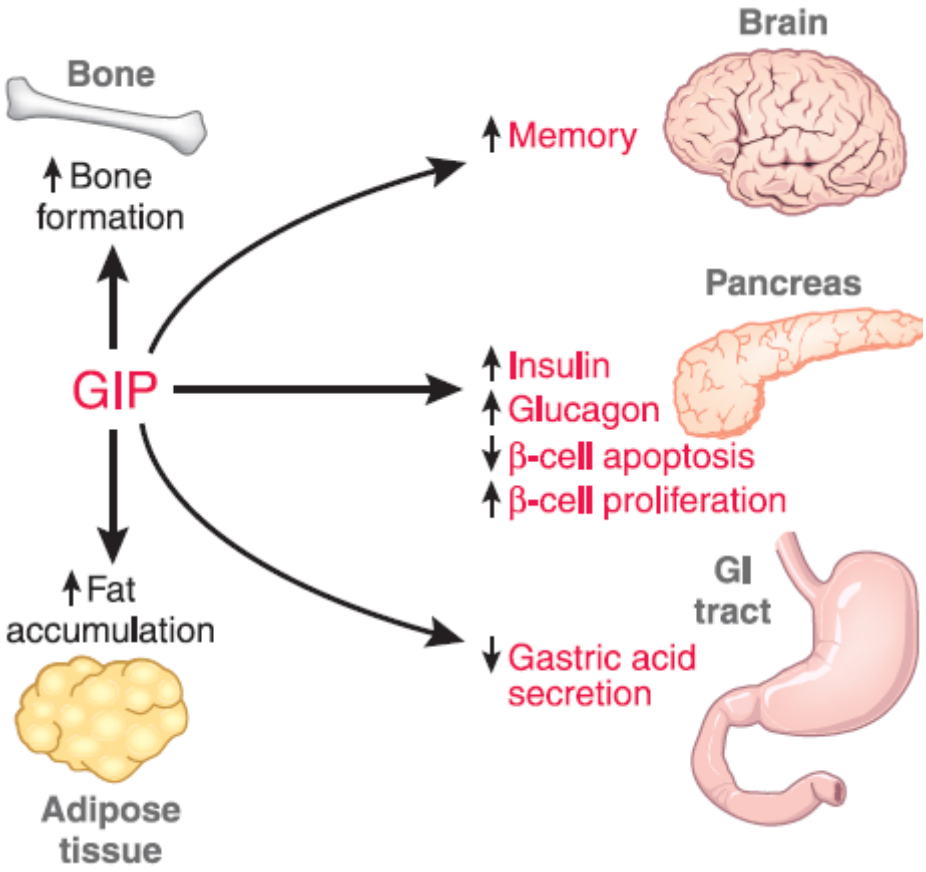


Figure 1.7 Diagram showing the effects of GIP on various organs and tissues. Adapted from Seino et al., 2010.

1.4.2.6. Glucagon like peptide-1

Glucagon like peptide-1 (GLP-1) is produced from L-cells in the distal small intestine (ileum) and colon, along with peptide YY (Holzer et al., 2012). Figure 1.8 shows the known effects GLP-1 has in the body.

Because of its effects on insulin it is known as an incretin hormone, which also includes gastric inhibitory peptide.

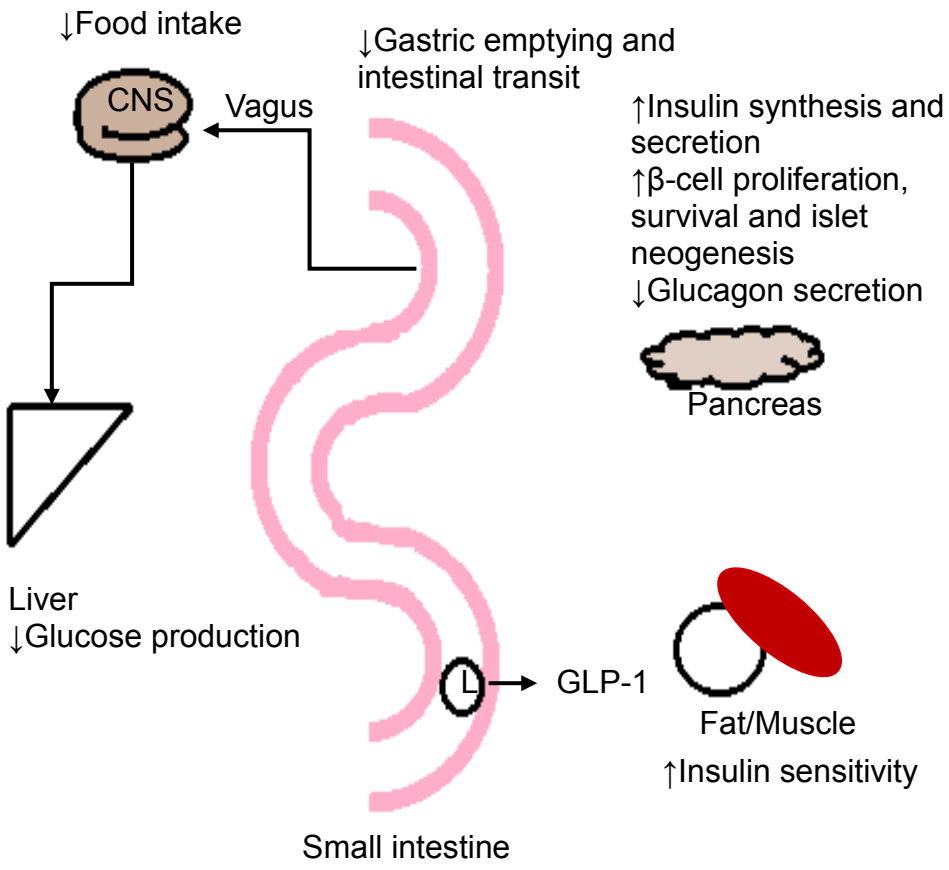


Figure 1.8 Effect that GLP-1 has on the pancreas, gut, brain and other tissues. Adapted from Brubaker, 2006.

1.4.2.7. Insulin

Insulin is released from the pancreas along with C-peptide and amylin from the β-cells located in the Islets of Langerhans. Its main role in the body is to maintain glucose homeostasis and is released in response to elevated blood glucose levels that occur after consumption of food. It exerts it affects by binding to cells and promoting the uptake of glucose

into the cells hence lowering blood glucose levels back to a normal range (Röder et al., 2016).

1.4.2.8. Leptin

Leptin is produced and released primarily from adipocyte cells that are located in white adipose tissue. Leptin is involved in maintaining the body's energy balance and is reflective of the amount of adiposity in the body, and is part of a negative feedback mechanism where it inhibits food intake (Oswal & Yeo, 2010).

1.4.2.9. Neurotensin

Neurotensin (NTS) is released from N-cells found in the jejunum and ileum, however it is classically known as a neurotransmitter (Boules et al. 2013; Zhao & Pothoulakis, 2006). The release of NTS from the GIT is in response to the stimuli of food being consumed, fat being the most potent nutrient responsible for this response (Zhao & Pothoulakis, 2006). Once released in the GIT it has effects on the motility of the digestive tract, more specifically it promotes the decrease of small intestinal motility and gastric emissions, but increases colonic motility, it is also known to be a satiety hormone, is involved in lipid digestion and regulates the release of hormones from the pancreas related to glucose homeostasis (Mazella et al., 2012; Zhou & Pothoulakis, 2006; Gui & Carraway, 2001).

1.4.2.10. Pancreatic polypeptide

Pancreatic polypeptide (PP) as it names suggests is primarily produced in the pancreas from F-cells that are located in the Islets of Langerhans of the organ (Holzer et al., 2012). However it has also been found in cells in the colon (Cox, 2007). PP is secreted postprandially, delaying gastric emptying, delaying insulin secretion and reducing feed intake (Schmidt et al., 2005; Schmidt et al., 2007; Holzer et al., 2012).

1.4.2.11. Peptide YY

Peptide YY (PYY) is produced from L-cells in the ileum and colon (Holzer et al., 2012). It is part of the pancreatic polypeptide family which also includes PP, which has already been discussed, and NPY which is expressed by enteric neurons in the gut but is also found in different locals in the gut-brain axis (Holzer et al., 2012). PYY circulates in the blood in two forms, PYY_{1-36} and PYY_{3-36} both of which are biologically active. After secretion from the L-cells the first two amino acids at the NH_2 terminal are cleaved through hydrolysis by dipeptidyl peptidase-IV (DPPIV) (Cooper, 2014). PYY's main known function is as a postprandial satiety hormone; it is released in response to food (and in particular fat) entering the digestive tract both at initial consumption and then elevates more when the digesta reaches the L-cells, entering blood circulation and acting on the arcuate nucleus in the hypothalamus to reduce feed intake and the feeling of hunger (Cooper, 2014). As well as responding to consumption of food PYY is also regulated by the release of CCK (Degen et al., 2007) which is located more proximally in the small intestine. Other

functions of PYY include inhibiting gut motility and slowing gastric emptying (Maljaars et al., 2008).

1.4.3. Summary

The gut hormones described here all have overlapping functions but also have individual functions, more of which are being discovered and their effects aren't just restricted to the GIT or appetite/feed intake regulation, although for the sake of this thesis these are the only actions that have been discussed. It is apparent that they play a large role in regulating energy homeostasis but also release of themselves and often the same stimuli is responsible for the production of more than one hormone, i.e. fat.

1.5. SUMMARY

Supplementation with xylanase could be one solution among many to help alleviate the pressures to improve animal performance, whilst using fewer resources and in a sustainable manner, in a bid to meet demands by the growing population. By using exogenous enzymes the nutrients stored in the resources that we use in non-ruminant feed can be fully utilised and so less will be wasted.

This literature review has outlined the research that has accumulated regarding non-ruminants and enzyme supplementation in feed, the main theories of which are shown in figure 1.9. It has been made clear that xylanase improves growth performance by decreasing the viscosity of the ingested feed by breaking down xylan and arabinoxylan, hemicelluloses found in the cell wall of the cereal endosperm. However this cannot be its only mode of action due to some cereals having lower levels of these NSPs and not creating viscous digesta. The activity of xylanase results in the production of xylooligosaccharides and arabinoxylooligosaccharides, whose prebiotic affects have been implicated in promoting the production of the gut hormone PYY in broilers. Furthermore the efficacy of the enzyme to exert performance effects has seen to be affected in broilers when supplemented at different ages.

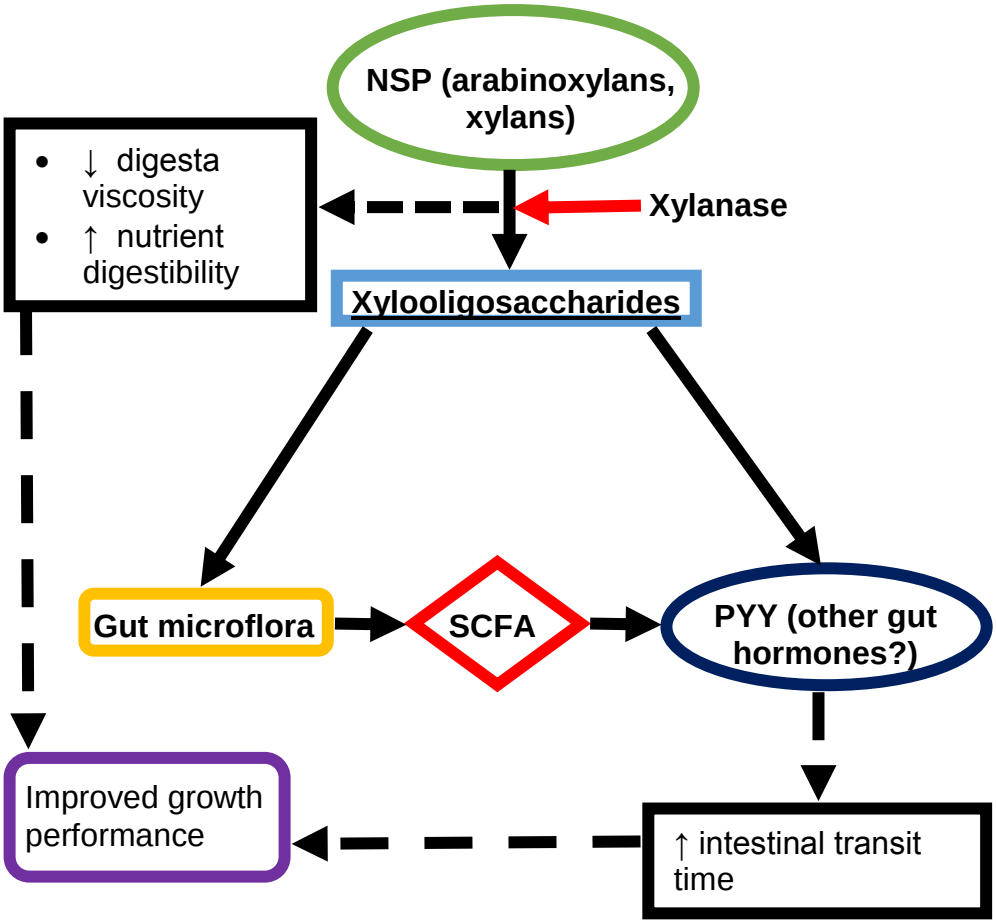


Figure 1.9 Summary of the main theories of the action of xylanase.

Therefore from the information gathered there are some gaps in knowledge that have been identified that need to be clarified. Firstly although there has been evidence showing that xylooligosaccharides act as a prebiotic and hence affect the population numbers of different bacteria there are some studies which did not see any effect at all or saw a reduction in beneficial bacteria. The age of the animal and the fat content of the diet have also been seen to interact with xylanases activity, however this has only been revealed in broilers therefore researching this in pigs would be beneficial in order to inform producers of the optimal conditions to use the enzyme under. Furthermore it has discussed here

that xylanase has been seen to affect intestinal transit time which many of the gut hormones have been found to regulate. Although there have been suggestions and some evidence that xylanase enhances the production of PYY, there needs to more research in order to consolidate this finding and also in pigs as the original study was carried out using broilers, as well as looking into xylanases potential effects on other hormones.

For these reasons a research programme has been designed in order to explore the possible mechanisms that xylanase acts through to improve growth performance in pigs, particularly focusing on its effects on gut hormone levels and to find what the optimum age is for use of xylanase in a commercial environment.

2. MATERIALS AND METHODS

2.1. ANIMALS AND HOUSING

For trial 1 entire male, Camb12 (Landrace x Large White x Duroc) pigs were delivered to the University of Nottingham, randomly allocated to one of four treatment groups (see chapter 3) and individually housed (pens measured 2m^2 and had concrete flooring with metal slating across the back wall floor for drainage, a rubber mat was placed in each pen as bedding) in the pig growth building. For all subsequent trials female, Camb12 piglets were used and delivered on day of weaning (28 days old) and individually housed in penning (measuring 1.5m x 2.2m with rubber slatted flooring) suitable for weaning pigs. The temperature was controlled in these rooms (which started at 25°C for weaned piglets then was gradually reduced as their age increased, but not reaching less than 16°C) and had a lighting regime of 12L:12D with a twilight period of 15mins before change. All animals in each trial were weighed on arrival. Water was available *ad libitum* from water troughs. All trials were designed and carried in accordance with conditions set out in EU Directive 2010/63/EU and the Animals (Scientific Procedures) Act 1986.

2.2. DIETS

The diets for trials 2-4 (chapters 4 and 5) were formulated by Primary diets (Ripon, UK) (see Appendix 1 for dietary composition) and ABN (Flixborough, UK) provided the diet for trial 1. Diets were in pellet form, were formulated to meet or exceed requirements as specified by the

NRC (National Research Council) and were fed on an *ad libitum* basis apart from in trial 1 (see chapter 3). In trials 2-4 half of the diets were supplemented with xylanase (16,000 BXU/kg, Econase XT, AB Vista, UK), an NSP degrading enzyme (see activity assay for each feed in Appendix 2).

2.3. ESTIMATING FEED INTAKE

During trials 2-4 feed intake (FI) was measured on a weekly basis at different time points of each trial. This was done by providing each animal a bucket for their feed ration (the ration was increased weekly in accordance to the animals increase in appetite) of a known weight and weighing back feed refusals. Feed refusals were captured by trays positioned underneath the feeding troughs. The weight of the wet feed on the refusal trays (urine and water contaminated dry feed) was estimated 'by eye' using pre-weighed amounts of dry feed and matching them in size to the wet feed accordingly. During trial 2 the feed refusals and hence FCR measurements were found to be highly variable and resulted in biologically inaccurate values being calculated for the FCR which was probably due to the feed being contaminated with liquid. Therefore for subsequent trials it was decided to modify the feeding troughs so that less feed would be spilled and hence potentially reducing the amount of contaminated refusals in the collection trays. In trial 3 the variability in FCR and FI decreased when comparing these results to the data collected from trial 2 (see chapter 4). Subsequently these troughs were also used for trial 4.

2.4. EXPERIMENTAL AND SLAUGHTER PROCEDURES

As well as being weighed on arrival, all animals were weighed every seven days and on their slaughter day. Two slaughter methods were performed in the trials:

- Commercial slaughter method (S): Each animal was electrically stunned (using head tongs), rendering them unconscious, suspended upside-down and exsanguinated by severing the jugular vein in the neck. Once most of the blood was drained from the animal they were then lowered into a scalding water tank heated to 60°C for 10 minutes, dehaired by rotating paddles as part of the scalding bath and then manually dressed and finally eviscerated.
- Non-commercial slaughter method (NS): Each animal was electrically stunned and exsanguinated then immediately eviscerated.

Slaughtering of the animals was undertaken by personnel who had ‘appropriate training’ to carry out this task to ensure ‘humane killing’ as specified by the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures.

In trial 1 both methods were used as part of the experimental design which was an examination of the slaughter methods (chapter 3). In trials 2 and 3 NS methodology was used and trial 4 were subjected to the S method.

2.5. SAMPLE COLLECTION

2.5.1. Blood collection

Blood was collected from the bleed out coming from the pigs at exsanguination, it was then immediately transferred into 10ml centrifuge tubes coated in EDTA for plasma or 50ml falcon tubes for serum and stored on ice. For trials 3 and 4 protease inhibitors were also added to the blood tubes (in 10ml of blood; 100µl of DDPIV inhibitor (Merck Millipore, Billerica, MA, USA), 10µl Protease Inhibitor cocktail (Sigma Aldrich, Dorset, UK), 10µl of Serine protease inhibitor (Pefabloc SC, Roche Diagnostics, Burgess Hill, UK)) in order to measure GLP-1, amylin and ghrelin which are otherwise quickly degraded in plasma and serum without the presence of these inhibitors. The samples were then centrifuged (Beckman GS-6R, Beckman Coulter, High Wycombe, UK) at 3000g for 10 mins at 4°C. The resulting plasma or serum supernatant was aliquoted in duplicate into 1.5ml microcentrifuge tubes and stored at -80°C.

2.5.2. Tissue collection

Once the animal was eviscerated jejunal, ileal and colonic tissues were dissected from the gut (pancreas and liver were also taken for trial 1). Samples taken from the GIT were longitudinal sections of the lumen approximately 3-4cm in length; jejunal samples were taken from the mid-point of the small intestine, ileum samples were taken 15cm anterior to the ileocecal junction and colon samples were taken from the distal colon 20cm anterior to the start of the rectum. Before being flash frozen in liquid

nitrogen the GIT samples were flushed with distilled water to get rid of any remaining digesta in the lumen. The samples were then stored at -80°C.

2.6. GENE EXPRESSION ANALYSIS

2.6.1. Tissue crushing

Each tissue sample was fractured into pieces using a metal mallet and a small piece was crushed to a powder in liquid nitrogen using a metal pestle and mortar and then stored at -80°C in 2ml microcentrifuge tubes.

2.6.2. RNA extraction

Roche Diagnostics High Pure RNA Tissue Kit (Roche Diagnostics, Burgess Hill, UK) was used to extract the total RNA from the liver and pancreas. All reagents were made up according to the manufacturer's instructions. The protocol involved weighing out approximately 25mg of the crushed tissue into a 1.5ml microcentrifuge tube and quickly adding 400µl of lysis buffer (4.5M guanidine-HCl, 100mM sodium phosphate, pH 6.6) then homogenising the tissue by disrupting with a manual plastic homogeniser. The resulting homogenate was then passed through a 20 gauge needle and sterile syringe until it was homogenous. The homogenate was centrifuged (Microfuge® 22R Centrifuge, Beckman Coulter, High Wycombe, UK) for 2 minutes at 14,000 rpm at room temperature before removing the resulting supernatant into another 1.5ml microcentrifuge tube. Two hundred µl of 100% ethanol was then

added to the supernatant and mixed through pipetting. A filter tube (double layered fiber fleece filter) was then placed inside a 2ml collection tube and 700µl of sample was pipetted into the filter tube and centrifuged for 30secs at 13,000rpm at room temperature. The eluted liquid in the collection tube was discarded and remaining sample was pipetted into the filter, centrifugation repeated and the eluted liquid discarded. Onto the filter 100µl of DNase solution was then pipetted and this was incubated at room temperature for 15mins. To the filter 500µl of wash buffer (5M guanidine-HCl, 20mM Tris-HCl, pH 6.6) followed by centrifugation at room temperature for 15 secs at 8000rpm. The eluted liquid was then discarded and an additional 500µl of wash buffer (20mM NaCl, 2mM Tris-Hcl, pH 7.5) was added to the filter and centrifuged as before. Again the eluted liquid was discarded and 300µl of the same wash buffer was added to the filter tube and centrifuged at 13,000rpm for 2mins. The filter tube was inserted into a clean 1.5ml microcentrifuge tube, 50µl of RNase free water added and centrifuged for 60secs at 8000rpm. The filter tube was discarded and the eluted liquid collected (containing RNA) was stored at -80°C.

Homogenates of jejunal tissue did not pass easily through the Roche column using this protocol, most likely because of its more fibrous composition (ileal and colonic tissue were expected to have the same issue) compared to the liver and pancreas, resulting in low RNA yields. To increase yields of RNA a digestion with proteinase K (QIAGEN, Hilden, Germany) was included. Comparison of the effectiveness of RNA

extraction from jejunum using an additional proteinase K digestion was made between the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) and the Roche Diagnostics High Pure RNA Tissue Kit (Roche Diagnostics, Burgess Hill, UK). Three RNA extraction methods were compared:

1. Roche Diagnostics High Pure RNA Tissue Kit
2. Roche Diagnostics High Pure RNA Tissue Kit plus proteinase K digestion step.
3. QIAGEN RNeasy Mini Kit plus proteinase K digestion step.

A single uniform jejunal sample was used for the extractions. The RNA concentration and quality was then assessed between the three methods (section. 2.6.3). Although there was no difference in the 260nm/280nm ratio between the two proteinase K linked RNA extraction methods the quantity of RNA extracted from the jejunum was significantly higher ($p<0.05$ when analysed using a T-test) using the QIAGEN RNeasy Mini Kit with a proteinase K digestion step (table 2.1). When the quality of the extracted RNA was compared (Agilent 2100 Bioanalyser, Agilent Technologies, Santa Clara, CA, USA) the RIN value (quality assessment) was significantly higher ($p<0.05$ when analysed using a T-test) for the QIAGEN RNeasy Mini Kit with a proteinase K digestion step (figure 2.1). A RNA extraction of the same sample of jejunum using Roche Diagnostics High Pure RNA Tissue Kit gave consistently lower yields and poorer quality RNA (although it was not possible to statistically test this). The same replicated sample had very distinct bands when

extracted using the QIAGEN RNeasy Mini Kit with a proteinase K digest compared to the other two methods. Therefore QIAGEN RNeasy Mini Kit with a proteinase K digestion was adopted for subsequent RNA extractions from the jejunum, ileum and colon.

Table 2.1 Effect of different RNA extraction procedures on RNA concentration and 260nm/280nm ratio from pig jejunum.

	260nm/280nm Ratio	**RNA Concentration/ ng/µl**
Roche (n=1)	1.9	59
Roche + proteinase K (n=3)	2.01 ± 0.024	107.7 ± 40.45
QIAGEN + proteinase K (n=3)	2.01 ± 0.006	342.0 ± 4.58

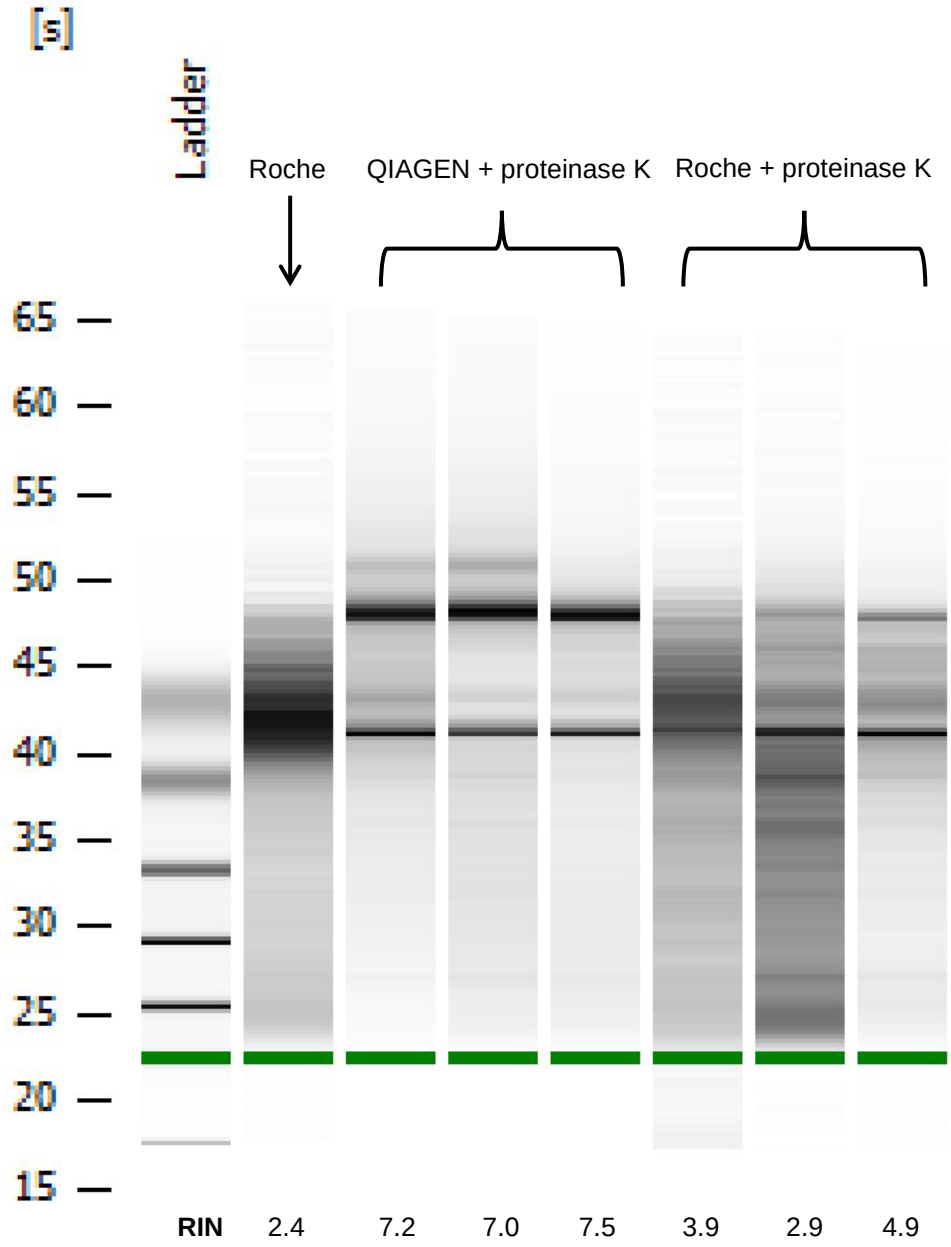


Figure 2.1 Effect of different RNA extraction procedures on RNA quality from pig jejunum. Gel like print out and RIN values from Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) system. The RIN value represents the band ratio of the 28S and 16S ribosomal RNA in each sample and the degree of degradation. The RIN values (the definition of which is explained in chapter 2.5.3) for each sample are provided below the gel impression.

The QIAGEN kit RNA preparation method required a maximum of 30mg of frozen tissue to be weighed out to which 300µl of lysis buffer (with 10µl β-mercaptoethanol per 1ml of lysis buffer) was immediately added. The tissue was homogenised as before when using the Roche kit. To this homogenate was added 590µl RNase free water along with 10µl proteinase K (>600 mAU/ml; QIAGEN, Hilden, Germany) and the mixture was incubated at 55°C for 10mins after which an additional 10µl of proteinase K was added and incubation repeated. The homogenate was then centrifuged (Microfuge® 22R Centrifuge, Beckman Coulter, High Wycombe, UK) for 3mins at 10,000g, the supernatant carefully removed (100µl at a time) into a new 1.5ml microcentrifuge tube then 450µl of absolute ethanol was then added and mixed by pipetting. To a filter column inside a 2ml collection tube 700µl of the supernatant/ethanol mix was added, then spun down for 15secs at 8000g at room temperature. This was then repeated with the rest of the remaining supernatant/ethanol mix, and any liquid that eluted from the column into the collection tube was discarded. To the filter column 350 µl of wash buffer was added, centrifuged for 15 secs at 8000g and the elute from the column discarded. Then 80µl of DNase digestion solution was added to the filter column and was incubated at room temperature for 15mins, after which another 350µl of the same wash buffer was added to the column and centrifuged at 8000g for 15secs at room temperature. The column was then put in a new 2ml collection tube and 500µl of a second wash buffer was added to the column and centrifuged at 8000g for 15secs. The eluted liquid was discarded and another 500µl of the wash

buffer was added and the solution was spun down again at 8000g for 2mins at room temperature. The column was then removed into a new 1.5ml microcentrifuge tube and 50µl of RNase free water was added and spun down for 1min at 8000g at room temperature. The filter column was then discarded and the eluted liquid, containing the isolated RNA, was stored at -80°C. This kit and protocol was also adopted for the ileal and colonic tissue in all trials as they are also quite fibrous.

2.6.3. RNA quantification and quality

The concentration of total RNA was quantified for each sample by loading 1.5µl onto a Nanodrop® ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). This machine recorded the 260nm/280nm ratio to gain an estimation of the purity of the RNA. Ratios greater than 2.0 being ideal. The samples were then diluted to 100 ng/µl (only pancreas) and 50 ng/µl (all other tissues). To 10µl of the RNA sample was added 2 µl of loading dye and these were loaded onto a 1% (w/v) horizontal agarose gel (1g agarose per 100ml 1x TAE buffer) and run at 100V for 40mins. The gel was then stained with ethidium bromide solution. The gel was examined for the integrity of the 28S and 18S ribosomal RNA bands ratio for any RNA degradation which would be evident by a ‘smearing’ of the bands. In addition the total RNA from trial 1 was also then assessed for quality using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA), which quantified the amount of degradation by classifying each sample with a RNA integrity number (RIN). The RIN system is a scale by which the quality of the RNA

is expressed; ten representing perfectly intact RNA and one representing completely degraded RNA, the method of which is expanded on in chapter 3.

2.6.4. cDNA synthesis

RNA (50 µg/µl) extracted from the liver (trial 1 only), jejunum, ileum and colon was synthesised into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Burgess Hill, UK) following the manufacturer's instructions. In brief, this involved adding 3µl of 60µM random hexamer primers to 10µl of RNA solution in a 200µl thin walled PCR tube. This mixture was incubated at 65°C for 10 minutes and then placed on ice. On ice the mixture was made up to 20µl by adding Transcriptor Reverse Transcriptase Reaction Buffer, Deoxynucleotide Mix, Transcriptor Reverse Transcriptase and Protector RNase Inhibitor (4µl, 2µl, 0.5µl and 0.5µl respectively). This final mixture was incubated at 25°C for 10 minutes, 55°C for 30 minutes, 85°C for 5 minutes then placed back on ice. The synthesised cDNA was diluted with 80µl of RNase-free water then stored at -20°C until further analysis.

2.6.5. cDNA synthesis verification and quantification

Before quantification, all cDNA was run with a pair of known working primers to make sure cDNA synthesis was successful. To check relative quanitity of cDNA generated a standard curve was used. The standard curve was generated by making a pool of cDNA from all samples. This was serially diluted by 2-fold successive dilutions down to a ratio of 1:63

cDNA to RNase free water. Total cDNA concentration was measured for all samples of RNA extracted from tissue samples on the LightCycler 480 (Roche Diagnostics, Burgess Hill UK) using a 384-well plate. The wells contained 5µl of cDNA plus 5µl of Oligreen (Invitrogen, Paisley, UK). Each sample was loaded onto the plate in triplicate which was then inserted into the LightCycler which followed a programme set to rise to 95°C at a rate of 0.11°C/s. The fluorescence was measured at 80°C and the relative amount of cDNA was calculated relative to the standard curve as described above.

2.6.6. Alignment of human and porcine gut peptides

In order to design primers for gene expression analysis the mRNA sequence for each target mRNA needed to be found. However some of the desired pig sequences were not full length or absent. As some of the sequences had a high degree of similarity to human cDNAs it was decided to align the human and pig cDNAs and then use the human mRNA sequence to find the complete sequence for the pig. First both the human and pig peptide sequences were found using the Protein Knowledge Uniprot (www.uniprot.org/uniprot) database and aligned against each other using ClustalW2 (now Clustal Omega; www.ebi.ac.uk/Tools/msa/clustalo). The next step was to find the n-terminus of each porcine mRNA for the peptides of interest. This was done by searching for the human peptide in the Unigene database on the National Center for Biotechnology Information site (www.ncbi.nlm.nih.gov) and using the mRNA sequence in the peptide

profile to search for similar sequences in the pig genome using the BLAST database (blast.ncbi.nlm.nih.gov/Blast.cgi). The corresponding pig mRNA was then translated (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and the peptide sequence generated was aligned, again using ClustalW2, to the pig peptide sequence that had already been procured. From this it could be confirmed if the correct mRNA sequence had been found and hence the n-terminus of the pig mRNA sequence. Now that the full mRNA and peptide sequences had been found, in order to generate primers for RT-qPCR the exon boundaries needed to be identified in the mRNA sequence. This was done by accessing the Ensembl database (http://www.ensembl.org/index.html). The alignment of the full human and pig peptide sequences to confirm sequence similarity, can be found in appendix 3.

2.6.7. Primer design

Both forward and reverse primers for the reactions were designed using Primer Express (Applied Biosystems, Paisley, UK) with either the forward or reverse primer being over an exon boundary to ensure specificity and were supplied by Sigma Aldrich (Dorset, UK). The sequences for the primers can be found in Appendix 4. The primers were prepared to 100μM according to manufacturer's instructions to create a stock and were diluted further to 10μM to create a working solution.

2.6.8. Real time q-PCR

RT-qPCR was conducted on the Roche LightCycler 480 using a 384-well plate. The wells contained 5µl of sample cDNA plus 7.5µl SYBR green master mix (Roche Diagnostics, Burgess Hill, UK), 0.45µl each of forward and reverse primer at a concentration of 10µM and 1.6 µl RNase-free water. To measure transcript abundance a standard curve was generated as before (described in 2.6.5). An ideal efficiency of 2 and standard curve negative gradient of the log base 2 of 10 (-3.322 to 3 decimal places) is optimal for quantification using RT-qPCR. Once in the LightCycler samples were incubated at 95°C for 5 min, and then went through 45 cycles of 95°C for 10 s, 60°C for 15s and 72°C for 15s. Melting curves were generated to check that only one product was detected.

2.7. ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

2.7.1. Insulin

A Porcine Insulin ELISA (Mercodia, Uppsala, Sweden) was used to measure the plasma insulin concentrations in trials 1 and 2 to compare with the Human Multiplex assay (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA) results. The kit had a 3.5% intra-assay and a 2.8% inter-assay coefficient of variation (CV). All reagents were prepared in accordance with the manufacturer's instructions. 25µl of undiluted plasma was pipetted in duplicate onto a mouse monoclonal anti-insulin coated 96-well plate along with a standard curve (provided by manufacturer) and a blank. Then 100µl of peroxidase

conjugated mouse monoclonal anti-insulin enzyme conjugate was added bringing all wells to 120µl in volume, the plate was then incubated at room temperature on a plate shaker for 2 hours. At the end of the incubation period the reaction liquid was discarded then the wells were washed to remove any unbound enzyme labelled antibody by adding 350µl of wash buffer which was then discarded, this was repeated 6 times in total. Two hundred µl of 3,3'-5,5'-tetramethylbenzidine (TMB) substrate was then added to the wells to detect bound conjugate, the reaction was allowed to occur for 15 mins at room temperature after which 50µl of 0.5M H_2SO_4 was added to stop the reaction, the solution was then mixed for 5 seconds using the plate shaker and the plate was read immediately on a microplate absorbance reader (Sunrise ™ Tecan, Männedorf, Switzerland) at 450nm. Concentrations of the samples were determined by creating a standard curve and rearranging the formula for the gradient of the curve (y=mx+c) to find x (concentration of samples).

2.7.2. CCK

The supplier for the insulin ELISA kit did not have an ELISA for CCK so another pig specific kit was sourced (Cusabio, Wuhan, China). Although it is suggested by the manufacturer to dilute the plasma samples by a factor of 5 the samples were run neat as tests on the kit showed that if the samples were diluted they did not show up on the standard curve. The intra-assay CV for this kit was <8% and <10% for inter-assay CV. All reagents were prepared as specifically directed by the manufacturer. One hundred µl of standard, blank and samples were pipetted in

duplicate onto a 96-well plate coated with an antibody specific for porcine CCK and incubated at 37°C for 2 hours. After the initial incubation the contents of the wells was decanted and 100µl of biotin-conjugated antibody, again specific for porcine CCK, was added and the plate incubated at 37°C for 1 hour. The wells were decanted again and were washed 3 times by adding 200µl of wash buffer and incubating at room temperature for 2 mins, at the end of which the liquid was removed. After this 100µl of avidin conjugated horseradish peroxidase was added to the empty wells and incubated again at 37°C for 1 hour. The plate was then washed as before repeating this process 5 times in total. 90µl of TMB substrate was then added and the plate incubated for 25 mins at 37°C. Finally 50µl of acidic stop solution was added to the reaction and the plate was read on a microplate reader (FLUOstar OPTIMA, BMG Labtech LTD, Buckinghamshire, UK) within 5 minutes. The plate was read at 450nm and at a correction wavelength of 540nm. A standard curve was generated and the concentrations for the samples calculated as with the insulin ELISA, but with the 540nm being subtracted from the 450nm reading first, before the average of the replicates was calculated and the blank subtracted.

2.8. MULTIPLEX ASSAY

A magnetic bead-based multiplex assay was used to simultaneously measure metabolic/gut hormones in all trials (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA). Details of the specific peptides measured can be found in subsequent chapters

the assay was used in (chapters 4 and 5). The multiplex system is similar to an ELISA method, however more than one analyte, in this case gut hormone, can be measured on a single 96-well plate, increasing time efficiency and decreasing the amount of sample needed. In brief the method involved incubating the samples (along with a blank, standards and two quality controls) in duplicate with a mixture of all of the analyte magnetic beads overnight (16-18 hours) at 4°C. Next the plate was washed so that all that remained was the beads attached to the peptides of interest, this was done using a magnet so that when the wells were decanted the sample was not lost. The peptide bound beads are then incubated with a biotinylated detection antibody and then with Streptavidin-Phycoerythrin conjugate. The plate was then read on the MAGPIX® System (Merck Millipore, Billerica, MA, USA) (xPONENT® Software (Merck Millipore, Billerica, MA, USA) was used to analyse the data), in which the beads pass through a laser which excites the internal dye of the bead which is linked to which antigen (peptide hormone) it is capturing, it is then passed through a second laser which excites the Streptavidin-Phycoerythrin conjugate so that the amount of peptide can be quantified.

3. TRIAL 1: GUT HORMONE PROTEIN LEVELS AND GENE EXPRESSION IN PIGS AFTER AN OVERNIGHT FAST: EFFECTS OF COMMERCIAL SLAUGHTER METHODS.

3.1. INTRODUCTION

At the time of planning the following study there was not a multiplex assay specifically designed to measure gut hormones in samples from pigs. When measuring proteins multiplex assays can reduce the time taken to analyse samples which would otherwise be assessed using separate ELISAs for each analyte, in this case gut hormones. As mentioned in chapter 2 the cDNA sequences for most of the hormones are highly conserved between the pig and human, therefore once complete copies of the pig mRNA of the gut hormones had been made, in order to make primers for use in RT-qPCR, the human and pig gut peptides were also aligned and were, as expected, highly similar (see appendix 3). Thus part of the aim of the current study was to analyse pig plasma using a human multiplex assay to ascertain if this was a valid way of measuring pig metabolic/gut peptides simultaneously.

As discussed in chapter 1 most of the hormones released from the gut and pancreas to aid digestion and subsequent absorption are released in response to certain dietary components entering the digestive tract; therefore in order to gauge the effectiveness of the human multiplex assay in measuring pig proteins animals in fed and fasted states were sampled. As a further means to test if the human multiplex assay was

successful in determining the gut/metabolic hormone levels in pigs, gene expression analysis was also undertaken to see if the same patterns as in the peptide data were obtained between the fed and fasted animals. In order to proceed with this the RNA needed to be extracted from tissues and be of high enough quality to be analysed using RT-qPCR. RNA is thought to begin to degrade immediately post mortem, so it is considered imperative to take tissue samples as soon as possible. In a commercial slaughter house it is routine that the carcasses undergo certain procedures that aid in food safety for the meat products derived from them to enter the food chain. Conventional slaughter procedures for pigs involve the use of a scalding tank at 60ºC followed by dehairing. This means it takes about 25 minutes after exsanguination (time of death) before tissue samples can be taken, whereas samples can be collected minutes after exsanguination if the scalding bath and dehairer are not used. The combination of length of time to collection and raised temperature associated with scalding for conventional slaughter is expected to increase RNA degradation, hence lowering the quality. If the quality of the RNA is too low then it cannot be used in downstream analyses.

As well as analysing gut hormone gene expression profiles in parts of the GIT, levels of PCK1 and PCK2 in the liver were also measured. Both of these genes code for enzymes that play key roles in gluconeogenesis which occurs, for one instance, when the body is in a state of fasting. PCK1 is upregulated when fasting occurs, however PCK2 is

constitutively produced; measuring these genes will help to determine if the fast was long enough and if the slaughter method has an effect on downstream analyses. Therefore the current trial was designed in order to test changes in gut hormone expression in response to fed and fasted states, and if this translated to circulating gut peptide levels, but also to observe if slaughter method affects RNA integrity.

3.2. OBJECTIVES

- Extract RNA from various sections of the GIT of pigs subjected to S or NS; S denoting commercial slaughter methods and NS denoting the non-commercial slaughter method. Half of these animals will also be subjected to an overnight fast.
- Assess the quality of the RNA using Agilent technology.
- If the quality of the RNA is suitable for RT-qPCR then it will be used to examine the differences in particular peptide gene expression levels.
- Analyse gut hormone levels in circulating porcine plasma using a human multiplex assay.

3.3. HYPOTHESIS

Total RNA extracted from animals subjected to S will be of lower quality than those that were subjected to NS. Gene expression of the measured gut hormones will decrease in the pigs subjected to an overnight fast. This trend will also be evident in plasma samples measured by a human multiplex assay measuring gut peptides. Gene expression of PCK1 and

PCK2 in the liver will differ in response to the fast, PCK1 increasing in response to the fast and PCK2 not being affected at all.

3.4. MATERIALS AND METHODS

The current section only describes in brief the methods undertaken for the current trial; for a more in depth methodology that is common in all trials please see chapter 2.

3.4.1. Animals and experimental design

The current study was a 2x2 factorial design with slaughter method and fed or fasted being treatment parameters. The pigs (n=24) were randomly assigned to one of four groups; S/Fed, S/Fasted, NS/Fed and NS/Fasted. One pig from group NS/Fed did not complete the trial due to ill health and so was not included in the analysis. After one week of being fed *ad libitum* (all animals were fed the same diet irrespective of treatment group). The night before slaughter, the animals in the fasted groups were had their food removed from them from 5pm, resulting in an average fasting time of 18 hours at slaughter. As pigs must be 'fed at least once a day' under the Welfare of Farmed Animals (England, Amendment Regulations 2003), the pigs had access to feed *ad libitum* until the point at which they had their feed removed. Slaughter was conducted over 2 days, day 1 included all animals for NS and day 2 those for S. All samples were collected after slaughter as described in Chapter 2.

3.4.2. RNA extraction and quality

All collected tissue (stored at -80°C) was crushed into a powder before total RNA isolation. RNA from liver and pancreas were extracted using Roche Diagnostics High Pure RNA Tissue Kit (Roche Diagnostics, Burgess Hill, UK) while RNA from the ileum, jejunum and colon were extracted using QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) as they are more fibrous than liver and pancreas tissue (see chapter 2). The concentration of RNA was measured on Nanodrop® ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and was then diluted and run on an agarose gel stained with ethidium bromide to check for degradation. A sample of diluted RNA was then run on the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to obtain a quantitative measure of RNA degradation. As mentioned previously (see Chapter 2) the Agilent system categorises each sample with a RIN which is indicative of RNA quality hence degree of degradation. Figure 3.1 shows representative electropherograms generated by the Agilent 2100 Bioanalyser. The presence of three peaks denotes intact RNA of good quality. The first peak represents the marker, the second is the 18S fragment and the third is the 28S fragment of the rRNA. The peaks appear earlier, broader and less distinctive when the RNA is degraded. The Agilent software also produces an image much like what is generated from an agarose gel showing what the degree of degradation could potentially look like on the gel (see chapter 2).

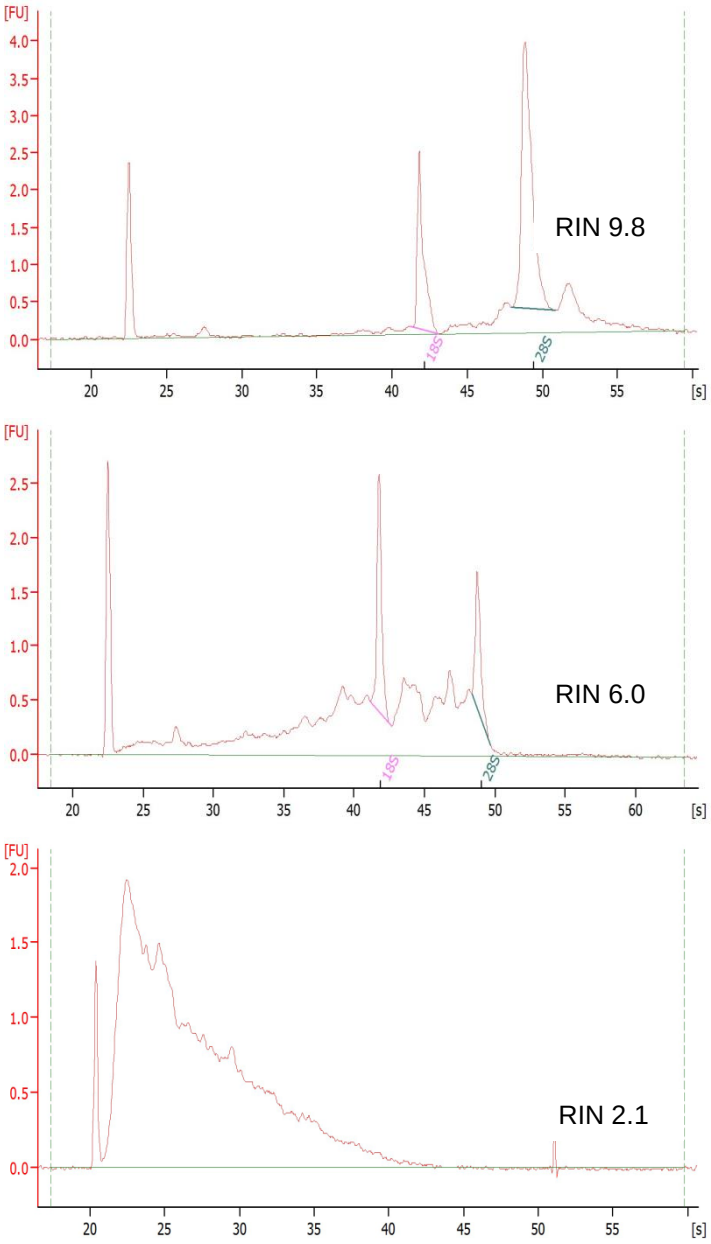


Figure 3.1 Example electrophereograms generated from the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

3.4.3. RT-qPCR

Gene expression analysis was performed on all tissues apart from the pancreas. The specific genes measured and tissue origin are listed in table 3.1. Primer sequences for each of these can be found in Appendix 4.

Table 3.1 Genes measured in the different tissues.

Tissue	Gene
Liver	PCK1, PCK2
Jejunum	CCK, GIP, NTS
Ileum	NTS, PYY
Colon	PP, PYY

Primers (Sigma Aldrich, Dorset, UK) were designed using Primer Express (Applied Biosystems, Paisley, UK) and all nucleic acid amplifications were performed on the Roche LightCycler 480 (Roche Diagnostics, Burgess Hill UK) using SYBR green (Roche Diagnostics, Burgess Hill, UK) master mix and normalised with total cDNA levels (determined using oligreen). Gene expression levels were quantified using a standard curve method.

3.4.4. Human multiplex assay, porcine insulin and CCK ELISAs

The multiplex assay (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA) was carried out as described in Chapter 2 measuring the following hormones; C-peptide, GIP, Insulin,

Leptin, PP and PYY. CCK and insulin were also measured separately using porcine specific ELISAs (Cusabio, Wuhan, China; Mercodia, Uppsala, Sweden). The insulin ELISA results were then compared to the multiplex assay insulin data.

3.4.5. Statistical analysis

A REML approach was used within the software package Genstat 15th Edition (VSN International Ltd, Hemel Hempstead, UK) to assess the statistical difference between the RINs of the four treatment groups. The treatments and tissue type from which the RNA had been extracted from were entered as fixed effects, whilst slaughter location and pig nested within slaughter location were entered as random effects (the S and NS methods had to be carried out in different locations due to slaughter house and meat entering the food chain legislation) (table 3.3). For the gene expression data a two-way ANOVA (Genstat 15th edition, VSN International Ltd, Hemel Hempstead, UK) was performed for each gut hormone in each tissue with slaughter method (NS and S) and fed or fasted as main effects.

A one-way ANOVA (C-Peptide and PYY) or Kruskal-Wallis (GIP, insulin, and PP) test (Genstat 15th edition, VSN International Ltd, Hemel Hempstead, UK) was used to assess the differences between the fed and fasted animals for each of the peptides measured using the multiplex assay in the blood plasma. Both the CCK and insulin ELISAs were analysed using a Kruskal-Wallis test. The Kruskall-Wallis test was

used when the data was not normally distributed whilst a one-way ANOVA was used when the data was normally distributed. When comparing the insulin results for the multiplex assay and ELISA the relationship was assessed using Spearman's rank order correlation (GraphPad Prism 6, GraphPad Software Inc., CA, USA) as well as Lin's concordance as a measure of reproducibility between the two variables (Lin, 1989;IBM SPSS statistics 22, International Business Machines Corp, NY, USA). Statistical significance was accepted at $p<0.05$.

3.5. RESULTS

3.5.1. Effect of slaughter method on RNA quality

There was no effect of conventional slaughter method nor feeding treatment on the RIN values for any tissue (Table 3.3; $p>0.05$ for first two rows) there was also no interaction between the slaughter method, fed or fasted treatment or tissue. However there was a significant difference in RIN between the tissues analysed due to the pancreas having consistently lower RIN values than the other tissues in each treatment group (Table 3.3; $p<0.001$) with mean values in table 3.2.

Table 3.2 RIN for trial 1 presented as mean ± SEM.

	Group			
Tissue	S/Fed (n=6)	S/Fasted (n=6)	NS/Fed (n=5)	NS/Fasted (n=7)
Liver	7.1±0.14	6.2±0.60	6.9±0.50	7.0±0.30
Pancreas	2.4±0.07	2.3±0.05	2.3±0.12	2.3±0.08
Jejunum	6.6±0.88	7.4±0.21	8.06±0.25	7.3±0.88
Ileum	7.7±0.32	8.7±0.18	8.5±0.10	8.0±0.37
Colon	8.8±0.53	8.9±0.28	8.5±0.61	8.7±0.34

Table 3.3 REML statistics table for RIN comparison in trial 1.

	Degrees of Freedom	P-value
NS vs. S	1	0.841
Fed vs. Fasted	1	0.433
Tissue	4	**<0.001**
NS vs. S * Fed vs. Fasted	1	0.448
NS vs. S * Tissue	4	0.542
Fed vs. Fasted * Tissue	4	0.771
NS vs. S * Fed vs. Fasted * Tissue	4	0.161

3.5.2. Gene expression of gut hormones

Gut hormone gene expression levels for all four treatment groups can be seen in figure 3.2 for the liver and jejunum and figure 3.3 for the ileum and colon. The only significant differences detected were for PCK1

expression in the liver and NTS expression in the jejunum. Levels of liver PCK1 mRNA increased in response to overnight fasting in both slaughter groups ($p<0.001$), whereas NTS mRNA in the jejunum decreased in response to the overnight fast in both slaughter groups ($p<0.01$). In each tissue, the slaughter method had no effect on gene expression ($p>0.10$; see table 3.4).

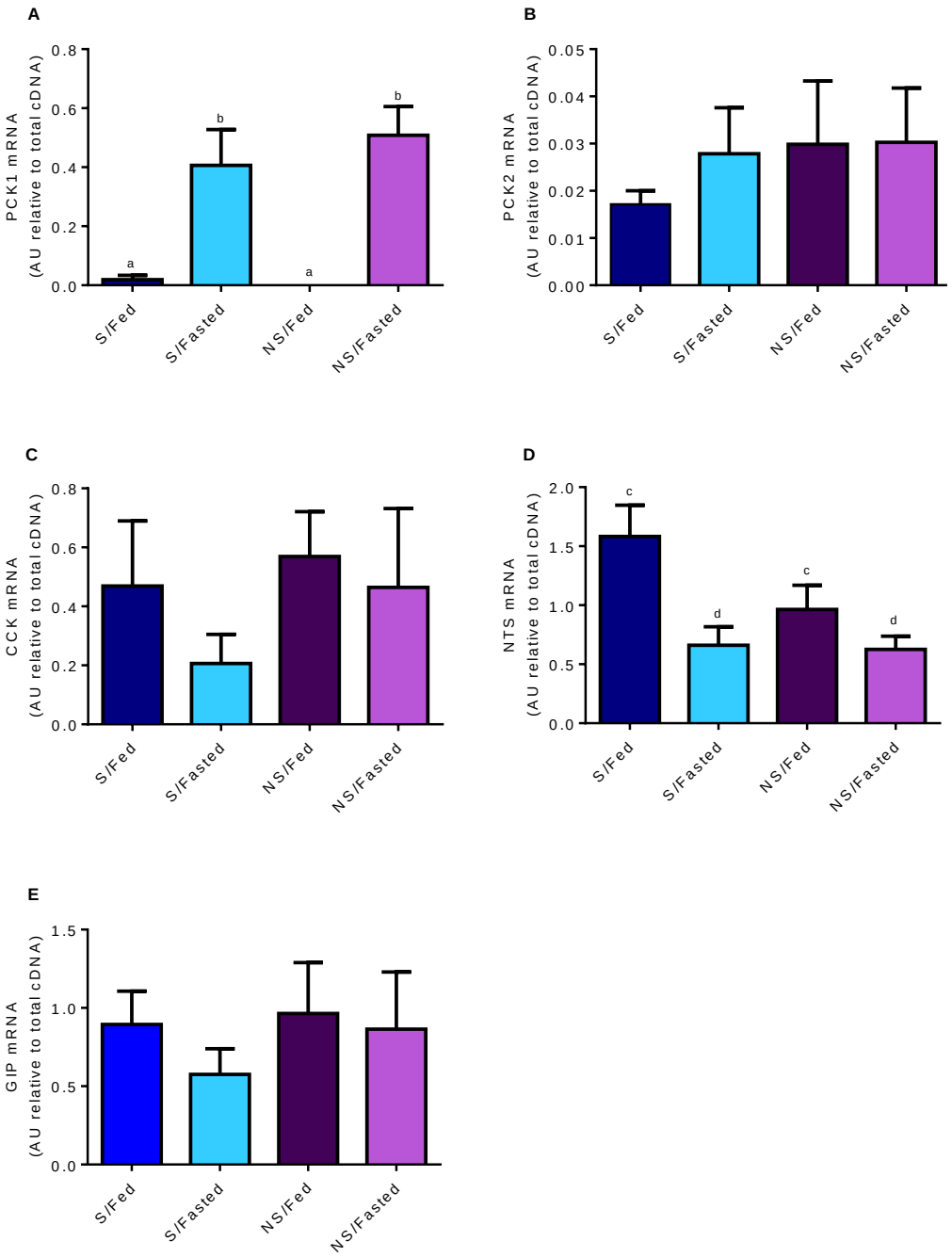


Figure 3.2 Images A-E show gene expression data for liver (A-PCK1 and B-PCK2) and jejunum (C-CCK, D-NTS and E-GIP) tissues (mean±SEM). Different lowercase letters in an image show significant differences between treatment groups (between a and b $p<0.001$; between c and d $p<0.05$). There were no significant interactions ($p>0.05$) between factors for any of the genes.

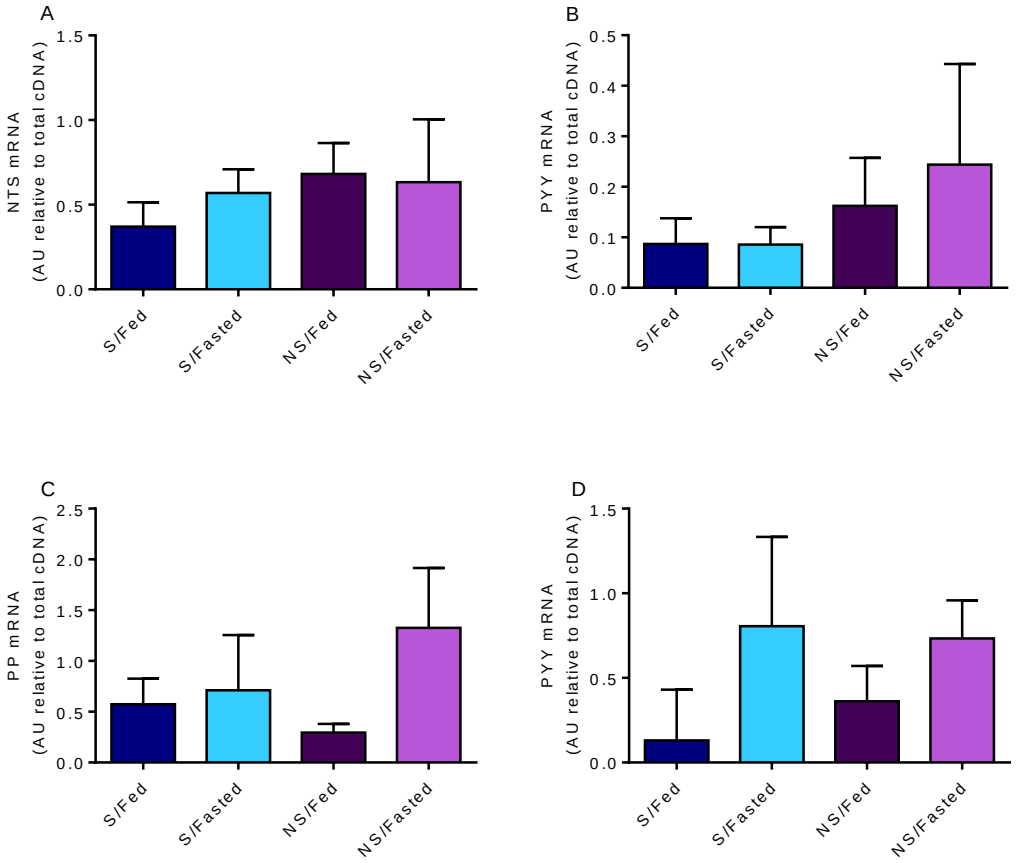


Figure 3.3 Images A-D show gene expression data for ileum (A and B) and colon (C and D) tissues (mean±SEM). There were no significant differences or interactions ($p>0.05$) between factors for any of the genes.

Table 3.4 Two-way ANOVA for gene expression data in trial 2 from each tissue analysed. P-values in bold show significance at p<0.05.

		P-value		
		Slaughter Method	**Fed vs Fasted**	**Interaction**
Liver	PCK1	0.484	**<0.001**	0.574
	PCK2	0.493	0.549	0.622
Jejunum	CCK	0.395	0.381	0.697
	GIP	0.597	0.534	0.741
	NTS	0.129	**0.004**	0.155
Ileum	NTS	0.116	0.433	0.826
	PYY	0.073	0.450	0.312
Colon	PP	0.674	0.151	0.293
	PYY	0.882	0.285	0.990

3.5.3. Gut hormones in circulating plasma

When analysing the data collected from circulating gut/metabolic peptide concentrations in plasma, slaughter method was not included as a factor as all blood samples were collected before the two slaughter methods differed and there was no difference in time taken to collect these samples. For the plasma gut hormone analysis there were significant differences for GIP, insulin and PP between the fed and fasted groups ($p<0.001$, $p=0.002$, $p=0.025$ respectively) using the multiplex assay, with the levels of both hormones decreasing in response to the overnight fast (figure 3.4). This significant decrease was also detected using the porcine ELISA for insulin (figure 3.5) but with a higher significance detected ($p<0.001$). Comparing the two methods the insulin results from the ELISA and multiplex assay were found to be significantly positively correlated ($r=0.9082$, $p<0.001$), as shown in the scatter plot in figure 3.6. However the Lin's concordance value was calculated as 0.261 suggesting that the reproducibility between the two assays is poor. Figure 3.7 shows the results from the CCK ELISA, with plasma CCK concentrations significantly decreased ($p<0.05$) in response to the overnight fast.

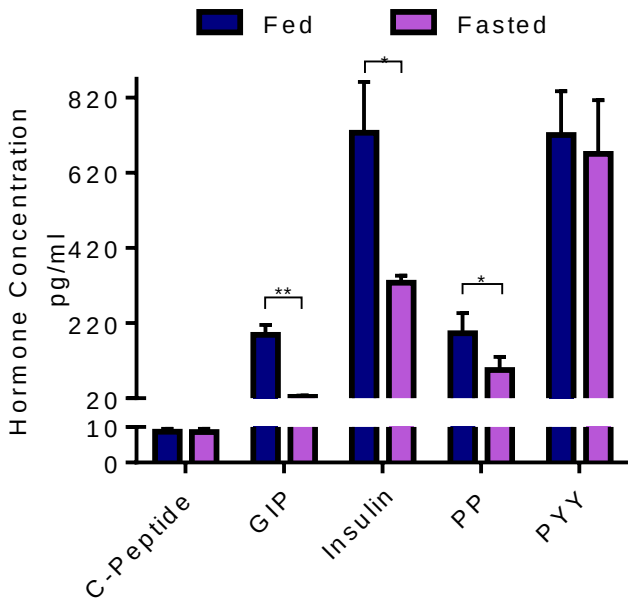


Figure 3.4 Plasma gut/metabolic hormone concentrations from trial 1 (mean±SEM) as measured from plasma using a multiplex assay (Human Metabolic Hormone Milliplex assay, HMHMAG34k kit, Merck Millipore, Billerica, MA, USA)..
* p<0.05
** p<0.001

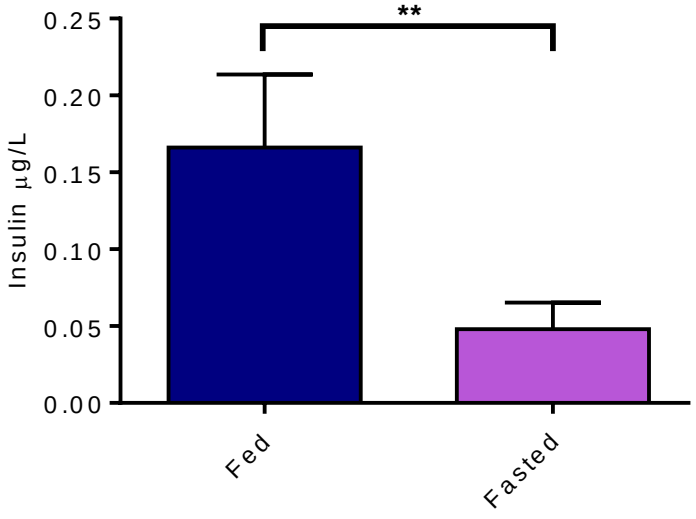


Figure 3.5 Porcine Insulin ELISA (Mercodia, Uppsala, Sweden) concentrations from blood plasma from trial 1 (mean±SEM).
** p<0.001

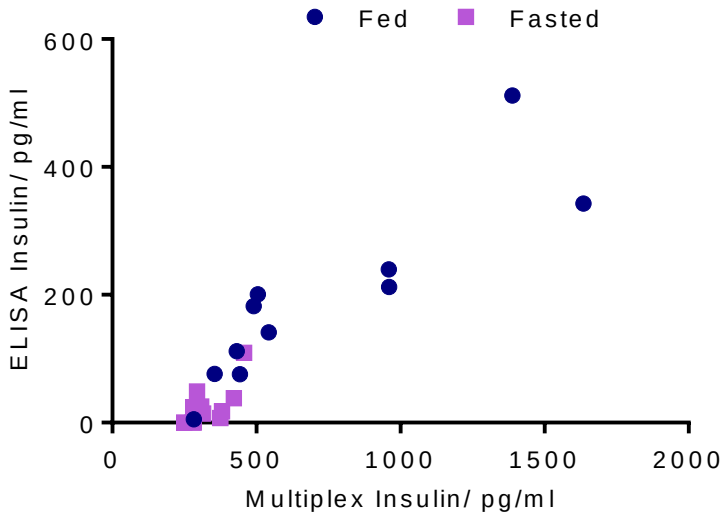


Figure 3.6 Correlation between the Porcine Insulin ELISA (Mercodia, Uppsala, Sweden) results and insulin concentrations from the Human Metabolic Hormone Milliplex assay (HMHMAG34k kit, Merck Millipore, Billerica, MA, USA) in trial 2. The two data sets were found to be significantly correlated ($r=0.9082$, $p<0.001$).

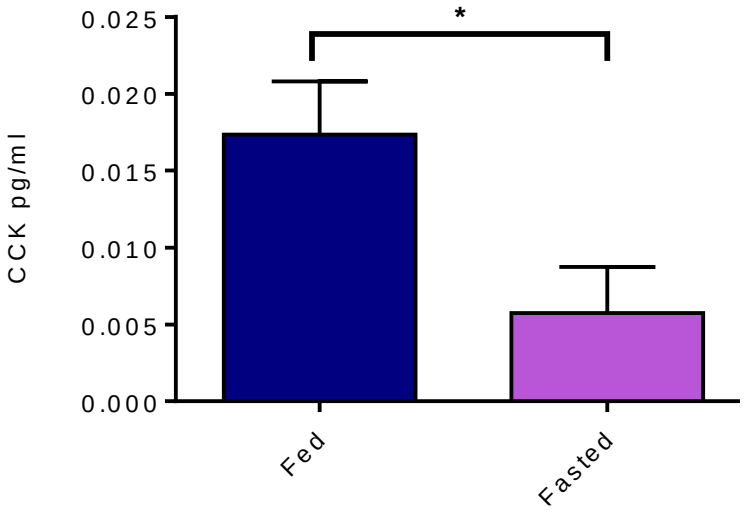


Figure 3.7 Porcine CCK ELISA (Cusabio, Wuhan, China) concentrations from blood plasma samples (mean±SEM).
* $p<0.05$

3.6. SUMMARY

Firstly, the hypothesis that animals subjected to S will have lower quality RNA extracted from their tissues, was shown to be incorrect as the quality of the RNA as assessed through RIN was not significantly lower than that from animals subjected to the NS method. Subsequently this was also mirrored in the gene expression data, with no gene expression levels being significantly affected by the slaughter method. This suggests that even though there is an increase in time of collection of sample and the carcasses from which the samples were obtained are subjected to 60°C heated water before being appropriated, total RNA isolated from S animals are of high enough quality to then be used in downstream analyses, in this case RT-qPCR, and there are no effects on the expression of the genes measured.

Furthermore when examining the gene expression data only two genes were affected by the overnight fast, these being PCK1 in the liver and NTS in the jejunum, with both reacting as expected as described in the hypothesis. As all of the gut hormones examined respond to food being present in the GIT, the fasting time may not have been long enough to clear the tract entirely of digesta, specifically in the distal part of the tract, therefore this may be the reason why the expression of gut hormone genes measured in the ileum and colon were not affected by the overnight fast.

When using the multiplex assay to measure the gut hormone peptides in circulating plasma only GIP, insulin and PP decreased in response to the fast as hypothesised. The ELISAs performed on the plasma showed that both insulin and CCK decreased in response to the fast which was also as expected. When comparing the two methods (multiplex vs. ELISA) of measuring plasma insulin there was a strong positive correlation between the two data sets, however through further analysis the reproducibility between the two methods was found to be poor. Therefore this suggests that the human multiplex assay can be used for analysing pig peptides, as the human and pig peptide sequences are similar enough that the antibodies, raised against the human peptides, are able to cross-react with the pig peptides, however the data needs to be viewed with some degree of caution as there seems to be a factorial difference between the two sets of data (potentially one assay has a higher accuracy than the other; the multiplex showed much higher concentration of insulin compared to the corresponding ELISA concentration), which has led to Lin's concordance to suggest that the reproducibility between the two methods is poor.

3.7. DISCUSSION

The measurement of tissue gene expression, for example in real time PCR analyses, is dependent upon obtaining RNA of good quality (Fleige and Pfaffl, 2006). It is widely accepted that RNA degrades easily and time has been identified as a factor in this. Jorbarteh et al. (2013) demonstrated that that as total processing time of sample increased, the

quality of RNA (measured using RIN) decreased linearly, but a RIN above 6 was still achievable up to a processing time of 60 mins, this is in agreement with the findings in the current study where RINs above 6 were acheivable after a processing time of 25 mins. Until now it was assumed that commercial slaughtering methods would not yield RNA of good quality, due to the carcasses being subjected to heat associated with scalding (certain RNases have been found to have an optimum activity level at 50°C and still work above 80% optimal activity at 70-75°C (Burka, 1969)), as well as the time delay in sample collection. The current study clearly shows that RNA quality was not affected by conventional slaughter nor if the pig was fed or fasted before slaughter, however it did significantly vary between the tissues types. It has previously been shown that pancreatic (Griffin et al., 2012) and jejunal (Fleige & Pfaffl, 2006) RNA are difficult to extract, which may relate to them having a high content of RNase enzymes and being a fibrous tissue (dependant on extraction method), respectively. This may be a contributing factor to the differences seen in mean RINs of the tissues in the current study. RNA samples with RIN values greater than 7 are considered of good quality (Udvardi et al., 2008) for use in most analysis methods (e.g. RT-qPCR), although some methods require RINs of 8 or more (e.g. deep sequencing; Guerau-de-Arellano et al., 2012). Most of the RIN values obtained were close to these benchmarks or marginally below. Therefore the RNA from the GIT tissues and liver was deemed of suitable quality to carry out RT-qPCR to assess gene expression in this instance.

The gut hormones chosen to be analysed are all up-regulated when food enters the GI tract, however in most of the tissues there was no statistical difference between the fed and fasted groups. The only gene that did change significantly with fasting was NTS mRNA in the jejunum, with expression being higher in the fed group, as expected. In the liver, PCK1 mRNA was seen to be dramatically affected by fasting, increasing significantly showing that these animals were in a fasted state. However, even with this being the case, the possible reasoning for not seeing a change in expression for the other genes may be that the fasting time (18 hours on average in the current study) was not long enough to completely empty the tract of digesta, specifically the ileum and colon, hence the fasted values are closer to the fed values as the hormones were still being expressed while digesta remained in these parts of the tract.

The current study also showed that porcine plasma can be analysed using a multiplex assay designed for use with human samples. As mentioned previously human and pig cDNAs and also peptide sequences are highly conserved (Appendix 3). Plasma GIP, insulin and PP concentrations significantly decreased in response to the fast, which follows what is known about these hormones from the literature review in chapter 1 (Seino et al, 2010; Holzer et al., 2012; Röder et al., 2016). PYY however did not and this may be accounted for by the fact that the fast may not have been long enough to completely clear the distal portion of the GIT of digesta. As PP is produced in the distal small intestine as

well as the pancreas, this may be why a significant difference was found for plasma PP concentrations compared to the no difference in fed and fasted animals for PYY. The insulin data were also compared to results obtained from a porcine insulin ELISA and it was found that the two data sets were highly correlated but had poor reproducibility, supporting the theory that the human multiplex assay can be used to measure porcine peptides, however as already indicated some caution over the accuracy of the results is needed.

In summary, the integrity of RNA extracted from tissue samples was not altered by conventional slaughter procedures, however it was dependent on the tissue it was extracted from. The intact RNA was then used to analyse gut hormone gene expression in GI tract tissues using RT-qPCR from both slaughter groups. Therefore RNA samples of good quality can be obtained from animals put through conventional slaughter and used in downstream analyses, and the carcass can then be sold on for human consumption, thereby lowering the cost of the study. Furthermore due to the high conservation of gut peptides sequences between humans and pigs, a human multiplex assay was used successfully to measure peptides from porcine plasma, reducing the time taken to obtain results (but may overestimate the concentration).

4. TRIALS 2 AND 3: THE EFFECT OF XYLANASE SUPPLEMENTATION ON GUT HORMONES IN NEWLY WEANED PIGLETS.

4.1. TRIAL 2

4.1.1. Introduction

As previously stated in chapter 1 the effects of xylanase have been found to be sensitive to the age at which the enzyme is supplemented to the animal. Therefore it seems plausible that the timing of supplementation is a key attribute in the enzymes efficacy to increase desirable performance factors. Unpublished data from another study supported by AB Vista showed that when piglets on a maize-based diet were supplemented xylanase (Econase XT, AB Vista, UK) from two weeks post-wean to six weeks post-wean, these animals were heavier than those that had been supplemented the enzyme immediately post-wean.

Singh et al. (2012) demonstrated in broilers that xylanase had an upregulating effect on serum peptide YY (PYY) levels when fed a maize-soybean based feed when measured at 42 days old. Another study also corroborated this finding in broilers fed a maize-soybean based feed, showing PYY was significantly increased ($p<0.001$) in blood plasma samples in response to the addition of xylanase in the feed (Masey O'Neill et al., 2012). PYY is mainly known as a satiety hormone, suppressing appetite in response to the stimulus of food entering the digestive tract. It also increases feed retention time by inhibiting digestive

tract motility and emptying of the stomach contents into the small intestine. It has been suggested that these mechanisms contribute to xylanases efficacy in improving feed digestibility and subsequently improved performance measurements (Singh et al., 2012; Masey O'Neill et al., 2014).

At the time of planning the current study there had yet to be any studies to explore if xylanase had any impact on gut hormones when supplemented in commercial pig diets. Hence the aim of the current study was, to **a)** recreate the previous AB Vista pig study but with a wheat-based diet and examine if the same effects on growth performance were seen; and **b)** investigate if any gut hormone levels were affected by measuring gene expression in the gut tissue and circulating peptide levels in blood plasma.

4.1.2. Objectives

- Monitor growth performance of newly weaned piglets throughout a six week period when supplemented with xylanase for differing periods of time and either starting at 0 or 2 weeks post-wean.
- Measure gut hormone levels in response to xylanase supplementation at six weeks post-wean, through circulating plasma protein levels and gene expression measurements in the GIT.

4.1.3. Hypothesis

Piglets supplemented with xylanase immediately post-wean will have the highest PYY concentrations, and will also increase other hormones associated with the inhibition of gut motility. Growth will also be affected, with piglets that are supplemented the enzyme from 2 weeks post-wean expected to have the heaviest end-point weight at 6 weeks post-wean.

4.1.4. Materials and Methods

The following description of the materials and methods used are just a brief outline. For a more in depth explanation please see Chapter 2.

4.1.4.1. Animals and experimental design

Thirty-two female weaned piglets (28d old) were randomly assigned to 1 of 2 diet groups on arrival (full diet composition can be found in Appendix 1). In period 1 (0-2 weeks post-weaning) a control diet (Co) or the same diet supplemented with xylanase (XS) (16,000 BXU/g, Econase XT, AB Vista, UK) was provided to the piglets. During period 2 (2-6 weeks post-weaning) half the pigs were kept on their original diet whilst the rest were swapped onto the other diet. This resulted in four treatment groups (2 x 2 factorial design; see figure 4.1) with an n of 8 animals in each treatment group where one group had been kept on the control diet for the full 6 weeks post-wean (Period 1: Co, Period 2: Co), another which had only been on the xylanase supplemented diet (Period 1: XS, Period 2: XS) and two groups that had been on both diets at different times and varying lengths of time (Period 1: Co, Period 2: XS; Period 1: XS, Period 2: Co).

The animals were weighed weekly and their feed was weighed out at the beginning of the week ready to be distributed daily. Feed refusals were measured daily to calculate weekly feed intake throughout the duration of the trial. This was also used along with weekly weight gain to calculate weekly feed efficiency, expressed as FCR (FCR= weekly feed intake/ weekly weight gain). At the end of period 2 all of the pigs were culled by electrical stun and exsanguination. It was at this point that all samples (blood and tissue) were collected for analysis and stored as described in Chapter 2.

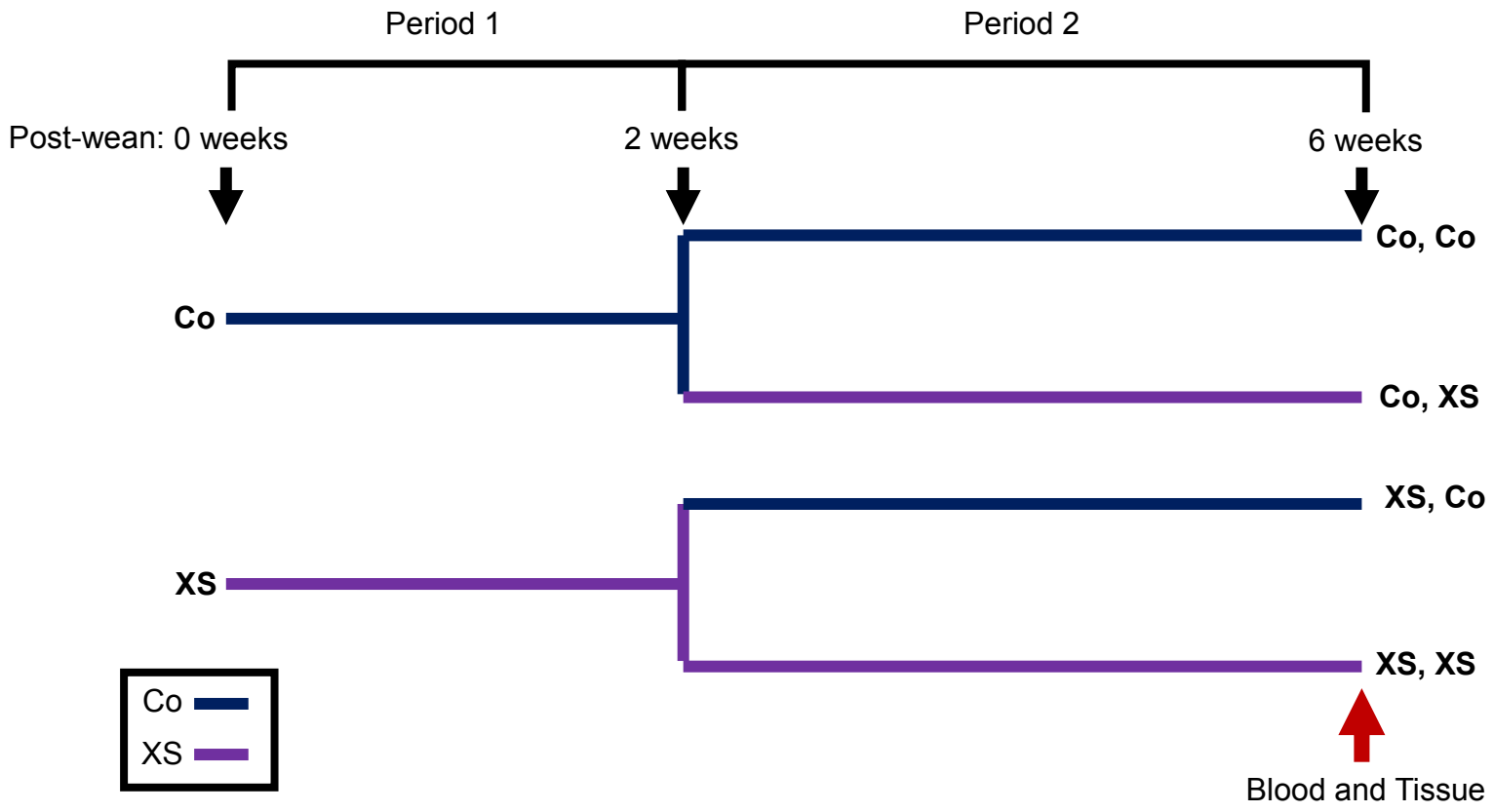


Figure 4.1 Experimental trial design for trial 2.

4.1.4.2. Gut hormone gene expression analysis

As described in chapter 2 the gut tissues (jejunal, ileal and colonic) were crushed and RNA extracted using QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) and quantified on the Nanodrop® ND1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA was then diluted and run on an agarose gel stained with ethidium bromide to check for degradation. First strand cDNA was synthesised using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Burgess Hill, UK) and was then used in RT-qPCR to identify the relative gene expression of the gut hormone genes in table 4.1. Primer sequences for these genes can be seen in Appendix 4.

Table 4.1 Tissue from which the expression of indicated genes were measured by RT-qPCR for trial 2 and 3.

Tissue	Gene
Jejunum	CCK, GIP, NTS
Ileum	NTS, PYY
Colon	PYY, GLP-1

4.1.4.3. Measurement of circulating gut hormones in pig plasma

Multiple metabolic/gut hormones were measured simultaneously using a human multiplex assay kit (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA). Aliquots of 100µl of plasma for each pig were defrosted on ice and 25µl, in duplicate, was

pipetted onto a 96-well plate along with standards and quality controls and incubated overnight with colour-coded antibody coupled beads for each analyte. The plate was then incubated briefly with a detection antibody and a fluorescence activated dye and then run on MAGPIX® System (Merck Millipore, Billerica, MA, USA) using xPONENT® Software (Merck Millipore, Billerica, MA, USA) to quantify the following metabolic hormones; C-Peptide, GIP, Insulin, Leptin, PP and PYY. The inter- and intra-assay precision for each analyte expressed as a coefficient of variation (CV) used in the kit can be found in table 4.2.

Insulin was also measured separately using a porcine specific insulin ELISA (Mercodia, Uppsala, Sweden) which had intra- and inter-assay CVs of 3.5% and 2.8% respectively. The multiplex assay insulin data were then compared to the data from the insulin ELISA.

Table 4.2 Inter- and intra-assay CV% for gut/metabolic peptides measured using a human multiplex assay (provided by the manufacturer).

Peptide	Inter-assay CV%	Intra-assay CV%
C-peptide	6	2
GIP	5	3
Insulin	6	3
Leptin	4	3
PP	7	4
PYY	11	2

4.1.4.4. Statistical analysis

Growth performance (including initial weight, final weight, total weight gain and carcass weight), gene expression and circulating metabolic/gut hormone peptides in plasma data were analysed using a two-way ANOVA (Genstat 15th Edition, VSN International Ltd, Hemel Hempstead, UK) factors being supplementation (Co vs. XS) and period of treatment (Period 1 vs. Period 2) . ADWG, FCR and ADFI were analysed using a repeated measures two-way ANOVA (GraphPad Prism 6, GraphPad Software Inc., CA, USA) with age of measurement and treatment group as factors. A Bonferroni multiple comparisons test was also performed for the FCR analyses. When comparing the insulin results for the multiplex assay and ELISA the relationship was assessed using Pearson's r correlation (GraphPad Prism 6, GraphPad Software Inc., CA, USA) as well as Lin's concordance as a measure of reproducibility between the two variables (Lin, 1989; IBM SPSS statistics 22, International Business Machines Corp, NY, USA).

4.1.5. Results

4.1.5.1. Animal performance

Final live weights for the pigs were not found to be significantly different nor carcass weight or total weight gain (table 4.3) between the four treatment groups. Furthermore there was no significant difference between the treatment groups for ADWG nor ADFI (figures 4.3 and 4.4) but were significantly different between age of measurement, which was expected (table 4.4). The analyses in table 4 shows that FCR had a

significant interaction between the factors (age at measurement vs. treatment). A multiple comparisons test revealed that when the pigs were 56d old group Period 1: XS, Period 2 XS was significantly different from all three of the other groups ($p<0.01$). Some FCR values recorded were not biologically possible as the animal would have been gaining more weight than it was consuming feed wise, this was put down to the animals spilling their feed and urinating on it or spilling water on it. This meant that some of the feed refusals had to be estimated based on size relative to weighed out portions of dry feed, so are likely to be inaccurate (see Chapter 2, section 2.3. Estimating feed intake).

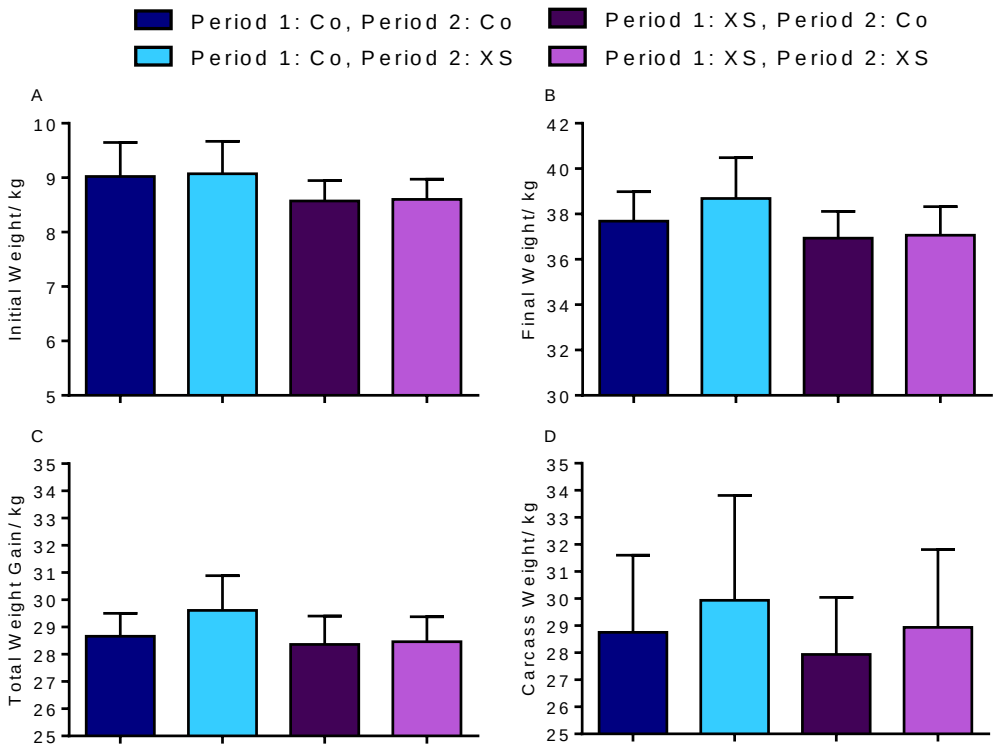


Figure 4.2 Animal growth performance data for trial 2, showing (A) initial weight, (B) final weight, (C) total weight gain and (D) carcass weight (mean±SEM). Analyses using a two-way ANOVA resulted in there being no interactions and no difference between the two periods.

Table 4.3 P-values from two-way ANOVA analyses for initial weight, final weight, total weight gain and carcass weight for trial 2.

	Initial weight	**Final Weight**	**Total weight gain**	**Carcass weight**
Period 1 (Co vs XS)	0.366	0.405	0.488	0.399
Period 2 (Co vs XS)	0.941	0.692	0.615	0.310
Interaction	0.980	0.758	0.683	0.930

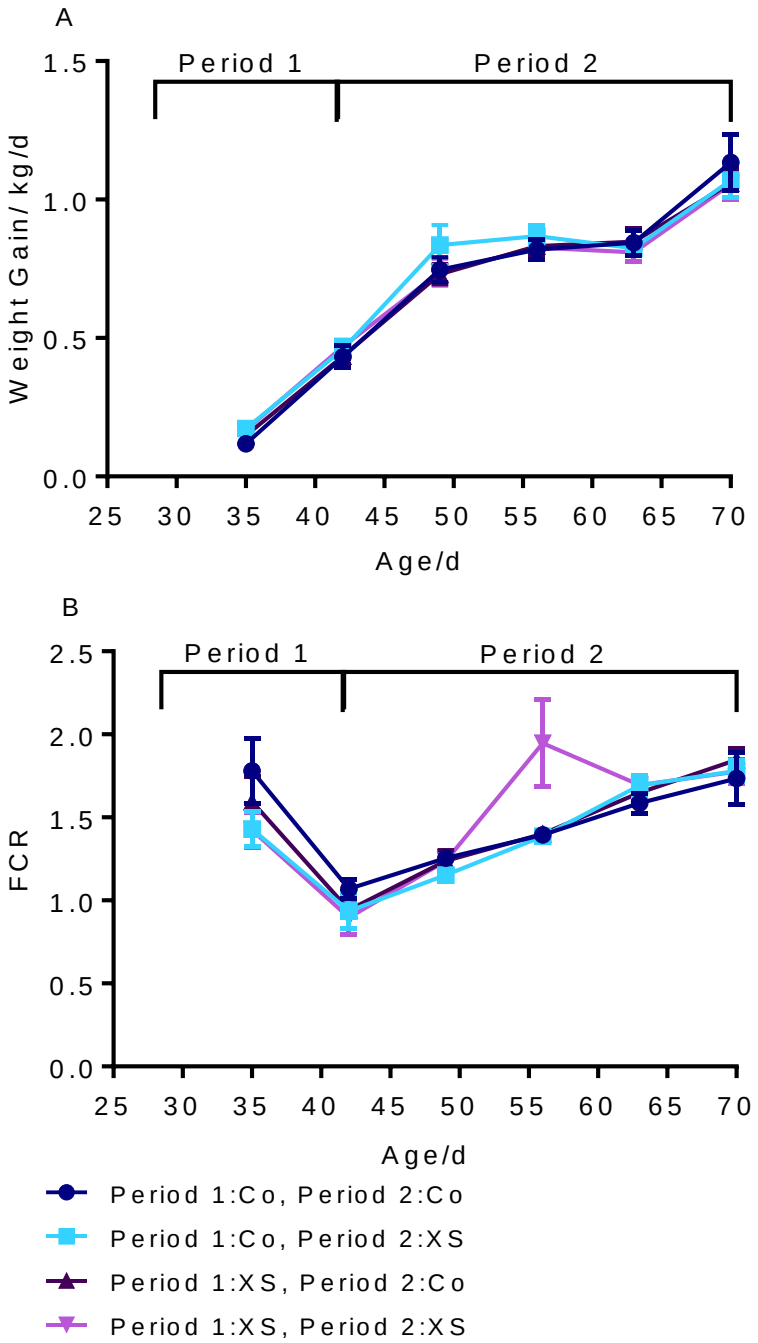


Figure 4.3 Line graphs depicting (A) weight gain per day/kg and (B) FCR for trial 2 (mean±SEM). Analyses using a repeated measures two-way ANOVA resulted in there being no difference detected between the groups at any time point.

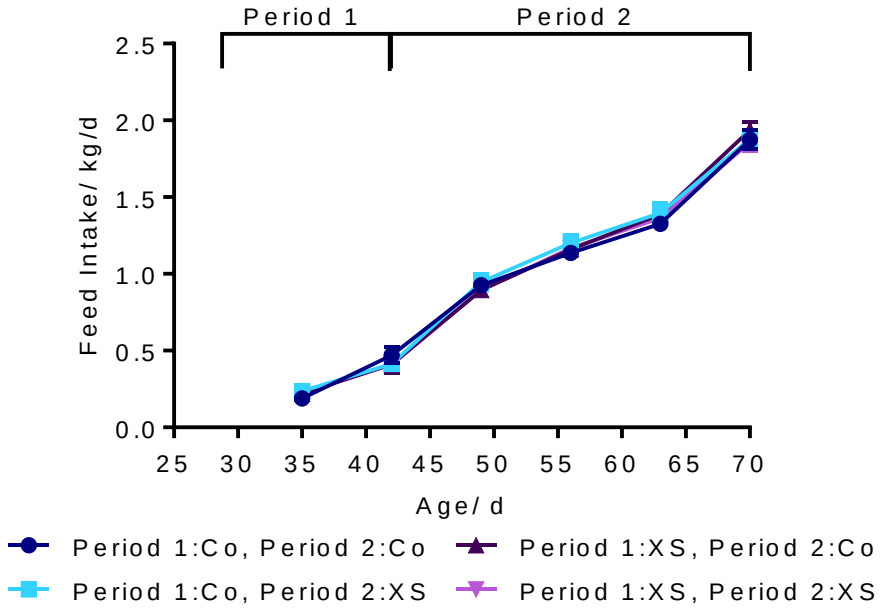


Figure 4.4 Daily feed intake data for trial 2 (mean±SEM). A repeated measures two-way ANOVA showed no difference between the four treatment groups at any time point.

Table 4.4 P-values from repeated measures two-way ANOVA for ADWG, FCR and ADFI for trial 2.

	P-value		
	ADWG	**FCR**	**ADFI**
Age	**<0.0001**	**<0.0001**	**<0.0001**
Treatment	0.8197	0.4901	0.9257
Interaction	0.9218	**0.0172**	0.8056

Table 4.5 P-values for Bonferroni multiple comparisons test for FCR measurement taken at age 56d.

Treatment comparison	P-Value
Period 1: Co, Period 2: Co vs. Period 1: Co, Period 2: XS	>0.999
Period 1: Co, Period 2: Co vs. Period 1: XS, Period 2: Co	>0.999
Period 1: Co, Period 2: Co vs. Period 1: XS, Period 2: XS	0.0016
Period 1: Co, Period 2: XS vs. Period 1: XS, Period 2: Co	>0.999
Period 1: Co, Period 2: XS vs. Period 1: XS, Period 2: XS	0.0013
Period 1: XS, Period 2: Co vs. Period 1: XS, Period 2: XS	0.0018

4.1.5.2. Effect of xylanase on piglets' gut hormone gene expression

The gene expression data for the jejunum can be seen in figure 4.5 and for ileal and colonic tissue figure 4.6. The statistical analysis for each of the genes can be found in table 4.7. There were no significant differences when supplementing xylanase in either period 1 or 2 and no interactions between the two periods of supplementation either.

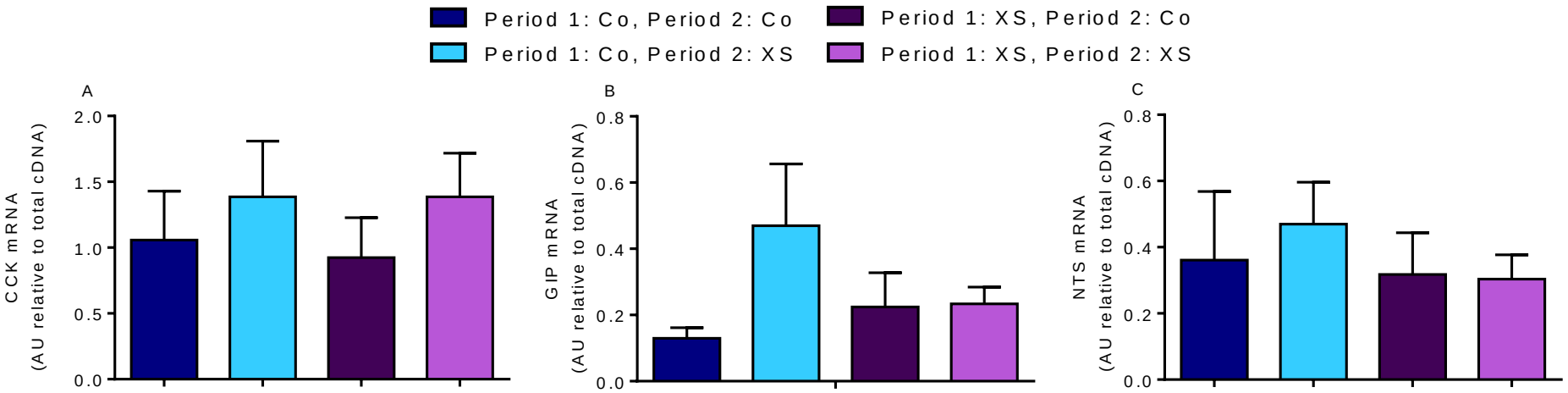


Figure 4.5 Gene expression data for trial 2 in the jejunum for (A) CCK, (B) GIP and (C) NTS (mean±SEM).

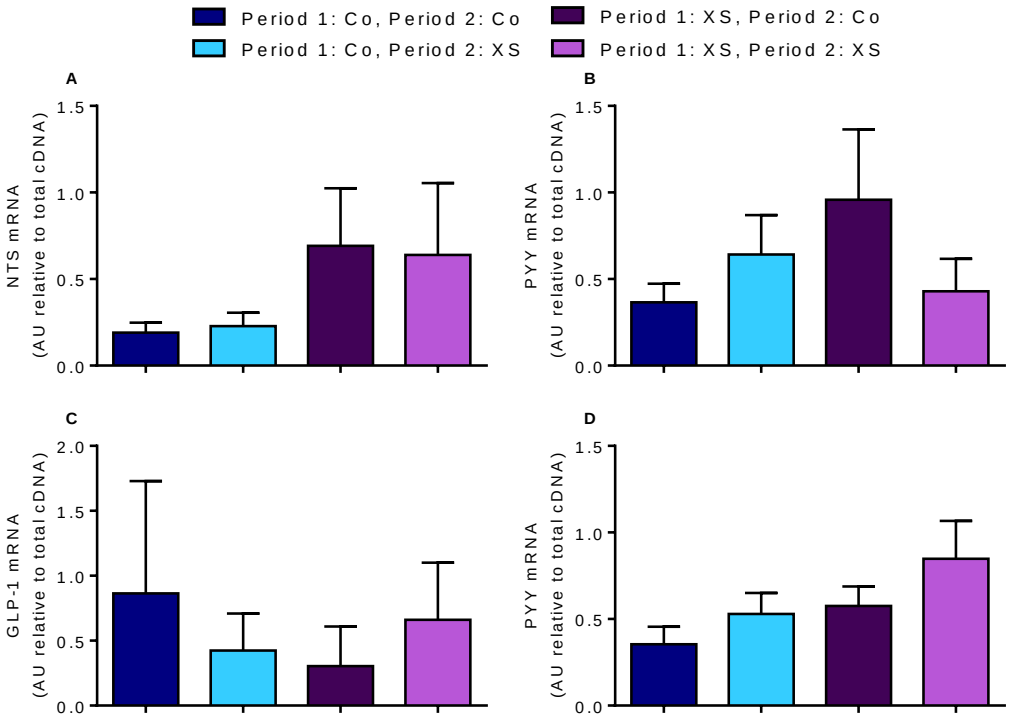


Figure 4.6 Gene expression data for trial 2 measuring (A) NTS and (PYY) in the ileum and (C) GLP-1 and (PYY) in the colon (mean±SEM).

Table 4.6 P-values for two-way ANOVA results for gene expression data in trial 2.

		P-value		
		Period 1 (Co vs XS)	Period 2 (Co vs XS)	Interaction
Jejunum	CCK	0.914	0.267	0.854
	GIP	0.549	0.126	0.146
	NTS	0.468	0.741	0.669
Ileum	NTS	0.102	0.979	0.869
	PYY	0.450	0.692	0.117
Colon	GLP-1	0.761	0.937	0.457
	PYY	0.075	0.138	0.742

4.1.5.3. Effect of xylanase on circulating gut hormone plasma levels

The end-point measurements of circulating metabolic/gut peptides are shown in figure 4.7, with the p-values from the statistical test in table 4.8. There were no interactions seen between the two xylanase inclusion periods for any hormone analysed (table 4.8). At 6 weeks post-wean, the plasma C-Peptide and insulin concentrations tended to be lower ($p=0.059$ and $p=0.071$ respectively), in the groups that were supplemented xylanase immediately post-wean for 2 weeks (i.e. in period 1), irrespective of the treatment in period 2. In contrast, PYY concentrations were lower ($p=0.008$) when xylanase was supplemented at 2-6 weeks post-wean (i.e. in Period 2), irrespective of treatment in period 1. When measuring insulin using a pig specific ELISA the data shown in figure 4.8 closely followed the pattern seen in figure 4.7 for insulin measured using the human multiplex assay. However when the ELISA data was analysed there was also no significant difference or tendency to be different when xylanase was supplemented in either period. When the relationship between the two sets of data (insulin multiplex and ELISA) were analysed the two sets of data were significantly correlated ($r=0.7542$, $p<0.001$), which can be seen in figure 4.9. However the Lin's concordance value was calculated as 0.096 suggesting that the reproducibility between the two assays was poor.

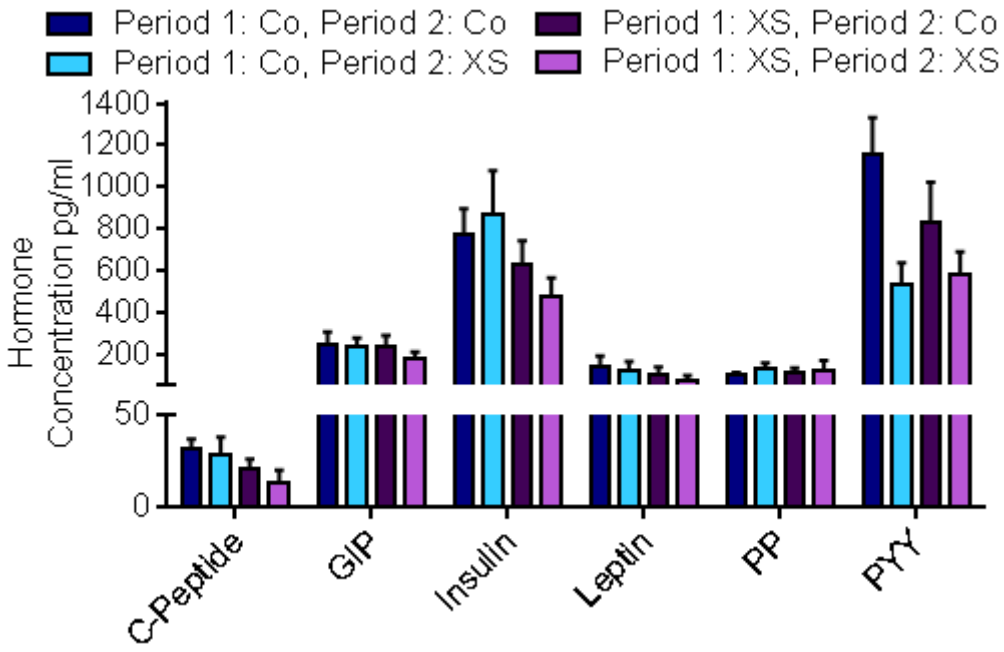


Figure 4.7 Circulating metabolic/gut peptide levels from trial 2 (mean±SEM), as measured from plasma using a multiplex assay (Human Metabolic Hormone Milliplex assay, HMHMAG34k kit, Merck Millipore, Billerica, MA, USA).

Table 4.7 P-values from the two-way ANOVA data for metabolic/gut peptides in trial 2, measured using a Human Metabolic Hormone Milliplex assay (HMHMAG34k kit, Merck Millipore, Billerica, MA, USA).

	P-value					
Factor	C-peptide	GIP	Insulin	Leptin	PP	PYY
Period 1 (Co vs. XS)	0.059	0.464	0.071	0.283	0.984	0.381
Period 2 (Co vs. XS)	0.447	0.554	0.831	0.523	0.447	**0.008**
Interaction	0.731	0.634	0.377	0.904	0.706	0.225

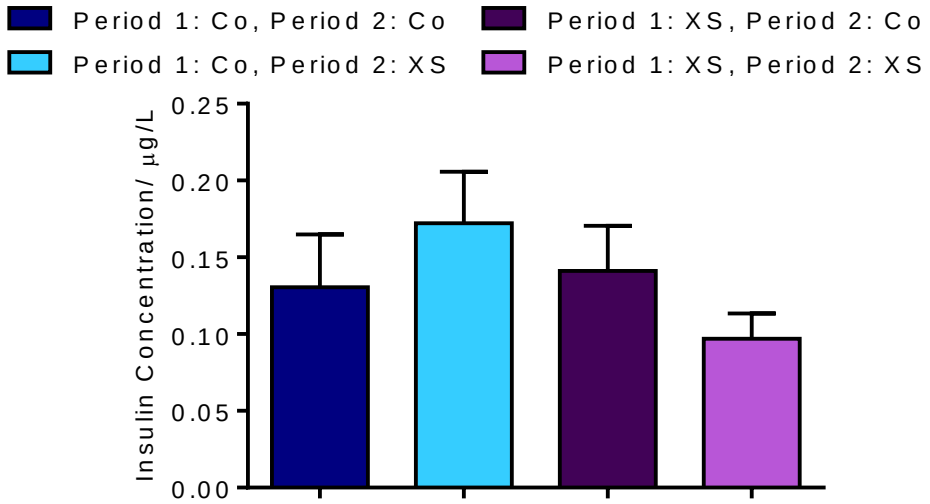


Figure 4.8 Insulin levels in circulating plasma for trial 2 (mean±SEM), measured using a porcine insulin ELISA (Mercodia, Uppsala, Sweden).

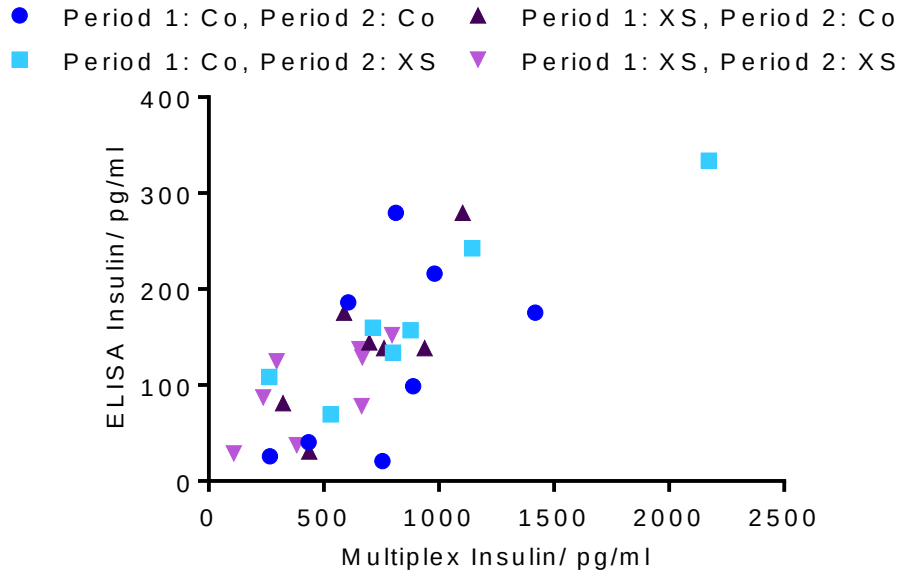


Figure 4.9 Scatter plot showing the relationship between insulin levels measured using a porcine insulin ELISA and a human multiplex assay in trial 2. The two data sets were found to be significantly correlated ($r=0.7542$, $p<0.001$).

4.1.6. Summary

From these results it can be concluded that when measured between 0 and 6 weeks post-wean xylanase supplementation has no significant effect on weight gain, FCR and other growth performance parameters in pigs fed on a wheat-based feed. In blood plasma samples taken at 6 weeks post-wean it was intriguing to find there was a decrease in PYY levels in the two groups where the piglets were supplemented the enzyme between weeks 2 and 6 post-wean, but there was no increase in feed intake detected which might be expected due to the hormones effects on satiety (see chapter 1). This may be due to the complications that arose when trying to measure feed refusals described in chapter 2.

The tendency for reduced levels of insulin and C-peptide (which are both produced in the pancreas from the same prohormone), seen in in the two groups where the piglets were supplemented xylanase in the first 2 weeks post-wean, suggest that the enzyme may affect pancreatic activity or development, which may not have been found to be significant due to the bloods being taken 4 weeks after the change over time point, particularly for the groups that was then put onto the control diet. The group supplemented xylanase for the entirety of the trial (Period 1: XS, Period 2: XS) had lower plasma concentrations of these two hormones than the group that was supplemented only immediately post-wean to 2 weeks post-wean (Period 1: XS, Period 2: Co) suggesting these animals were able to ‘catch-up’ in the 4 weeks prior to blood collection back to levels similar to the groups fed the control diet in period 1. Lastly as in

trial 1 (chapter 3) insulin data for both the human multiplex assay and porcine ELISA were found to be significantly correlated however reproducibility was poor, again suggesting that the human multiplex assay can measure porcine peptides but results should be viewed with caution due to the factorial difference between the two sets of data.

4.2. TRIAL 3

4.2.1. Introduction

The results in trial 2 looking at supplementation of xylanase from wean to 6 weeks post-wean suggest that supplementation in the first 2 weeks post-wean may have an effect on the activity of the pancreas. It was found that there was a tendency for insulin and C-peptide, hormones that are produced and secreted simultaneously from the β-cells of the pancreas, to be decreased when animals were supplemented with xylanase during this early period (0-2 weeks post-wean). Insulin is a key pancreatic hormone involved in glucose homeostasis and a decrease in its production or secretion during a fed state may mean that there may be change in the animals' insulin sensitivity (Arnoff et al., 2004).

Furthermore in trial 2, PYY was found to decrease when xylanase had been supplemented from 2-6 weeks post-wean, but not when it was supplemented before this point. Therefore it was of interest to go back to the 2 weeks post-wean time point and analyse the levels of the gut hormones to see if there was an explanation for the slight decrease in levels of insulin and C-peptide, and to corroborate that there would be no change in PYY levels between xylanase supplemented and non-supplemented piglets.

4.2.2. Objectives

The objectives of trial 3 were similar to those in trial 2:

- Monitor growth performance of newly weaned piglets throughout a two week period when provided with a wheat-based feed either containing the xylanase enzyme or not.
- Measure gut hormone levels in response to xylanase supplementation at two weeks post-wean, through circulating plasma protein concentrations and gene expression measurements.

4.2.3. Hypothesis

Piglets supplemented the enzyme xylanase in the first two weeks post-wean will have lower insulin and C-peptide levels than those fed with the control diet. PYY levels on the other-hand will not be affected as well as growth performance parameters.

4.2.4. Materials and Methods

The following description of the materials and methods used are just a brief outline. For a more in depth explanation please see Chapter 2.

4.2.4.1. Animals and experimental design

Sixteen female weaned piglets (28d old) were assigned to one of two diet groups upon arrival, either Co or XS (16,000 BXU/g, Econase XT, AB Vista, UK) which were the same diets used in trial 2. Unfortunately one piglet from each treatment group had to be culled due to ill health. Feed

was provided twice daily to ensure *ad libitum* access, and the weights of the piglets were measured weekly and feed intake was monitored through measuring daily feed refusals only in the second week of the study. After 2 weeks the piglets were culled through electrical stunning and exsanguination (NS). It was at this point that all blood and tissue samples for analysis were collected and stored as described in Chapter 2.

4.2.4.2. Gene expression analysis using RT-qPCR

The genes and the tissues in which they were measured in can be seen in table 4.1. As previously described total RNA was extracted (QIAGEN RNeasy Mini Kit, QIAGEN, Hilden, Germany) from each tissue that had previously been crushed into a frozen powder, quantified (ND1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA) and was then diluted and run on an agarose gel stained with ethidium bromide to check for degradation. From the RNA samples first strand cDNA was prepared (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Burgess Hill, UK) and used to measure relative abundancies of each gene through RT-qPCR.

4.2.4.3. Human multiplex assay and porcine CCK ELISA

The peptides that were measured using a multiplex assay (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA) and the inter- and intra-assay CVs can be seen in table 4.9. More peptides could be analysed (amylin, GLP-1 and ghrelin) due to the

addition of protease inhibitors in blood collection tubes in this trial (see Chapter 2, section 2.5.1. Blood collection). CKK was also measured using a porcine specific ELISA (Cusabio, Wuhan, China) and had an intra- and inter-assay CV of <8% and <10% respectively.

Table 4.8 Inter- and intra-assay CV% for peptides measured using the human multiplex assay in trial 3 (provided by the manufacturer).

Peptide	Inter-assay CV%	Intra-assay CV%
Amylin	33	2
C-peptide	6	2
Ghrelin	8	2
GIP	5	3
GLP-1	10	7
Insulin	6	3
Leptin	4	3
PP	7	4
PYY	11	2

4.2.4.4. Statistical analysis

Since some of the hormone data was found to not be normally distributed (amylin, ghrelin, GLP-1, insulin, leptin and CCK) they were analysed using a Kruskal-Wallis test, whereas the hormones that were normally distributed (C-peptide, GIP and PYY) were analysed using a one-way ANOVA. All animal growth parameters (table 4.10) were normally distributed and therefore analysed using a one-way ANOVA, apart from

carcass weight which was analysed with Kruskal-Wallis as the data were not normally distributed. The gene expression data that were not normally distributed (jejunal CCK and NTS and colonic GLP-1) were also analysed by Kruskal-Wallis and the rest were analysed by one-way ANOVA as they were normally distributed (jejunal GIP, ileal NTS and PYY and colonic PYY). Pearson's r correlation (normally distributed) and Spearman's rank order (not normally distributed) were used to assess the relationship between the gene expression data collected and gut peptide plasma concentrations, for both trial 2 and 3.

4.2.5. Results

4.2.5.1. Animal Performance

As seen in table 4.10 there was no significant effects of xylanase supplementation when looking at growth performance parameters. When compared to results from the end of period 1 (0-2 weeks post-wean) in trial 2 the variability measured in CV% of feed intake and FCR at the end of the second week in trial 3 was reduced by 17.6% and 13.4% respectively (figure 4.10) when using the modified troughs described in chapter 2.

4.2.5.2. Gene expression analysis of piglets' gut hormone levels

There were no effects of xylanase supplementation (table 4.11) on any gene, in any of the three tissues when measured at the end of the two week trial (figure 4.11).

Table 4.9 Mean animal growth performance measurements for trial 3.

	Co n=7	XS n=7	SED	P
Initial live weight/ kg	9.17	9.31	0.526	0.790
Live weight week 1/kg	10.44	9.99	0.478	0.358
Final live weight/ kg	14.33	13.76	0.606	0.364
Total weight gain/ kg	5.16	4.44	0.450	0.138
ADWG week 1 / kg/d	0.139	0.108	0.0308	0.340
ADWG week 2/ kg/d	0.555	0.539	0.0537	0.766
ADFI/ kg/d	0.568	0.542	0.0359	0.491
FCR	1.05	1.02	0.078	0.686
Carcass weight/ kg	10.57	10.29	0.559	0.743

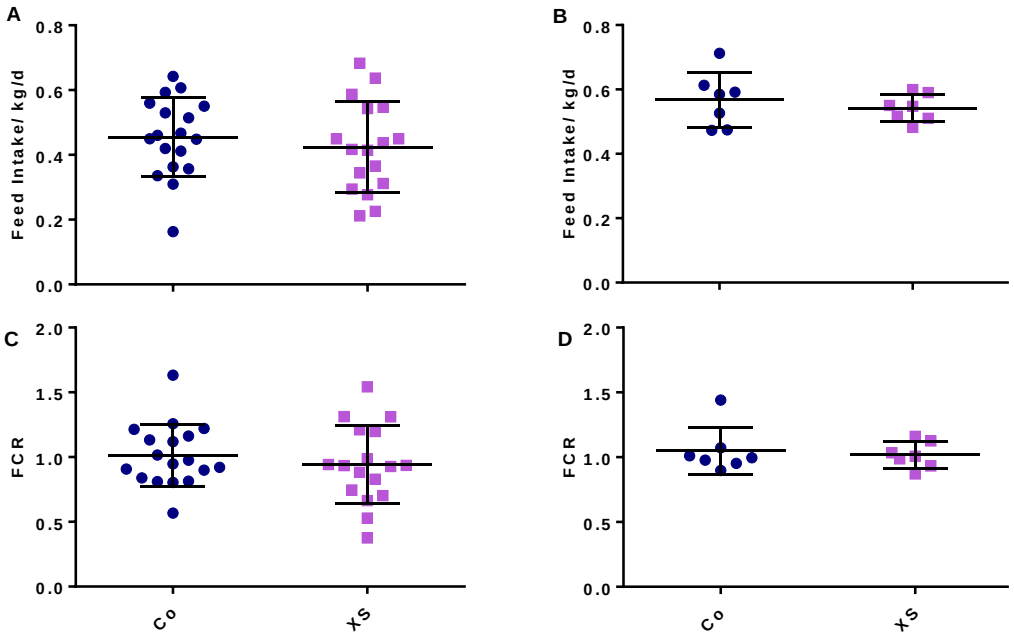


Figure 4.10 Mean feed intake measurements for trial 2 (A) and trial 3 (B), and FCR measurements for trial 2 (C) and trial 3 (D) taken at the end of week 2 post-wean for both trials.CV% for each are: (A) 29.7, (B) 12.1, (C) 27.6 and (D) 14.2. Error bars represent SEM.

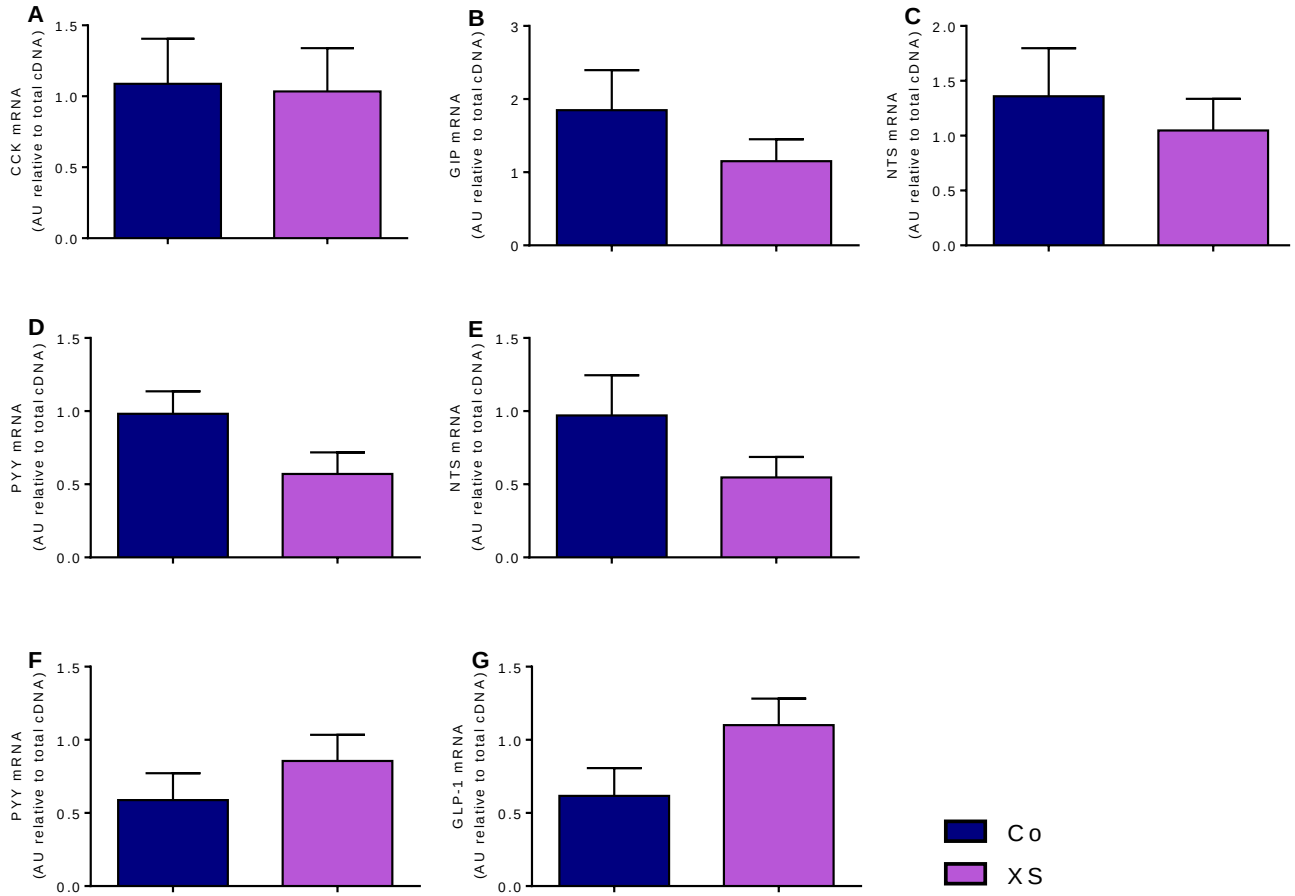


Figure 4.11 Relative abundance of genes measured using RT-qPCR in the jejunum (A: CCK, B: GIP, C: NTS), ileum (D: PYY and E: NTS) and colon (F: PYY and G: GLP-1) for trial 3 (mean±SEM).

Table 4.10 P-values for one-way ANOVA (jejunal CCK and NTS and colonic GLP-1) and Kruskal-Wallis (jejunal GIP, ileal NTS and PYY and colonic PYY) results for gene expression data in trial 3.

		P-Value
		Co vs. XS
Jejunum	CCK	0.886
	GIP	0.288
	NTS	0.848
Ileum	NTS	0.177
	PYY	0.078
Colon	GLP-1	0.225
	PYY	0.318

4.2.5.3. Circulating gut peptide levels in plasma

Figure 4.12 shows the concentrations of the metabolic/gut hormone measured in circulating plasma using the multiplex assay. The only significant differences found between Co and XS were for GIP, GLP-1 and PP (all $p<0.05$), with each of them having lower circulating levels in the XS group. When analysing plasma CCK concentrations in the two groups no significant difference was found ($p>0.05$; figure 4.13).

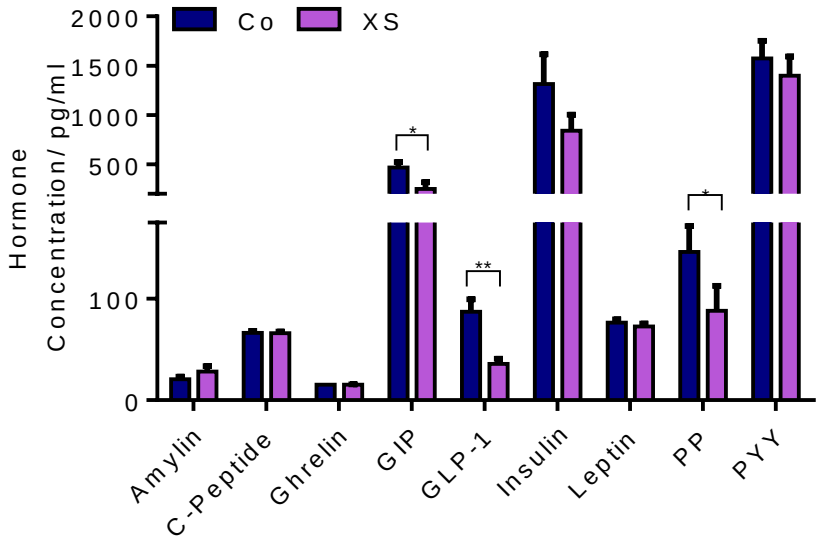


Figure 4.12 Concentrations of metabolic/gut hormones for trial 3, measured using a human multiplex assay (mean±SEM).
* p<0.05
**p<0.01

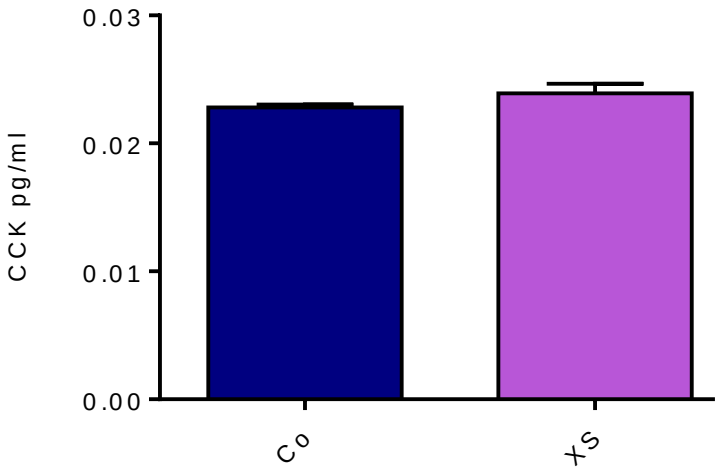


Figure 4.13 Blood plasma CCK concentrations from trial 3, measured using a porcine CCK ELISA (mean±SEM).

4.2.5.4. Gene expression data vs. gut peptide plasma concentrations

Figure 4.14 shows the relationships between the relative abundance gene expression data and corresponding gut peptide concentration in plasma from trials 2 (A-C) and 3 (D-H). There was no significant relationship detected between gene expression and peptide plasma concentration for either trial ($p>0.10$).

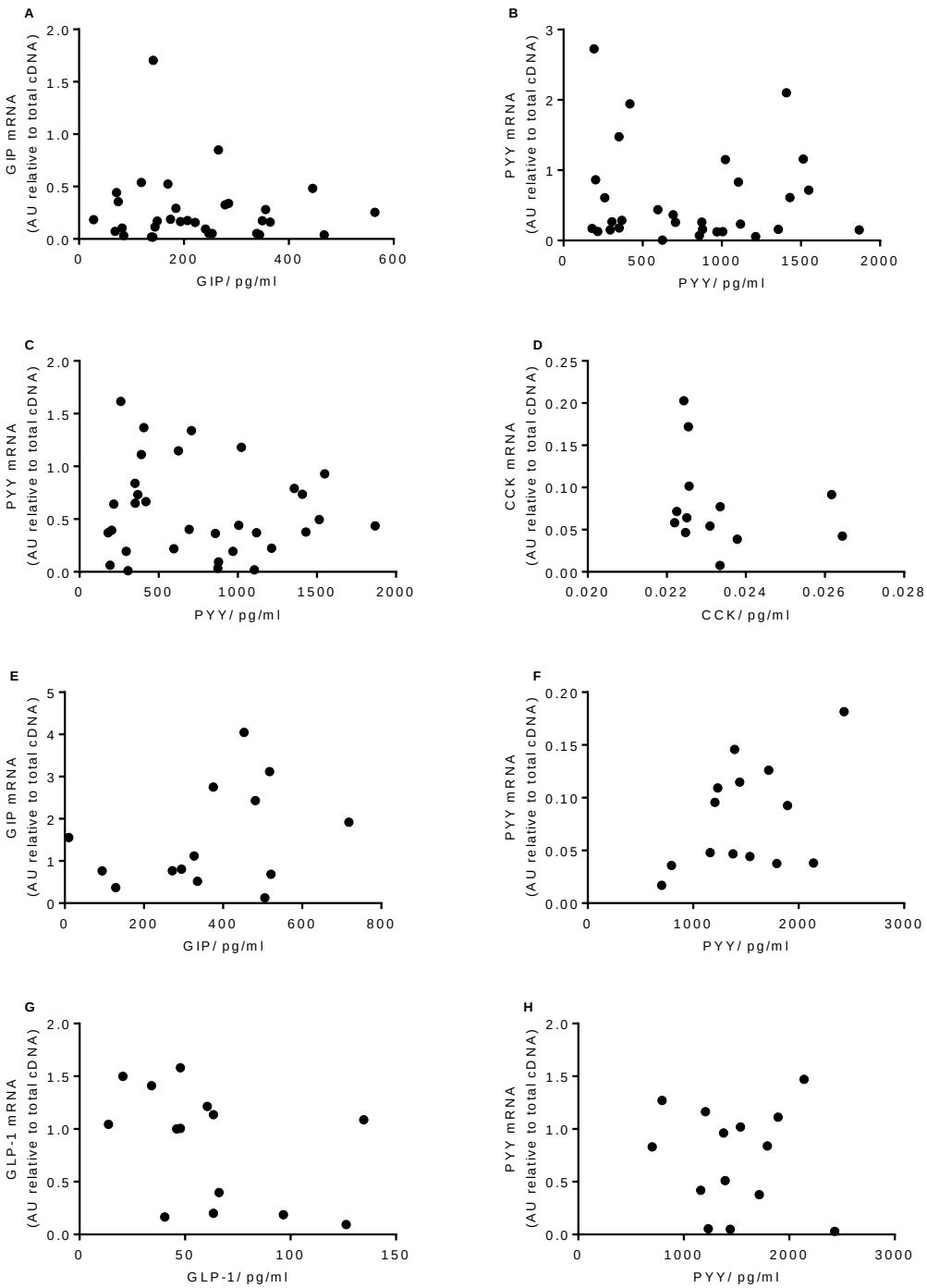


Figure 4.14 Correlation data for trials 2 and 3 (see chapter 4) between gene expression data and corresponding peptide plasma concentrations. Letters A-C are from trial 2 and D-H from trial 3.

Trial 2: (A) GIP (mRNA from jejunum) r=-0.0103, p=0.9555.
(B) PYY (mRNA from ileum) r=-0.0412, p=0.8290.
(C) PYY (mRNA from colon) r=0.0260, p=0.8876.

Trial 3: (D) CCK (mRNA from jejunum) r=-0.3077, p=0.3603.
(E) GIP (mRNA from jejunum) r=0.3460, p=0.2256.
(F) PYY (mRNA from ileum) r=0.3582, p=0.2090.
(G) GLP-1 (mRNA from colon) r=-0.4053, p=0.1484.
(H) PYY (mRNA from colon) r=-0.0111, p=0.7069.

E and H were analysed using Pearson's r correlation and Spearman's rank order correlation was used for the rest as the data was not normally distributed.

4.2.6. Summary

When measured at 2 weeks post-wean the xylanase treatment had no effect on ADWG nor FCR, which supports the results from trial 2 when these measurements were taken at the end of period 1 (2 weeks post-wean). Furthermore xylanase supplementation also had no effect on other measured growth parameters (table 4.9), which is what was anticipated by the hypothesis. Although most hormones were not affected by the treatment, there were three that were significantly reduced when the enzyme was present in the piglets' feed, these being GIP, GLP-1 and PP. As detailed in chapter 1 GIP and GLP-1 are incretin hormones and therefore influence the production of insulin from the pancreas, and PP is primarily produced from F-cells in the pancreas. Therefore supplementing xylanase at this point in the piglets' development may impact on pancreatic function and subsequently production of other metabolites that it releases such as insulin, but at this stage in growth seems to have no effect on performance traits examined in this study.

4.3. DISCUSSION

The age at which non-ruminants' feed is supplemented with xylanase has been found to be a factor that affects the ability of xylanase to exert beneficial performance effects (Masey O'Neill & Cowieson, 2013). In the present trials it was found that there were no significant effects on weaner performance traits. This confounds previous evidence in piglets from Omegbenigun et al. (2004), who found supplementation with exogenous

enzymes improved feed efficiency as well as weight gain, and Owusu-Asiedu et al. (2010), who found that only feed efficiency was improved. However both of these studies used xylanase alongside other exogenous enzymes, therefore they may have seen an additive effect on growth performance that was not present in the current trials. Woyengo et al. (2008b) reported in grower pigs that xylanase supplementation had no effect on either weight gain nor feed efficiency, in concordance with the present studies, however they supplemented the enzyme in the grower phase and not from weaning.

Weaning is a highly stressful time for young animals as they are subjected to many changes in their environment (Levast et al., 2010) these include; being separated from their mother, having to adjust to how their food is now delivered to them and in a normal commercial setting having to become accustomed to new animals from different litters (Moeser et al., 2007). At this time in the animal's life its digestive tract is also being challenged due to the change from liquid feed to solid feed (Moeser et al., 2007) which can have detrimental effects on the intestinal mucosal barrier (Smith et al., 2010) in turn altering growth performance due to nutrient absorption being affected. Supplementing xylanase at this critical time point may not be beneficial in terms of weight gain and overall animal performance in a commercial setting, however this will not be apparent until a wean to slaughter trial is conducted to deter if the pigs supplemented immediately post-wean have impeded growth outputs (such as weight gain and feed efficiency) than those that are not.

Furthermore, poultry do not have to go through the process of weaning which may contribute to why their performance benefits from the supplementation of xylanase immediately post-hatch, which has been suggested as being microflora driven (Masey O'Neill & Cowieson, 2013). Courtin et al. (2008) showed that the xylooligosaccharides produced from the action of xylanase affect the microbial population in the digestive tract of young broilers, increasing the population of bacteria deemed to be beneficial to the host.

It is known that through the fermentation of undigested oligosaccharides the microbial community in the distal intestines influences the release of metabolic/gut hormones as well as other peptides in the endocrine system (Neuman et al., 2015). Singh et al. (2012) showed a significant increase in the serum concentration of PYY in 42d old broilers, however found no influence of xylanase on microbial populations. In the present studies there was no significant effect of xylanase supplementation on plasma PYY concentrations when measured at the end of trial 3 (2 weeks post-wean). This fitted with the hypothesis based on the gut/metabolic multiplex data from trial 2, which suggested no difference in PYY level in period 1 of the experiment (0-2 weeks post-wean) when xylanase was included in the diet. However when xylanase was supplemented from 2 to 6 weeks post-wean there was a significant decrease in PYY levels. This was opposite to what was hypothesised based on studies carried out with broiler chickens, where PYY levels were significantly increased in serum and plasma samples when xylanase was supplemented in their

feed from hatch until 42d of age (Singh et al., 2012; Masey O'Neill et al., 2012).

The decrease in porcine PYY does not fit in with the mechanism that xylanase produces prebiotics that are fermented by microbiota in the large intestine producing SCFA that drive the production of PYY. As PYY is a satiety hormone you would anticipate an increase in feed intake if its levels were significantly decreased during the consumption of feed, however this was not the case in trial 2. None of the feed intake values between the groups were found to be significantly different between the treatment groups, although intake increased significantly with age. Although a difference in feed intake was not detected as would be expected with changes in PYY levels this may be explained by the problems encountered whilst trying to measure the feed refusals in trial 2 (see chapter 2).

In trial 2 a slight decrease in insulin and C-peptide levels were detected ($p<0.10$), in the animals where xylanase was provided in the first 2 weeks post wean. This spurred the question of what was happening at this time point to cause a decrease in these two pancreatic hormones when measured 4 weeks later, and was postulated that these two hormones would likely be significantly lower when measured at this earlier time point, as it was thought due to measuring the hormones at this later date and not at 2 weeks post-wean the effect may have decreased over time. When plasma gut/metabolic hormone concentrations were measured in

trial 3 after weaned piglets were again fed either a Co or XS feed it was discovered that at this time point there was a significant decrease in GIP, GLP-1 and PP but not insulin or C-peptide, interestingly though there is a link between these hormones. GIP and GLP-1 are classed as incretin hormones meaning that their release promotes the release of insulin, and as C-peptide is produced at the same time as insulin as it is part of the same prohormone (see chapter 1) it is logical it would be affected by these hormones also. PP is a pancreatic hormone produced by F-cells in the pancreas and has been found to delay insulin secretion (Schmidt et al., 2005). All three of these hormones are also linked to gastric motility and satiety signalling, all promoting a reduction in feed intake. Feed intake in the XS group was not significantly higher than the Co group at the end of trial 3, but due to the errors that were encountered when measuring this parameter it is not certain if the differences in the peptide concentrations affected the satiety of these animals.

Although there were significant differences in gut/metabolic hormone levels there were no differences in gene expression when the corresponding gene levels were measured in the appropriate gut tissues. This is likely down to variation in the measurement due to animal to animal, sampling site and density of the secretory endocrine cells variability, although as much care as possible was taken to take tissue samples from the same section of intestine as described in chapter 2. Furthermore the turnover rate of epithelial cells (including enteroendocrine cells) is extremely fast and ‘new’ villus epithelium are

turned over every 2-6 days in mature mammals (Knudsen et al., 2015; Williams et al., 2015). It is likely that because of these variables the quantity of mRNA for the genes of interest differed in abundance dependant on which part of the GIT was sampled and eventually processed for analysis. When exploring the relationship between the relative abundance of the gene expression data and concentrations of corresponding gut peptides, it was found that there was no significant correlation ($p>0.10$). Due to post-translational processing this is expected and shows that there are potentially other pathways, which don't include changes in mRNA expression, via which the gut peptides in question are downregulated. One potential is the role of miRNAs in endocrine cell development, of which one (miR-375) has previously been found to inhibit the development of endocrine cells releasing different gut peptides (Knudsen et al., 2015).

5. TRIAL 4: LONG-TERM SUPPLEMENTATION OF XYLANASE AND ITS EFFECTS ON GUT/METABOLIC HORMONE CONCENTRATIONS IN PIGS GROWN TO A COMMERCIAL SLAUGHTER WEIGHT.

5.1. INTRODUCTION

Studies focussing on the outcomes of supplementing commercial pig feeds with xylanase have been carried out separately over the main distinct periods during pig growth (weaning, grower and finisher) but have not often crossed over these three periods, and at most have been carried out over two of the periods, weaner-grower or grower-finisher (Barrera et al., 2004; Woyengo et al., 2008b; Mc Alpine et al., 2012; Prandini et al., 2014). Many studies have also used more than one NSP degrading enzyme in combination and not just xylanase as with the studies detailed in the current thesis.

In the weaner trials described in the current thesis (see chapter 4) it was found that the duration or timing of dietary supplementation had an impact on metabolic/gut hormone production. In trial 4 dietary xylanase supplementation from 0-2 weeks post-wean lowered the plasma concentrations of incretin hormones (GLP-1 and GIP), being named so because of their influence on insulin production (Seino et al., 2010), and PP which is produced in the pancreas (Holzer et al., 2012). Similar effects were detected in trial 3 as insulin and C-peptide circulating plasma concentrations were decreased in animals fed diets supplemented with

xylanase prior to the 2 week post-wean time point irrespective of whether or not the enzyme continued to be supplemented after this point, although this observation was not statistically significant ($p<0.08$). This suggests that dietary supplementation of xylanase immediately post-weaning might have an effect on the function of the pancreas. Consequently, the decreased circulating insulin concentrations may have an effect on the uptake of glucose into tissues and protein synthesis which could affect growth performance. The satiety hormone PYY was also seen to decrease ($p=0.008$) in trial 2 when the pigs were supplemented xylanase from 2-6 weeks post-wean.

Therefore the aim of the current trial was to see if xylanases effects on the gut/metabolic peptides were sustained over a longer period of supplementation and if growth performance was subsequently effected due to the elongated exposure to the NSPase.

5.2. OBJECTIVES

Measure levels of gut/metabolic hormones in pigs at 13 weeks post-wean (proposed time to reach a commercial slaughter weight) and monitor growth performance parameters, when the animals are fed a wheat-based feed either supplemented or not with the exogenous enzyme xylanase, for the whole of this time period or a combination of these two feeds in this time, either before or after the 2 week post-wean time point.

5.3. HYPOTHESIS

For animals fed a Co diet from wean to 2 weeks post-wean and then supplemented xylanase from this point to 13 weeks post-wean will have heavier final body weights than pigs fed diets supplemented with xylanase at other time points and for a different length of time. PYY plasma concentrations will be lower in pigs fed diets supplemented with xylanase for the whole of the trial and from 2 weeks post-wean.

5.4. MATERIALS AND METHODS

The following description of the materials and methods used are just a brief outline. For a more in depth explanation please see Chapter 2.

5.4.1. Animals and experimental design

Thirty-six female weaned piglets (28d old) arrived in two batches (n=18) one week apart and were assigned to one of two diets, either a Co or XS (16,000 BXU/kg; Econase XT, AB Vista, Marlborough, UK) diet (composition in Appendix 1). After 2 weeks on this diet, referred to as period 1 (0-2 weeks post-wean), half of the piglets stayed on the original feed they were already assigned to or were swapped onto the other diet (Co or XS) until slaughter (2-13 weeks post-wean), this being referred to as period 2-Ext. This resulted in four groups as in trial 2, one that was on the Co diet from wean to slaughter (Period 1: Co, Period 2-Ext: Co), another that was on the XS for the same amount of time (Period 1: XS, Period 2-Ext: XS), and two groups that were crossed over at the end of period 1 (Period 1: Co, Period 2-Ext: XS and Period 1: XS, Period 2-Ext)

(see figure 5.1). Before the end of the second week of the trial for batch 1 two of the piglets were removed from the trial due to ill health which resulted in the numbers of pigs in each group being unequal due to the assignment pattern for both batches; n numbers for each group can be seen in table 5.1 below.

Table 5.1 n values for each group in trial 4.

		Period 2-Ext	
		Co	XS
Period 1	Co	7	10
	XS	8	9

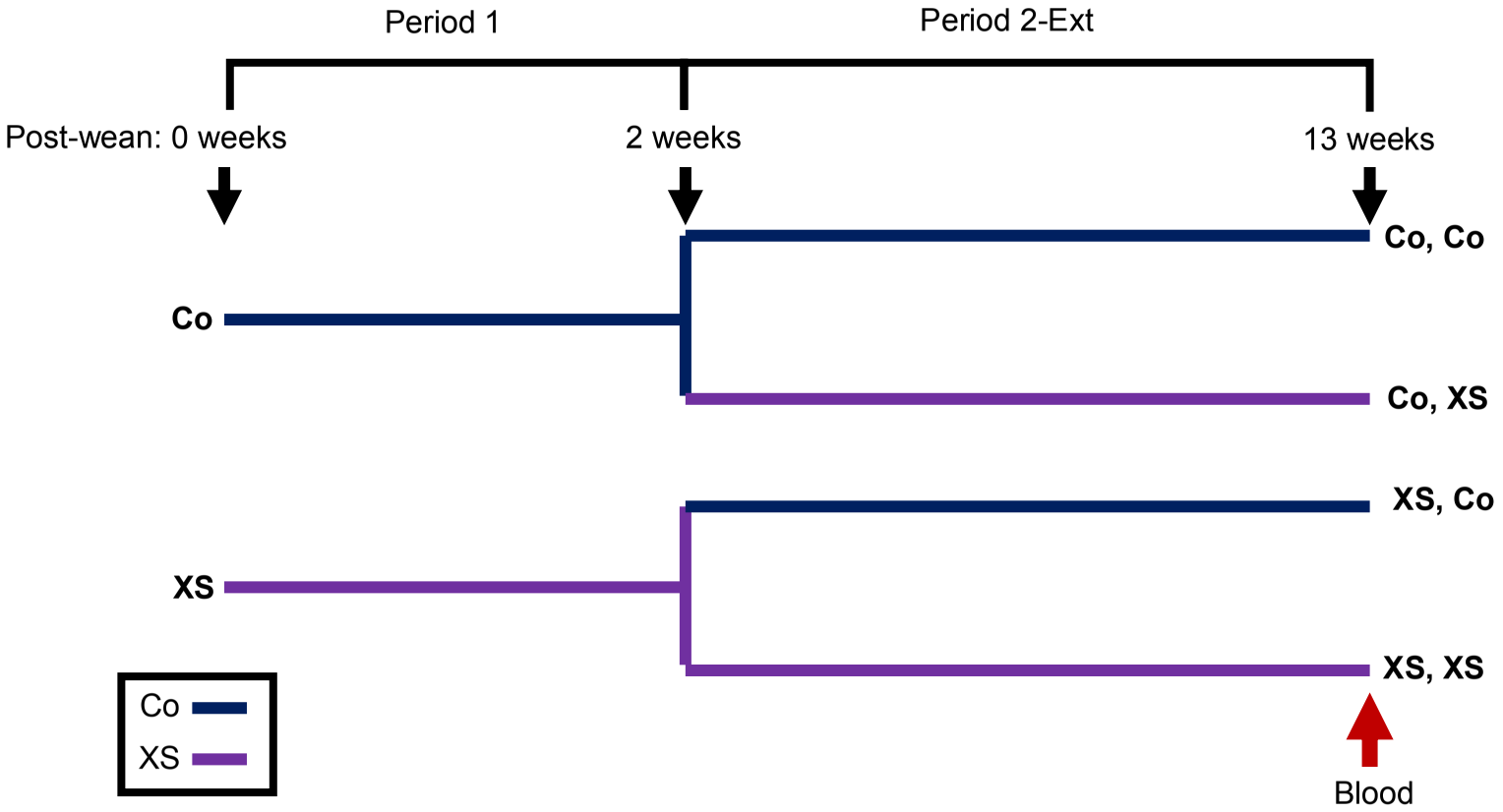


Figure 5.1 Trial design for trial 4.

The animals were weighed weekly, they were fed twice daily to ensure *ad libitum* access to feed from a bucket which contained a known amount of feed that was weighed out at the beginning of each week and feed refusals were measured daily for a week at a time at different points during the trial (Weeks 2, 4, 7, 10 and 13). At the end of period 2-Ext (end of week 13) both batches of pigs were culled by electrical stun and exsanguination (S). It was at this point that blood samples were collected for analysis and stored as described in Chapter 2.

5.4.2. Gut/metabolic hormone levels in pig plasma

A number of gut/metabolic hormones were simultaneously measured in the pig plasma samples, collected at 13 weeks post-wean, using a human multiplex assay (Human Metabolic Hormone Milliplex HMHMAG34k kit, Merck Millipore, Billerica, MA, USA). The peptides measured and their inter- and intra-assay CV% can be seen in table 5.2. In brief the method carried out consisted of, pipetting 25µl of each plasma sample, in duplicate, onto a 96-well plate along with standards and two QC's that were provided with the kit. After an overnight incubation at 4°C with analyte specific colour-coded antibody coupled beads, the samples and beads were incubated briefly at room temperature with a detection antibody, then a fluorescence activated dye and read on the MAGPIX® System (Merck Millipore, Billerica, MA, USA) using xPONENT® Software (Merck Millipore, Billerica, MA, USA).

5.4.3. Statistical analysis

A repeated measures two-way ANOVA (GraphPad Prism 6, GraphPad Software Inc., CA, USA) was used to analyse ADWG, FCR and ADFI, with age of measurement and treatment group as factors. Other growth performance parameters (including initial weight, final weight and total weight gain) and circulating metabolic/gut hormone peptides in plasma data were analysed using a two-way ANOVA (Genstat 15th Edition, VSN International Ltd, Hemel Hempstead, UK) factors being supplementation (XS) or no supplementation (Co) in Period 1 or Period 2.

Table 5.2 Inter- and intra- assay CV% for peptides measured using a human multiplex assay (provided by the manufacturer).

Peptide	Inter-assay CV%	Intra-assay CV%
Amylin	33	2
C-peptide	6	2
Ghrelin	8	2
GIP	5	3
GLP-1	10	7
Insulin	6	3
Leptin	4	3
PP	7	4
PYY	11	2

5.5. RESULTS

5.5.1. Animal performance

Means for each group for initial weight, final weight and total weight gain can be seen in figure 5.2 and the analyses of the data can be seen in table 5.3. There were no significant interactions detected ($p>0.50$) and no significant effect of supplementation in either period ($p>0.30$) for each

variable. Results for ADWG, FCR and ADFI can be seen in figures 5.3 and 5.4, respectively. As seen in table 5.4 there were no significant interactions between treatment groups or age of measurement for any of the variables. There were also no significant effects on the growth parameters between any treatment groups, however there was a significant effect on each of the variables between age of measurement ($p<0.001$).

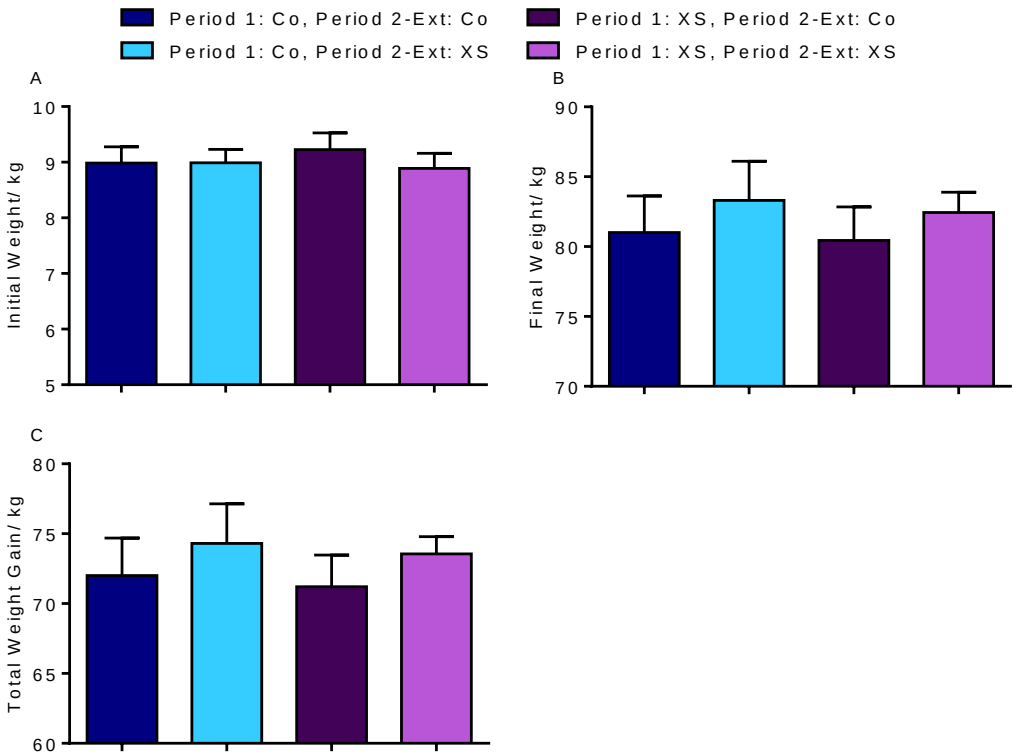


Figure 5.2 Animal growth performance data for trial 4, showing (A) initial weight, (B) final weight and (C) total weight gain. Each bar represents the mean for each group with error bars representing SEM. A two-way ANOVA showed and no interaction between the two dietary supplementation periods ($p>0.50$) and no difference between treatment groups in each period ($p>0.30$).

Table 5.3 P-values from two-way ANOVAs for initial weight, final weight and total weight gain for trial 4.

	Initial Weight/ kg	**Final Weight/ kg**	**Total weight gain/ kg**
Period 1 (Co vs XS)	0.832	0.725	0.702
Period 2-Ext (Co vs XS)	0.542	0.372	0.328
Interaction	0.545	0.952	0.992

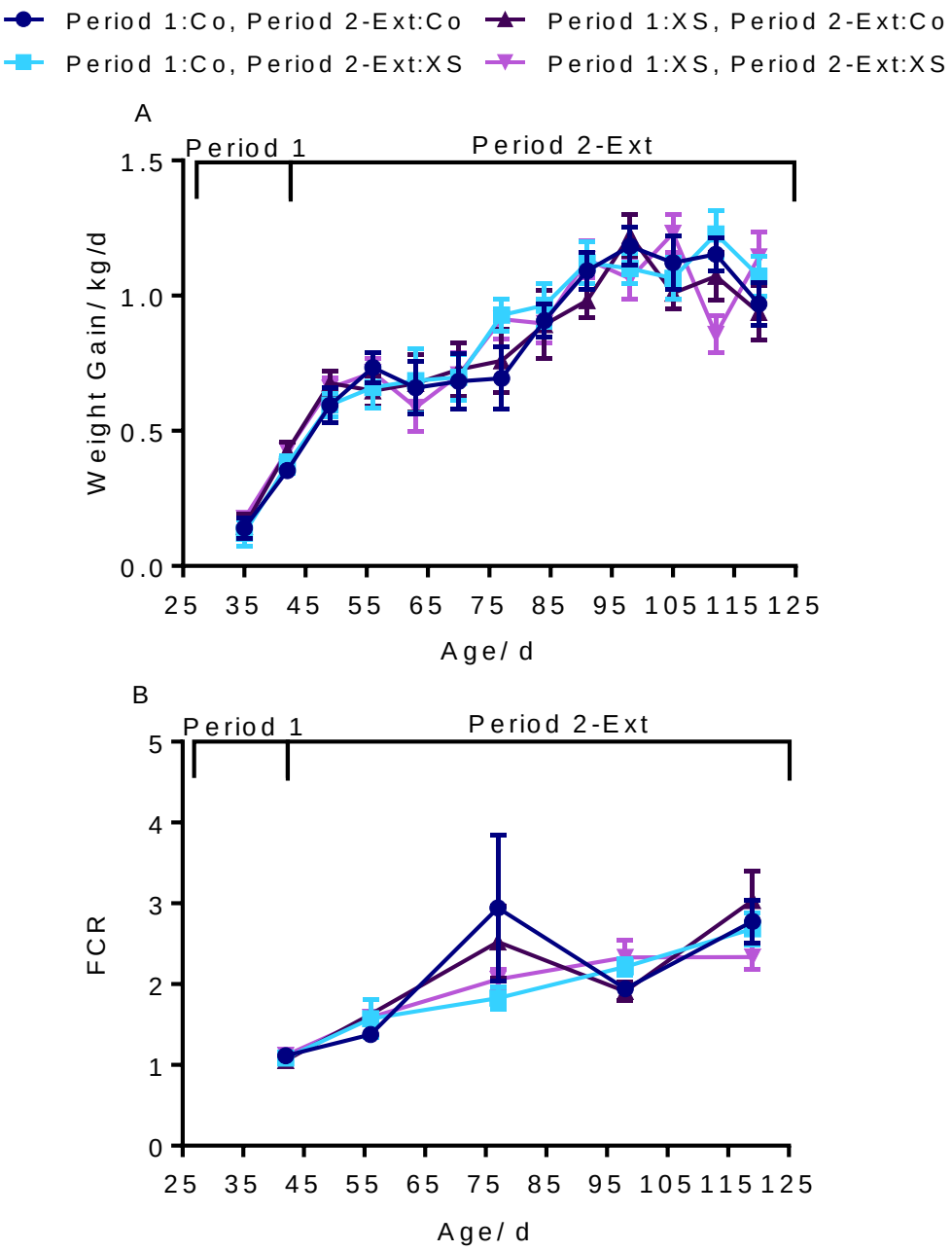


Figure 5.3 ADWG (A) and FCR (B) for trial 4. Each point represents the mean value taken at each time point for the group and error bars represent SEM. A two-way ANOVA was conducted at each time point for both (A) and (B), see tables 5.4 and 5.5.

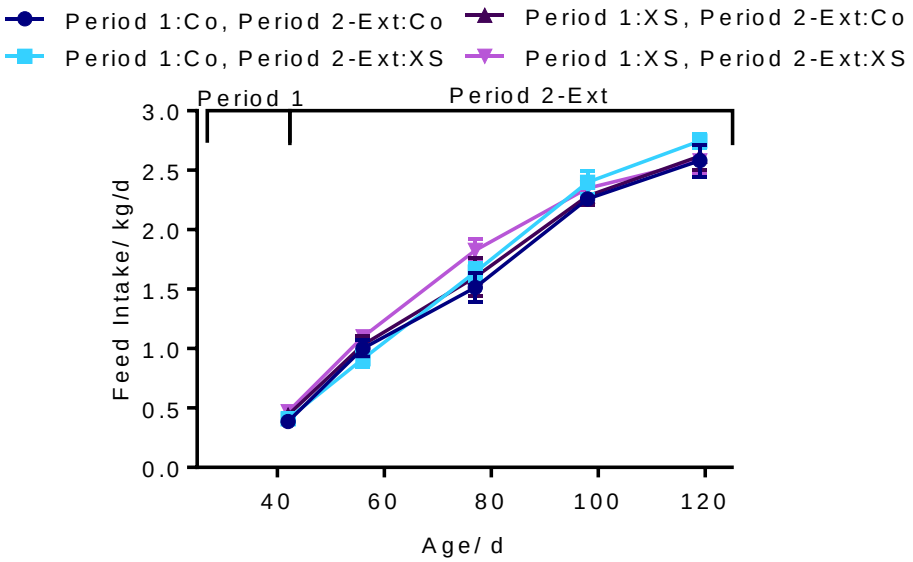


Figure 5.4 Average daily feed intake for trial 4. A two-way ANOVA was performed for each time point shown in table 5.6. Each point represents the mean value taken at each time point for the group and error bars represent SEM.

Table 5.4 P-values for repeated measures two-way ANOVA for ADWG, FCR and ADFI for trial 4.

	P-value		
	ADWG	**FCR**	**ADFI**
Age	**<0.0001**	**<0.0001**	**<0.0001**
Treatment	0.7782	0.5201	0.5313
Interaction	0.2942	0.2054	0.3089

5.5.2. Gut/metabolic hormone circulating plasma levels

There were no significant interactions detected between the two periods of supplementation for the gut/metabolic hormones, apart from for GLP-1 ($p=0.05$). In period 2-Ext there was no effect of xylanase supplementation on the concentrations of the gut/metabolic peptides, however interestingly in period 1 xylanase supplementation increased the concentrations of ghrelin and leptin ($p<0.05$). Amylin also tended to be increased in these groups ($p<0.07$).

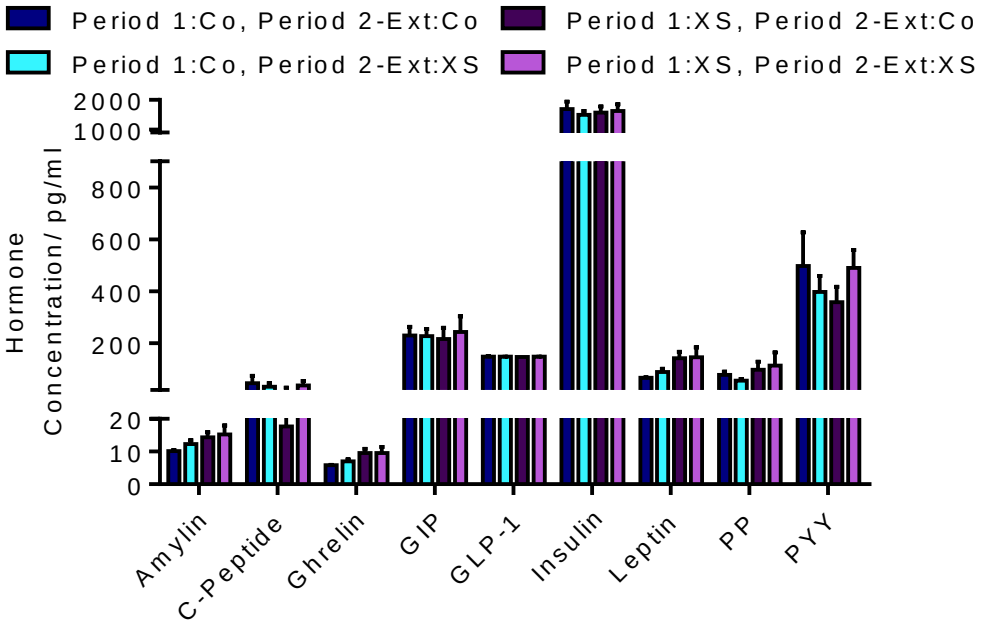


Figure 5.5 Gut/metabolic concentrations from plasma samples in trial 4. Each bar represents the mean for each group with error bars representing SEM. A two-way ANOVA was performed for each peptide shown in table 5.7.

Table 5.5 Two-way ANOVA for metabolic/gut hormones in trial 4, measured using a Human Metabolic Hormone Milliplex assay (HMHMAG34k kit, Merck Millipore, Billerica, MA, USA). P-values in bold show significance at p<0.05.

	Amylin	**C-peptide**	**Ghrelin**	**GIP**	**GLP-1**	**Insulin**	**Leptin**	**PP**	**PYY**
Period 1 (Co vs XS)	0.065	0.713	**0.021**	0.955	0.142	0.914	**0.015**	0.310	0.890
Period 2-Ext (Co vs XS)	0.425	0.543	0.637	0.659	0.530	0.836	0.620	0.403	0.861
Interaction	0.745	0.407	0.649	0.842	**0.050**	0.632	0.707	0.248	0.143

5.6. SUMMARY

Overall dietary xylanase supplementation had no effect on final measurements of growth performance parameters, Supplementation for more than 6 weeks post-wean did not sustain the effects seen in the previous trials in this thesis on gut/metabolic hormones. PYY was not of a lower concentration in those fed diets supplemented with xylanase from 2 weeks post wean or the full duration of the trial as had been hypothesised. However significant effects ($p<0.05$) were seen in ghrelin and leptin, being increased in pigs fed diets supplemented with xylanase immediately post-wean to 2 weeks irrespective of the feed they had after this period, from 2 to 13 weeks (Period 1: XS, Period 2-Ext: XS and Period 1: XS, Period 2-Ext: Co).

5.7. DISCUSSION

Gene expression data was not collected for this trial due to the high variability in the data collected in the previous studies detailed in this thesis (trials 2 and 3). When conducting correlation analysis to match gene expression and corresponding protein levels (see chapter 4) it was found that the data collected was not significantly correlated, as would be expected, and this was put down to the high variability of the gene expression data in the previous trials and the potential that protein levels were being influenced by other factors that did not change the mRNA expression of the genes associated with the gut peptides.

Although xylanase has previously been found to have both a muting and amplifying effect on the circulating levels of PYY, in the current thesis as well as in other non-ruminant trials (Singh et al., 2012) respectively, in the current trial this was neither the case (chapter 4). This suggests that the effect supplemental xylanase exerts on gut/metabolic hormones is timing dependent and not so much on the duration of the addition of the enzyme. This is opposite what was expected as it was thought that the effects would be cumulative over time. Surprisingly in the current trial dietary supplementation of xylanase significantly increased both plasma leptin and ghrelin levels (in fed state animals) when it was provided in period 1 (0-2 weeks post-wean). Ghrelin is known as the hunger hormone and is elevated in the fasted state to induce feed intake and rapidly decreases when food enters the proximal digestive tract (Dong et al., 2009). Leptin exerts the opposite effects to ghrelin and inhibits the intake of feed (Oswal & Yeo, 2010), and increased levels of this hormone suggests increased adiposity in the animals fed diets supplemented with xylanase in this period, although this parameter was not measured in the current study. Therefore it is anomalous that these hormones would be increased in the same groups due to their antagonistic effect on food intake (Klok et al., 2007).

In the current study there was no significant effect of dietary xylanase supplementation on growth performance, such as final weight. Studies carried out on grower-finisher pigs have also found this, either when solely supplementing xylanase or when used in combination with other

NSPases, in varying cereal based diets (Barrera et al., 2004; Olukosi et al., 2007; Woyengo et al., 2008b). However there have been studies where xylanase has improved growth performances (Kiarie et al., 2012; Wilamil et al., 2012), potential reasoning for which was discussed in chapter 1.

6. GENERAL DISCUSSION

Before the current body of work was undertaken very little was known about the effect of exogenous xylanases on gut/metabolic hormones when supplemented in commercial feeds for non-ruminants, although it had previously been eluded to due to its influence on gut microbiota through the production of xylooligosaccharides (Courtin et al., 2008) and retention time of digesta (Esmaeilipour et al., 2011). Work carried out by Singh et al. (2012) confirmed this in 21d old broiler chickens; PYY concentrations in serum were found to be significantly increased in chickens that were fed diets supplemented with 16,000 xylanase BXU/kg in a maize-soybean-based feed. However the role of PYY and other potential hormones involved in gut motility had yet to be explored in pigs when investigating the effects of dietary xylanase supplementation. Therefore the work in the current thesis aimed to bridge this knowledge gap within the field on exogenous xylanases effects on gut/metabolic hormones in pigs.

6.1. PERFORMANCE PARAMETERS

Studies into dietary supplementation of xylanase, whether alone or in combination with other NSPases, have shown that they can improve growth performance parameters (Barrera et al., 2004; Omogbenigun et al., 2004; Prandini et al., 2014). In all of the trials presented in the current thesis performance parameters were not significantly affected by the supplementation, whether it was for the duration of the trial or at differing

time points and durations; this lack of effect is however supported by other studies (Woyengo et al., 2008b; Owusu-Asiedu et al., 2010; Agyekum et al., 2015). The response to dietary xylanase supplementation as reported in studies is highly variable, and may be in part due to varying levels of inclusion and the underlying diet used in previous studies. For instance the amount of the pentosans (arabinose and xylose), that are the substrates for the xylanase enzyme, present in the diet could potentially not be high enough to impede significantly on growth performance, therefore supplementation of the enzyme would not be seen to exert any beneficial effects on the growth parameters. Furthermore different cereals have varying levels of the pentosans (Hashimoto et al., 1987).

In the current studies the total amount of pentosans (g) per kg of total feed has been estimated (see Appendix 1); in trial 2 the animals received 29.31 g/kg of pentosan initially and this increased to 43.27 g/kg, in trial 3 they received 29.31g/kg for the duration of the study and for trial 4 they initially had 29.31 g/kg and this increased to 48.03 g/kg by the end of the trial. Of the other literature reported in this study many do not report pentosan content of the feeds used therefore it is hard to make an accurate comparison. Using measurements provided by Saini and Henry (1989) and Hashimoto et al., (1987) it was estimated that in Bedford and Classen's (1992) study in broilers that when the diet contained 40.26 g/kg total feed (corresponding diet of 0g/kg of rye) that when supplemented with NSP degrading enzymes there was an increase in weight gain and

feed efficiency improved. Therefore they saw improvements in performance when providing the broiler levels of pentosan that were in the same range of the current trials. However Barrera et al. (2004) provided 64.92 g/kg total feed (estimated as described before) to pigs and only saw improvements in weight gain when xylanase was supplemented. As these are only estimations it is difficult to make straight comparisons, but potentially the amount of pentosan in the feed of the current studies was not high enough for xylanase supplementation to improve performance results. In future in-depth diet analysis should be performed and report pentosane concentration provided by the basal diet.

6.2. GUT/METABOLIC HORMONE CONCENTRATIONS

It was presented in the current thesis that when xylanase is supplemented in the 2 week period immediately post-wean that this significantly decreased the concentrations of GIP, GLP-1 and PP in plasma. The possible effects at 2 weeks of the decrease in the two incretin hormones (GIP and GLP-1) was observed at 6 weeks post-wean when insulin and C-peptide concentrations tended to be lower in groups where the piglets were fed diets supplemented xylanase 0-2 weeks post-wean. This suggests that xylanase inclusion at this time point influences the function or development of the pancreas as three hormones produced in the organ are decreased, and potentially others such as glucagon, which were not measured in the current trials. Due to its role in glucose homeostasis, a decrease in circulating insulin levels may

reduce the uptake of glucose into tissues and protein synthesis and, due to the decrease in hormones involved in inhibiting gut motility (GLP-1, PYY and PP), may increase circulating glucose concentrations. It has been recognised that supplementation with exogenous enzymes improves glucose absorption in grower pigs, but no supplementation effects were seen on insulin production (Agyekum et al., 2016).

PYY concentrations did not follow the same pattern as seen in broiler chickens fed diets supplemented with xylanase whose levels increased (Singh et al., 2012), whereas in pigs levels decreased when measured at 6 weeks post-wean when the enzyme was supplemented into diets from 2 weeks post-wean irrespective of supplementation status between weaning and 2 weeks post-wean. This difference could therefore be microbially driven as chicks do not have to be weaned and therefore their microbiome is established after hatching. Therefore, in chicks, xylanases products from breaking down the NSPs can exert their prebiotic effects creating a potentially beneficial bacterial populous immediately. However, piglets begin to establish their microbiome foremost whilst on maternal milk which then undergoes changes when the young animal is weaned onto a solid feed (Frese et al., 2015) therefore prebiotic effects that xylanase may exert have to first override the established gut microbiome. This will result in the two species having a very different gut microbial profile and hence may affect hormone productions differently; the effects of the gut microbiome on the endocrine system have been well-established (Neuman et al., 2015). However the response of PYY

seen in pigs does not does not fit in with the proposed mechanism that xylooligosaccharides produced from xylanases enzymatic action have prebiotic effects on the guts bacterial population. By increasing the production of SCFA from the fermentation of these products by the bacteria and increasing PYY production and potentially other gut hormones.

Taylor et al. (2016) found that there was no significant effect of dietary xylanase supplementation on PYY plasma levels when measured peripherally or from the portal vein. This was in weaner-grower pigs fed a wheat-barley-based diet and supplemented xylanase at 8,000, 16,000 and 32,000 BXU/g feed, supplementation commenced at 3 weeks post-wean (assuming weaning was at 4 weeks of age) and blood plasma was collected at 8 weeks post-wean. This corroborates with the findings in the current study that the effects of dietary supplemental xylanases on hormone production are not cumulative over time and are dependent on timing and/or duration of supplementation.

Ghrelin and leptin were significantly increased 13 weeks post-wean in pigs fed diets supplemented xylanase immediately post-wean for 2 weeks. An increase in leptin levels suggests an increase in adiposity in these animals but this parameter was not measured in trial 4. The two hormones are the antithesis of each other in terms of their effects on appetite and hence food intake, with ghrelin promoting feed intake and leptin supressing it. However there were no effects on feed intake

detected at any point in the trial in the animals fed diets supplemented xylanase 0-2weeks post-wean. The fact that both of these hormones increased significantly is confusing and more investigation is needed in order to fully elucidate the effects that have been seen in the study presented in the current thesis.

A limitation of the peptide measurements, is that in each trial the measurements were only taken at a single time-point. To get a true understanding of how the hormones responded to the enzyme measurements needed to be taken at more than one point (at different ages) and monitored for over time for fluctuations in release to be accounted for. For instance PYY has an initial release within 15 mins of a meal being ingested but then has another increase in production when the nutrients reaches the site of production of the peptide (distal ileum and colon) and can be released for up to 6 hours after peak concentrations are reached 60-90mins post-meal consumption (Cooper, 2012).

6.3. MAIN CONCLUSIONS FROM THE THESIS

In summary the current thesis has added to the previous body of work by finding:

- Commercial slaughtering methods do not affect RNA integrity or subsequent downstream analysis of gene expression from liver and GIT tissue (as discussed in chapter 3).

- A human multiplex assay can be used to measure pig gut/metabolic hormones, but reproducibility from the ELISA was poor.
- Dietary xylanase supplementation decreases incretin hormone (GIP and GLP-1) and PP concentrations in circulating plasma measured at 2 weeks post-wean, when it is provided in feed immediately post-wean.
- This has a knock-on effect on insulin and C-peptide levels when plasma is analysed for these metabolites at 6 weeks post-wean. Also at this time-point PYY concentrations are decreased in animals fed diets supplemented xylanase from 2 weeks post-wean, regardless of supplementation prior to this time-point.
- These changes in peptide production, however, are not sustained when xylanase is supplemented for a longer period of time (over 6 weeks post-wean) and there is no detected difference in C-peptide, GIP, GLP-1, insulin, PP or PYY when measured at 13 weeks post-wean.
- At 13 weeks post-wean ghrelin and leptin concentrations are higher in pigs fed diets supplemented xylanase in the period 0-2 weeks post-wean. This suggests higher adiposity in these animals but further investigation is needed to determine the importance of the effects of these results.
- Dietary supplementation of the exogenous enzyme had no effect on growth performance parameters when measured at any age when fed on a wheat-based diet.

6.4. FUTURE WORK

Future work that should be considered in order to further knowledge in this area include:

- Continuing to explore and monitor gut/metabolic hormones in response to dietary xylanase supplementation in both broilers and pigs. The focus of this research should not be on one hormone (e.g. PYY) but should encompass others due to their effects on each other and their shared functions, such as regulation of appetite and gastric motility, and hence potential effects on production efficiency. It would also be useful to gather this information along the course of the study rather than just looking at end-point concentrations of peptides.
- Further validation protocols of the Human multiplex metabolic hormone assay need to be carried out to further asses its reproducibility using porcine samples. Alternatively, and preferably, porcine specific multiplex assays need to be developed for metabolic hormones.
- There should be more long-term studies looking into dietary supplementation of exogenous enzymes in young pigs to elucidate the effects might have on their growth performance all the way through to slaughter.
- The potential method of designing diets that regulate gut hormone production to favourably effect feed efficiency should be explored.

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APPENDIX 1

Diet composition for trials 2-4 (Chapters 4 and 5) shown as a percentage of total feed composition.

	Diet							
	A1 age 28-42d	**A2 age 43-57d**	**A3 age 58-72d**	**A4 age 73d +**	**B1 age 28-42d**	**B2 age 43-57d**	**B3 age 58-72d**	**B4 age 73d +**
Micronised barley	10	10	10	15	10	10	10	15
Wheat raw whole meal	19.56	40	48.9	50.71	19.55	39.99	48.89	50.71
Micronised wheat meal	10	5	-	-	10	5	-	-
Porridge oat	7.5	2.5	-	-	7.5	2.5	-	-
Wheatfeed	-	1.25	5	5	-	1.25	5	5
Herring meal	7.5	5	2.5	-	7.5	5	2.5	-
Soya hypro	15.8	22	27.5	23.75	15.8	22	27.5	23.75

ABN pig starter (Vitamin/mineral premix)[1]	0.5	0.5	0.5	-	0.5	0.5	0.5	-
Soycomil	2.5	1.25	-	-	2.5	1.25	-	-
Dried skimmed milk powder	7.5	-	-	-	7.5	-	-	-
Whey powder	12.6	6.5	-	-	12.6	6.5	-	-
Sugar/sucrose	0.625	0.625	-		0.625	0.625	-	-
L-lysine	0.155	0.335	0.298	0.45	0.155	0.335	0.298	0.45
DL-methionine	0.149	0.168	0.132	0.07	0.149	0.168	0.132	0.07
L-threonine	0.13	0.176	0.121	0.12	0.13	0.176	0.121	0.12
L-tryptophan	0.02	0.02	-	-	0.02	0.02	-	-
Vitamin E	0.015	0.001	0.001	-	0.015	0.001	0.001	-
Quantum	0.05	0.05	0.05	0.025	0.05	0.05	0.05	0.025
Econase XT 25 trial	-	-	-	-	0.01	0.01	0.01	0.01
Sucram	0.01	0.01	-	-	0.01	0.01	-	-

Vanilka	0.02	0.02	-	-	0.02	0.02	-	-
Benzoic acid	0.5	0.5	0.5	-	0.5	0.5	0.5	-
Limestone flour	0.178	0.275	0.37	0.25	0.178	0.275	0.37	0.25
Dicalcium phosphate	0.388	0.984	1.13	0.25	0.388	0.984	1.13	0.25
Salt	-	0.156	0.63	0	-	0.156	0.63	0.25
Binder (lignobond DD)	-	-	0.625	0.625	-	-	0.625	0.625
Soya oil	4.3	2.68	1.743	1.55	4.3	2.68	1.743	1.55
PDS 3 supp	-	-	-	1.75	-	-	-	1.75

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[1]The pig starter Vitamin and mineral premix contained per kg of diet: 11,500 IU of vitamin A, 2,000 IU of vitamin D3, 100 IU of vitamin E, 4 mg of vitamin K, 27.5 µg of vitamin B12, 15 mg of pantothenic acid, 25 mg of nicotinic acid, 150 µg of biotin, 1.0 mg of folic acid, 160 mg of CU ($CUSO_4$), 1.0 mg of Iodine (KI, $Ca(IO_3)_2$), 150 mg of Fe ($FeSO_4$), 40 mg of Mn (MnO), 0.25 mg of Se (BMP-Se), 110 mg Zn ($ZnSO_4$).

Grams of total pentosan content (arabinose + xylose) per kg of total feed.

	Diet (g pentosan per kg total feed)							
Feed component	**A1**	**A2**	**A3**	**A4**	**B1**	**B2**	**B3**	**B4**
Micronised barley[1]	7.1	7.1	7.1	10.65	7.1	7.1	7.1	10.65
Wheat raw whole meal[1]	13.12	26.84	32.81	34.03	13.12	26.83	32.81	34.03
Micronised wheat meal[1]	6.71	3.36	0	0	6.71	3.36	0	0
Porridge oat[1]	2.38	0.79	0	0	2.38	0.79	0	0
Wheat feed[1]	0	0.84	3.36	3.36	0	0.84	3.36	3.36
Total	29.31	38.93	43.27	48.03	29.31	38.92	43.26	48.03

[1]Cereal pentosan (arabinose + xylose) concentrations were taken from Hashimoto et al. (1987) and used to calculate total pentose content in each feed component.

APPENDIX 2

Analysis results of enzyme activity for trial 2.

Diet	Xylanase activity (ELISA BXU/kg)	CV %
A1	8700	15
A2	<2000	-
A3	<2000	-
B1	26000	6
B2	18600	6
B3	17800	2

Analysis results of enzyme activity for trials 3 and 4.

Diet	Xylanase activity (ELISA BXU/kg)	CV %
A1	<2000	-
A2	<2000	-
A3	<2000	-
A4	<2000	-
B1	14300	10
B2	30900	3
B3	49400	6
B4	18200	5

This work was carried out by Enzyme Services & Consultancy (ESC, UK).

APPENDIX 3

Human and pig peptide sequence alignments, aligned using Clustal W2.

Amylin

```
HUMAN  MGILKLQVFLIVLSVALNHLKATPIESHQVEKRKCNTATCATQRLANFLVHSSNNFGAIL
PIG    ---------------------------------CNMATCATQHLANFLDRSRNNLGTIF
                                        ** ******:***** :* **:*:*:

HUMAN  SSTNVGSNTYGKRNAVEVLKREPLNYLPL
PIG    SPTKVGSNTY-------------------
       * *:******
```

Whole sequence homogeneity: 30.23%

Available sequence homogeneity: 72.22%

C-peptide

```
 HUMAN  EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ
 PIG    EAENPQAGAVELGGGL--GGLQALALEGPPQ
        ***: *.* ******   *.** *****  *
```

Whole sequence homogeneity: 64.52%

Ghrelin

```
HUMAN  -------MPSPGTVCSLLLLGML-WLDLAMAGSSFLSPEHQRVQQRKESKKPPAKLQPRA
PIG    PGRPAEAMPSTGTICSLLLLSVLLMADLAMAGSSFLSPEHQKVQQRKESKKPAAKLKPRA
              *** **:******.:*   ***************:********** ***:***

HUMAN  LAGWLRPEDGGQAEGAEDELEVRFNAPFDVGIKLSGVQYQQHSQALGKFLQDILWEEAKE
PIG    LEGWLGPEDSGEVEGTEDKLEIRFNAPCDVGIKLSGAQSDQHGQPLGKFLQDILWEEVTE
       * *** ***.*:.**:**:**:***** ********.* :**.* ************..*

HUMAN  APADK-------------------------------------------
PIG    APADK*LSLRPATSVLPAS*GLTWLPGRFHYHTQL*GTLAWEVNKHSDW
       *****
```

Whole sequence homogeneity: 118.64%

Available sequence homogeneity: 77.97%

GIP

```
HUMAN  MVATKTFALLLLSLFLAVGLGEKKEGHFSALPSLPVGSHAKVSSPQPRGPRYAEGTFISD
PIG    MVAMKTFCLLLVSLLLAVALGEKEEGHFLSLPSLPLRFHTKASGSQPRGPRYAEGTFISD
       *** ***.***:**:***.****:**** :*****:  *:*.*. **************

HUMAN  YSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQREARALELASQANRKEEEAVEPQSSPA
PIG    YSIAMDKIRQQDFVNWLLAQKGKKSDWKHNITQREARALELAHQSNRKE-EAREPQGSLP
       ********:***************.***************** *:**** ** ***.*

HUMAN  KNPSDEDLLRDLLIQELLACLLDQTNLCRLRSR
PIG    QNPGDDDVLKDVLTRELLAWMVEQLELCRLR*-
       :**.*:*:*:*:* :**** :::* :*****
```

Whole sequence homogeneity: 72.55%

Available sequence homogeneity: 74%

GLP-1

```
HUMAN  ------------------------------------------------------------
PIG    MKTIYFVAGLFVMLVQGSWQRSLQNTEEKSRSFPAPQTDPLDDPDQMTEDKRHSQGTFTS


HUMAN  -------------------------------HDEFERHAEGTFTSDVSSYLEGQAAKEFI
PIG    DYSKYLDSRRAQDFVQWLMNTKRNKNNIAKRHDEFERHAEGTFTSDVSSYLEGQAAKEFI
                                      ****************************

HUMAN  AWLVKGRG-------------------------------------------------------
PIG    AWLVKGRGRRDFPEEVTIVEELRRRHADGSFSDEMNTVLDNLATRDFINWLLHTKITDSL
       ********

HUMAN  ------------
PIG    *EVCH*SRSCSX
```

Whole sequence homogeneity: 316.66%

Available sequence homogeneity: 100%

Insulin

```
HUMAN  -------------------MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYL
PIG    PSGTSCVPRPPASRSSPPAMALWTRLLPLLALLALWAPAPAQAFVNQHLCGSHLVEALYL
                         **** ************.* ** *****************

HUMAN  VCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICS
PIG    VCGERGFFYTPKARREAENPQAGAVELGGGL--GGLQALALEGPPQKRGIVEQCCTSICS
       ************:*****: *.* ******   *.** *****  ***************

HUMAN  LYQLENYCN-------------------
PIG    LYQLENYCN*AAPEGACCSPHPKTQ*X
       *********
```

Whole sequence homogeneity: 117.27%

Available sequence homogeneity: 87.27%

Leptin

```
HUMAN  MHWGTLCGFLWLWPYLFYVQAVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGL
PIG    MRCGPLCRFLWLWPYLSYVEAVPIWRVQDDTKTLIKTIVTRISDISHMQSVSSKQRVTGL
       *: * ** ******** **:**** :****************.**** *******:****

HUMAN  DFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLP
PIG    DFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLP
       ********:*:*********:********:********************:** **** **

HUMAN  WASGLETLDSLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC-------------
PIG    QARALETLESLGGVLEASLYSTEVVALSRLQGALQDMLRQLDLSPGC*SLEGLSPHSRGK
        * .****:********* *************:***** ********

HUMAN  ------------------------------------------------------------
PIG    KPELPGVCWRREPVRTSSLQVCGPFLSRSAKLLFQRQKTPRHDTKDRKAWFRAHRERGAR


HUMAN  ------------------------------------------------------------
PIG    PANGGLDFGFSTNVFLPVPSPAHRVLQRDRGDFRAFRPSSRVPSENSGEHGEGYRHTQLD


HUMAN  ------------------------------------------------------------
PIG    APTQHKLEAFLYLLCGVFWLDLKQNTSLSRLSGVSRG*GEAPEVLFPVPSMGLAEANPF*


HUMAN  ------------------------------------------------------------
PIG    VT*GLSRSFSRDWLCFYCD*L*NCSVCTGIALRGSRRARFS*KEEDELCQGCVRGDRKCV


HUMAN  ------------------------------------------------------------
PIG    GGRGMDPECVFLV*WTFV*GALWTG*GHCLIFVVFMREGDDSFTGVVGFCQPPVQGVGKG


HUMAN  ------------------------------------------------------------
PIG    LKPKTVGGRGELCLLQLLEAGLSHQGVRVSRWRQ*SPGRGSLPWPSEWSGLIPH*CHNRE


HUMAN  ------------------------------------------------------------
PIG    VPWFGICMLTQSKGLLPIHLGRIFSSRGRVKALGGLWALADGPKSWVSESRDSVTGLEEP


HUMAN  ------------------------------------------------------------
PIG    PSPYRSCSIGQTRRHGFPTILPL*CSHRTTGGRSVQGNLNLSNHFQD*ASIVLSPNWCYG


HUMAN  ------------------------------------------------------------
PIG    LREAHQINIKIQSCLQGPCIPDDNTSPTPRLQRLSFHHGNRAAETQCGPQQVERLS*GGQ


HUMAN  ------------------------------------------------------------
PIG    CPGAHRLTLLALGSIFTVRGASASITEKPHPFEAG*LRL*K*ENTRVPLWHRGLRIQCCC


HUMAN  ------------------------------------
PIG    CSSLGHGCGKGSIPGLGTFTCCRQGQKK*INKKKKKK
```

Whole sequence homogeneity: 474.25%

Available sequence homogeneity: 85.63%

PP

```
HUMAN  ------------MAAARLCLSLLLLSTCVALLLQPLLGAQGAPLEPVYPGDNATPEQMAQ
PIG    MESEGGCQEAGTLMAARRCLFLLLLSTCVALLLQPPLGARGAPLEPVYPGDDATPEQMAQ
                  : *** ** ************** ***:***********:********

HUMAN  YAADLRRYINMLTRPRYGKRHKEDTLAFSEWGSPHAAVPRELSPLDL-------------
PIG    YAAELRRYINMLTRPRYGKRDEEDLLDLK-CSSLHAAAPRELSPMGA*CHLLLL*LQGPC
       ***:****************.:** * :.  .* ***.******:

HUMAN  ------------------------------
PIG    RPSFPVHTLGSGQACSLLPNRLNKASQDHX
```

Whole sequence homogeneity: 132.63%

Available sequence homogeneity: 77.89%

PYY

```
HUMAN  MVFVRRPWPALTTVLLALLVCLGALVDAYPIKPEAPREDASPEELNRYYASLRHYLNLVT
PIG    MVTVRRPWPAMATVLLGLLICLGTLVDAYPAKPEAPGEDASPEELSRYYASLRHYLNLVT
       ** *******::****.**:***:****** ***** ********.**************

HUMAN  RQRYGKRDGPDTLLSKTFFPDGEDRPVRSRSEGPDLW----------------------
PIG    RQRYGKRDSPDALLSKLLFPEGEDRPVKSWPEGAYLW*PPPRLLGDMLIHRSHLHVCSRS
       ********.**:**** :**:******:*  **  **

HUMAN  --
PIG    QX
```

Whole sequence homogeneity: 106.19%

Available sequence homogeneity: 80.41%

APPENDIX 4

Primer sequences for use in RT-qPCR.

Gene (*Peptide*)	Accession Number	Forward Primer	Reverse Primer	Amplicon length
PCK1¥	NM_001123158.1	CTGAGCCACATGGAGGAAGAG	GATGACCGTCTTGCTTTCAATTC	114
PCK2¥	NM_001161753.1	ATCCGAAAGCTCCCCAAGTAC	CAATCACCGTCTTGCTTTCTACTC	88
CCK	NM_214237.2	CAGGCTCGAAAAGCACCTTCT	ATGTAGTCCCGGTCACTTATTCTGT	92
GIP	NM_001287408.1	CGCCAACAAGACTTTGTGAACT	TGTGTTTCCAGTCGCTCTTCTTC	64
GCG (*GLP-1*) *	NM_214324.1	CAAGAGGAACAAGAATAACAT	AAGAACTTACATCACTGGTA	86
NTS	XP_003481806.1	CCAGCTCCGAAAAATCTGTCA	AACATCTTCTTGAATCAACTCCCAAT	77
PP	XM_003131372.1	GGCCCAGGTACGGGAAAA	TGCATGTAAGGAGCTGCACTTC	79
PYY	NM_001256528.1	CTCGGCAGAGGTATGGGAAA	AGGGAAGAGCAGTTTGGAGAGA	62

* taken from paper by van der Wielen et al., (2014).
¥ kindly provided by Z. Daniel.