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Amino acid balance and appetition in weaned pigs

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Executive Summary

Current feeding practices in pig production include the use of safety/excess margins of the most limiting nutrients for growth, such as essential amino acids (EAA). While safety margins increase feed costs, the consensus is that they may help minimise growth variations within a herd. In addition, diets are frequently formulated disregarding specific non-essential amino acids (NEAA) while keeping a minimum of crude protein (CP) in the diet. NEAA are generally regarded as dispensable provided sufficient dietary CP is offered. Feed formulation practices pay little attention to dietary excess of EAA or NEAA. However, dietary excess of protein and/or some AA have been shown to negatively impact feed intake and growth in pigs. To the best of our knowledge, to date there has been no systematic study on the effect of dietary supplementation of the twenty proteinogenic AA on appetite in pigs. Furthermore, little is known about the mechanism associated with the appetite modulatory effect of AA in pigs. This project aimed at testing the effect of the proteinogenic AA on appetite regulation, feed intake and growth in pigs. The project was organized in four phases.

The first phase was used to test the hypothesis that high levels of dietary limiting EAA would result in an anorexigenic effect mediated by the release of CCK in the duodenum and/or GLP-1 in ileum. This would mainly refer to Lys and Met, the branched chain AA (BCAA; Ile, Leu and Val), and the aromatic AA (Trp and Phe). In addition, given that intestinal release of GLP-1 has been linked to glucose sensing, we hypothesized that high levels of glucogenic AA in the diet would also impact feed intake by stimulating GLP-1 release. Phase 2 and 3 were designed to test: a) if the impact of CCK on feed intake would be short-termed (by triggering satiation) and associated to diet selection for AA balance; and b) if the impact of the GLP-1/insulin release on feed intake would be long-lasting associated with the maintenance of energy homeostasis. Finally, the last phase (4) of the project was used as a final proof-of-concept to test if dietary excess of one selected anorexigenic AA (from the previous three phases) would negatively impact feed intake and performance following a linear dose-dependent correlation in piglets.

The first phase aimed at screening the anorexigenic capacity of the 20 proteinogenic AA, based on CCK and GLP-1 secretion. The experiment consisted of developing an ex-vivo model where porcine intestinal samples were incubated while exposed to one of the 20 proteinogenic AA at physiological relevant doses (10 mM). We anticipated that the most limiting amino acids (Lys and Met) together with the BCAA (Ile, Leu and Val) and aromatic AA (Phe and Trp) would show the strongest stimulatory effect on CCK and GLP-1. In addition, gluconeogenic AA would specifically stimulate GLP-1. The results showed that CCK and GLP-1 secretions were the highest in the duodenum and the ileum of young pigs, respectively ($p \leq 0.05$). Phe, Gln and Asn were the most significant stimulants of CCK ($p \leq 0.05$) while minimal or no effect was observed associated to the most limiting dietary AA (e.g., Lys, Met, Thr, Trp or BCAA). In addition, Arg, Glu, Met, Trp, Leu, and Ile significantly triggered GLP-1 release in ileum ($p \leq 0.05$). While the first two are gluconeogenic AA, the last four are generally regarded as potentially limiting dietary AA. Contrary to the hypothesis, the outcome of the first phase indicated that GLP-1 was more responsive to dietary limiting AA than CCK. In addition, CCK and GLP-1 were preferably released from different intestinal segments: more proximal for CCK (duodenum) and more distal for GLP-1 (ileum).

Consistent with the findings from phase 1, Phe, Leu, Ile, and Glu were selected to test in-vivo in phases 2 and 3. The AA of interest (at 3 mmol.kg-1) were administered through oral gavage following an overnight fasting in young pigs. In addition, Lys was also selected as a reference AA being the most limiting essential AA in pig diets, and the appetite modulation effects described in previous literature. On the one hand, blood samples were collected at set intervals before and after the oral gavage to measure the effect on CCK and GLP-1 levels. On the other hand, pigs were video recorded

immediately after the gavage to study the impact of the selected AAs on feeding behaviour. The results showed that Lys and Leu significantly ($p \leq 0.05$) lowered feed intake including a reduction on cumulative feed intake up to three or two hours post gavage, respectively. In addition, Lys altered the meal pattern by lowering the duration of the first meal ($p \leq 0.05$) and increasing ($p \leq 0.1$) the inter-meal interval, whereas Leu significantly ($p \leq 0.01$) increased the interval between meals and lowered ($p \leq 0.05$) the number of meals within the first two hours post-treatment. Importantly, the changes in meal pattern due to Lys and Leu gavages were associated with increased plasma levels of CCK. Phe, Ile, and Glu did not significantly affect feed intake or meal patterns. However, Phe raised GLP-1 plasma levels, which, in turn, were positively correlated with plasma CCK concentrations. In conclusion, for phases 2 and 3 of the project, Lys and Leu showed a significant anorexigenic effect associated with the release of CCK. In addition, the research provided evidence of a potential feedback mechanisms where GLP-1 would promote CCK secretion in pigs.

The integration of the results from phases1 to 3, Lys was identified as the dietary AA with the strongest and more consistent anorexigenic properties was elected for the final stage (phase 4) of the project. Phase 4 consisted in a dose-response performance experiment in post-weaning pigs testing Lys at 20%, 50% or 100% dietary excess relative to a control group offered feed formulated to meet the NRC requirements. In addition, the effect of Lys on CCK was re-tested with the same ex-vivo intestinal primary culture used in phase 1 using a wider array of concentrations tested. The performance data showed a negative linear response to dietary Lys levels on feed intake ($p \leq 0.05$) during the third- and fourth-week post weaning. In addition, a negative linear response to dietary Lys levels was observed on average daily gain the third week post weaning. In the ex-vivo test, Lys at 20 mM significantly ($p \leq 0.05$) stimulated CCK secretion from proximal jejunum. The latter is compatible with the results from the phase1 since Lys was not tested in jejunum. The main findings in phase 4 confirmed that dietary excess of Lys inhibited feed intake, and that this effect is associated with CCK secretion in pigs.

In summary, this research project studied the appetite modulatory properties of dietary AA in pigs. Firstly, an ex-vivo model of primary intestinal cultures was used to identify the most anorexigenic free AA leading to in-vivo studies. Lys was not identified as anorexigenic in the first round of ex-vivo tests, however, was selected for the in-vivo studies as a reference given its nutritional relevance and appetite modulatory effect. The in-vivo results identified a strong reduction of feed intake following the gavage of Lys and the BCAA Leu and Ile. In addition, Leu and Lys altered the meal pattern reflecting both short- and long-term anorexigenic effects. These changes in feeding behaviour triggered by Lys and Leu were linked with the secretion of the gut hormone CCK. Overall, this study has provided strong evidence of the impact of individual AA (particularly Lys, Leu, and Ile) on appetite modulation in pigs. Pig producers should closely monitor and avoid when possible, dietary excess of these key anorexigenic AAs. Other dietary amino acids identified to potentially affect appetite such as Phe or Glu, warrant further investigations.

Table of Contents

List of Tables

List of Figures

1. Background to Research

Post-weaning diets are formulated to contain low crude protein using highly digestible and palatable protein sources supplemented with growth-limiting essential AA (EAA) to stimulate early intake and prevent diarrhoea. However, the aftermath of the post-weaning process consistently shows a dry matter intake-driven plateau in growth after 3 to 5 days (Bark et al., 1986; Brooks and Tsourgiannis, 2003) (Figure 1). This event has been related to overconsumption, impaired intestinal function and often, but not always, to diarrhoea (Makkink, 1993; Bruininx et al., 2001; King and Pluske 2003; Vente-Spreeuwenberg and Beynen, 2003). However, relatively little attention has been paid to the satiation effect of proteins and amino acids in piglets. In general, the highly satiating effect of protein has been observed under ad libitum regimes (Eisenstein et al., 2002). More precisely, the inclusion of dietary protein at 25% of total calories has been established to promote early satiation and weight loss in humans (Jensen et al., 2014). The type and amount of dietary protein sources and non-protein bound AA may have a significant impact of satiation that needs to be considered (Westerterp-Plantenga, 2003).

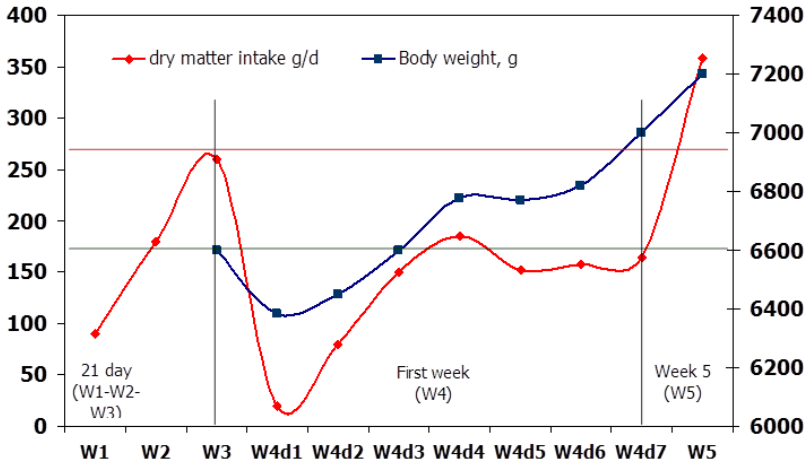

Figure 1.1. Average feed intake and growth plateau around 3-4 days post-weaning in piglets. (Sources: Bark et al., 1986; Brooks and Tsourgiannis, 2003)

Previous results from our group (Pork CRC 6A-101) showed that some AA seem to increase piglet's appetite in the first 48 hours post-weaning, but that is followed by an increase in plasma levels of cholecystokinin (CCK), a satiety hormone which accounts for a subsequent decrease in feed intake and growth over the following days. In pigs, while classical formulation preserves the EAA balance in weaner diets based on the ideal protein principle (Wang and Fuller, 1989), it does not account for excess amino acids and how they may affect satiation and satiety. Edmonds and co-workers (1986), reported that pigs avoid diets containing excess EAA (Lys, Met, Thr and Trp) compared to a balanced diet in a multiple-choice setup. In addition, Guay and Trottier (2006) showed that partial replacement of dietary CP with EAA reduced growth and protein accretion in muscle. The presence of amino acids in the oral cavity, stomach and small and large intestines are sensed by G-protein coupled receptors and carrier transporters located on enteroendocrine cells which, in turn, are responsible for the release of hormones such as CCK, Glucagon Like Peptide-1 (GLP-1) and Peptide YY (PYY). CCK, GLP-1 and PYY are known to mediate the onset of satiation and satiety by reaching some of the key areas in the brain mainly the hypothalamus and the brainstem (Black et al, 2009; Chaudhri et al., 2008; Gribble 2012; Tolhurst et al. 2012). Thus, the association between excess AA with feed intake and the appetite/satiety status in piglets warrants further investigation.

In contrast, unpublished results related also to the Pork CRC project 6A-101 uncovered that some EAA and non-EAA such as arginine and aspartic acid among others had very significant appetite enhancing properties in piglets. The traditional division of AA into EAA or non-EAA was mostly based on growth or nitrogen balance and needs to be reviewed (Hou et al., 2015). The consideration of

responses to dietary "non-EAA" would imply a recognition that farm animals seem to have dietary needs for non-EAA to achieve optimal growth, development, lactation, reproduction, and health (Hou et al., 2015). Thus, the innate sensory-motivated preference of the amino acids as uncovered by Pork CRC 6A-101 may be an indication of a more balanced requirement of AA for optimal growth, gut development, reduced weaning stress and modulation of immune responses and prevention of infectious diseases in piglets (Koopmans et al., Ren et al., 2014; Wu et al., 2013). By regulating the free AA profile in the GIT, one could potentially reduce postprandial satiating properties of feeds. The current proposal capitalized on these previous findings to develop the following objectives and hypotheses of the project.

2. Objectives

The objectives of the project are outlined below:

a) To map CCK and GLP-1 secretion sites along the gastrointestinal tract of post weaning pigs.

b) To screen the 20 proteinogenic AA evaluating their impact on the secretion of the most vastly described gut anorexigenic hormones, CCK and GLP-1, using an "ex vivo" porcine intestinal model.

c) To characterise the impact of oral gavages of selected satiating AA (based on the "ex vivo" screening) on feeding behaviour in young pigs.

d) To evaluate the impact of oral gavages of selected satiating AA (based on the "ex vivo" screening) on plasma CCK and GLP-1 levels in young pigs.

e) To evaluate the effect of dietary excess of an identified satiating AA (based on the ex-vivo and in-vivo results) on the growth performance of post weaning pigs.

The outcomes of the project include:

a) Gut hormone data showing the anorexigenic properties of the 20 proteinogenic AA at physiological relevant doses in post weaning pigs using a high throughput ex-vivo intestinal model.

b) Gut hormone and feeding behaviour data in live pigs detailing the appetite modulatory effect of the oral administration of five AA, in young pigs.

c) The performance impact of providing dietary excesses of selected AA identified as a strong gut hormone secretagogue in pigs.

3. Introductory Technical Information

Feeding represents the major cost of pork production (Patience et al., 2015). Commercial farms operate based on least-cost feeding strategies with the objective of minimizing dietary costs without compromising pig's growth and welfare (Solà-Oriol and Gasa, 2017). To do so, it is necessary to understand the nutritional requirements of the pig together with the main factors involved in modulating hunger and satiety that determine nutrient intake and growth (Nyachoti et al., 2004; Li and Patience, 2017). The least-cost feed formulation is based on the concept of providing a balanced diet that meets all the nutritional requirements of the pig (which, in turn, is a function of age, gender, and productive stage, among other factors) at the lowest cost possible. The formulation requires an accurate assessment of the nutritional value of the ingredients on a per cost unit bases. Thus, small deviations/errors in the estimates of nutrient availability from the diet may have significant impacts on the final formulation and may result in high risk of causing marginal deficiencies for some of the most limiting nutrients, particularly essential amino acids (EAA). Consequently, formulating diets with safety/excess margins for the most limiting AA has become common practice. Thus, pigs are frequently exposed to diets with higher AA content than required (Wang and Fuller, 1987; Presto Åkerfeldt et al., 2019).

The provision of excess dietary AA may have relevant effects on pig performance. Several AA have been identified as deterrents of feed intake and growth when offered in excess (Edmonds and Baker 1987; Edmonds et al., 1987; Kwon et al., 2019). The appetite modulatory effect of AA may be related to the secretion of anorexigenic gut hormones such as CCK and GLP-1 (Veldhorst et al., 2008). Phe, Trp, Ile, or Leu, among other AA, have been shown to stimulate CCK release in pigs indicating that EAA may have a prevalent role in modifying feed intake (Zhao et al., 2018, Tian et al., 2019, Feng et al., 2019). However, the impact and the mechanisms of action of dietary EAA on the hunger-satiety cycle remains largely unknown in pigs. In addition, NEAA such as Arg have also been involved in gut peptide secretion (Wang et al., 2018). In fact, the overall nutritional relevance of NEAA has been gaining momentum related to mediating metabolic pathways relevant to energy homeostasis (glucogenesis and fatty acid synthesis) and protein accretion (Wu et al., 2014). The role of NEAA on appetite control and growth remains elusive and merits further attention in pigs.

The main organs involved in the regulation of feed consumption and appetite are the hypothalamus and the brainstem, which respond to pre-absorptive and post-absorptive gut signals (Cumming and Overduin, 2007; Ahima and Antwi, 2008). During a meal, the hypothalamus and hindbrain receive information on the amount and nutrient content of the feed consumed through sensory signals from the oral cavity, stomach, or small and large intestine (de Graaf et al., 2004; Cumming and Overduin, 2007). One of these key signals are hormones secreted by enteroendocrine cells (EEC) scattered along the gastrointestinal tract (GIT), from which more than 20 different types have been identified including CCK and GLP-1 (Furness et al., 2013). CCK has been described as one of the main anorexigenic hormones in mammalian species, including the pig (Gibbs et al., 1973; Anika et al., 1981; Liverse et al., 1995). Similarly, GLP-1 secretion has been linked with reduced energy intake (Flint et al., 1998; Renner et al., 2018). In addition, GLP-1 contributes to the glycaemic control by stimulating insulin release and inhibiting glucagon secretion, among other effects (Steinert et al., 2017). Dietary protein and AA have been shown to be one of the main stimulants of CCK and facilitate sustained releases of GLP-1 in rodents and humans (Hopman et al., 1985; Liddle et al., 1985; Carr et al., 2008; Mace et al., 2012; Daly et al., 2013). A similar link between protein intake and anorexigenic hormone release has been shown also in pigs but further and more precise evidence would be critical to help nutritionists improve the formulations (Cuber et al., 1990). Understanding the role of individual AA on appetite in pigs could help improve growth performance and reduce feeding costs.

4. Research Methodology

The project consisted of 4 phases. The research methodology for all phases is described in detail below:

4.1. Phase1: Effect of amino acids on porcine gastrointestinal satiety hormone secretions (a screening method).

4.1.1. Aim

To use an "ex vivo" model with a high throughput potential to determine and quantify the ability of the 20 proteinogenic AA to trigger the release of satiety hormones (CCK and GLP-1) from porcine gastrointestinal tissues.

4.1.2. Animals, housing, and diet

Six 25-day-old male piglets (Domestic Landrace x Large White; BW= 6.94 ± 0.29 kg) were sourced from Sunpork farm (Westbrook, Queensland), and moved into a room with environmental automatic control and plastic fully slatted floor pens at Herston Medical Research Centre (HMRC) (Herston Campus, The University of Queensland). Piglets were weighted, marked, and randomly assigned to three adjacent pens (2 pigs/pen). Temperature in the nursery was thermostatically set between 27-28°C. Pigs had "ad libitum" access to water and feed for the duration of the experiment. The starter diet offered contained a higher concentration of crude protein (25%) as compare to standard weaner diets of (18-20%; NRC, 2012), to meet or exceed all EAA requirements without the need for synthetic AA supplementation. This dietary strategy was chosen to avoid the overexpression of only a few AA transporters as this might influence gut peptide secretion (Reimann et al., 2004; Tolhurst et al., 2011; Morales et al., 2017). The composition of the experimental diet is shown in Table 4.1. Following a 7-day adaptation period, piglets were euthanized to collect intestinal samples from duodenum, jejunum, and ileum. Intestinal samples were later used to measure CCK and GLP-1 secretion in response to free AA.

4.1.3. Primary intestinal cell culture

On day 7 of the trial, animals were humanely euthanized using Lethobarb. Next, the small intestine was removed completely (from the pylorus to the ileocecal valve) and its total length measured. Intestinal segments were then immediately collected from duodenum (5 cm distal from the pylorus), proximal jejunum (at 10% of the total length of the small intestine starting from the pylorus) and ileum (5 cm proximal to ileocecal valve) within 10 minutes. The sampling locations were selected based on previous data published by Adeola and King (2006), regarding intestinal morphometry in young pigs. Intestinal segments were stored in ice cold KRB/HEPES buffer bubbled with O2 /CO2 (95 %/5 %), to prevent ischemia, and transported within 20 minutes to Lab C213 at the St Lucia Campus (The University of Queensland) to be used in a porcine primary cell culture adapting the method published by Voortman et al. (2012).

Table 4.1. Composition of experimental diet (as fed basis).

Item		Item	
Ingredients	%	**Ingredients**	%
Wheat	60.8	*Analysed Composition*	
Soya bean full	16.0	Crude protein	25.13
Blood meal	3.0	Moisture	8.19
Meat meal	6.55	Ash	5.31
Fish meal	4.25	Crude fibre	2.69
Chocolate milk powder	5.0	Ether extract	6.9
Single cell protein	2.5	Lysine	1.33
Vegetable oil	1.5	Methionine	0.42
Salt	0.15	Threonine	0.95
Choline chloride 60%	0.0375	Tryptophan	0.3
Vitamin and mineral premix[1]	0.2	Glycine	1.34
Calculated Nutrient Content		Histidine	0.74
Crude protein	24.97	Arginine	1.53
Digestible energy (MJ/kg)	15.25	Alanine	1.45
Calcium	1.18	Tyrosine	0.7
Phosphorus	0.86	Valine	1.22
Lysine	1.40	Serine	1.14
Methionine	0.44	Phenylalanine	1.28
Threonine	0.94	Isoleucine	0.88
Tryptophan	0.28	Leucine	1.88
Met/Lys	0.31	Glutamic acid	4.47
(Met + Cys)/Lys	0.60	Proline	1.69
Trp/Lys	0.20	Hydroxyproline	0.27
Thr/Lys	0.67	Aspartic acid	2.1

[1] Premix composition (ad-fed basis): vitamin A (10,000 IU/kg), vitamin D3 (1,800 IU/kg), vitamin E (100 mg/kg), vitamin K3 (5 mg/kg), vitamin B1 (3 mg/kg), vitamin B2 (6 mg/kg), niacin (30 mg/kg), pantothenic acid (30 mg/kg), pyridoxine (4 mg/kg), biotin (0.3 mg/kg), folic acid (2.5 mg/kg), vitamin B12 (0.04 mg/kg), iron (100 mg/kg), iodine (0.7 mg/kg), manganese (45 mg/kg), selenium (0.3 mg/kg), zinc (120 mg/kg), cobalt (0.3 mg/kg), copper (10 mg/kg).

Tissue segments were rinsed with cold KRB buffer (to clean the tissues from any debris), cut open longitudinally and had their outer muscle layer strip off using tweezers (Figure 4.1A). Equally sized round tissue samples (approximately 1.13 cm2) were punched out with a 12 mm biopsy punch (Figure 4.1B, C). Sixty-three samples were taken from each segment to test each treatment (20 AA + control) in triplicates within each tissue (duodenum, jejunum, and ileum) and animal (n=6). Each circular tissue sample was then transferred into a 24-well plate filled with 500 μl ice-cold KRB/HEPES buffer at pH 7, 4 (Figure 4.1D). The plates were brought to room temperature within 30 min, followed by a pre-incubation at 37°C for 1 hour in a humidified incubator at 5 % v/v CO2. The KRB/HEPES buffer was then replaced by a pre-warmed KRB/HEPES buffer (37 °C, 500 μl with pH 7.4) without glucose but containing one of the 20 AA or a control (buffer with no added AA or glucose) and incubated for an additional 1 hour at 37 °C and 5 % v/v CO2. All AA were tested at 10 mM except Tyr which was tested at 2.5 mM due to low solubility. After incubation, the media from each well was collected into eppendorf tubes and stored at -80°C for future hormone analysis. Likewise, tissue samples were transferred into eppendorf tubes filled with RNAlater and left at room temperature for 24 hours before being placed at -80°C for future mRNA analysis. Tissue viability was assessed based on lactate dehydrogenase (LDH) activity (cytosolic enzyme released following cell abrasion) compared to the positive controls (tissue samples treated with 1% of Triton-X 100). The collected media for the evaluation of LDH was stored at 4°C until analysis.

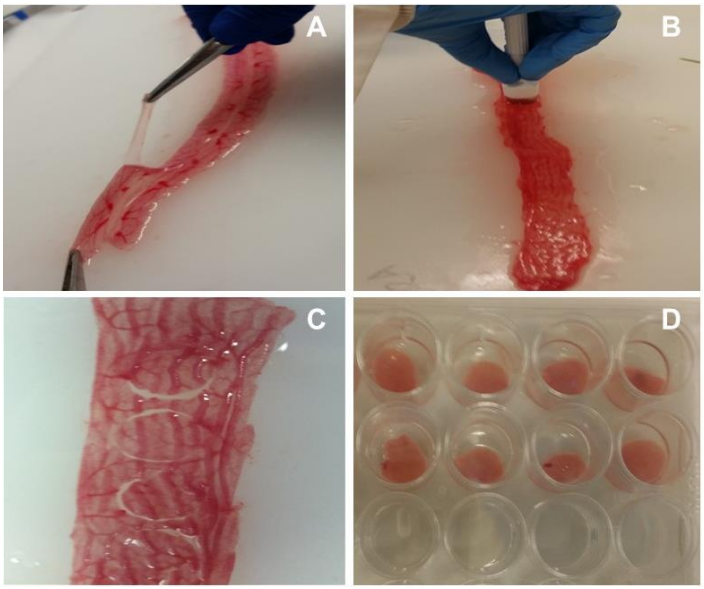

Figure 4.1. Photographs depicting the "porcine primary intestinal cell culture" preparation process: removal of the outer muscle layer (A), sample collection with biopsy punch (B and C), placement of tissue in a 24-well plate filled with cold KRB/HEPES buffer before incubation (D).

4.1.4. Hormones and lactate dehydrogenase analysis

Concentrations of total CCK were analysed using a Porcine Cholecystokinin ELISA kit (MBS264395) from MyBioSource (San Diego, California, USA). The inter-assay coefficient of variation for the CCK kit was 7.5% and the intra-assay coefficient of variation was 3.2%. GLP-1 supernatant levels were analysed using the Glucagon-Like Peptide-1 (Total) ELISA kit (EZGLPT1-36K) from Millipore (Burlington, Massachusetts, USA). Inter-assay coefficient of variation was 7.8%, whereas the intra-assay

coefficient of variation was 2.1%. The optical density of the ELISA plate wells was measured in a BMG FLUOstar OPTIMA Microplate Reader (BMG Labtech, Mornington, Victoria, Australia). LDH activity, on the day of the experiment, was determined using a Roche LDH reagent kit PLUS (Sigma-Aldrich, Castle Hill, New South Wales, Australia.).

4.1.5. RNA extraction and RT-qPCR analysis

Initial extraction of RNA from the intestinal mucosa samples was performed using Trizol® Reagent (Cat. No. 15596026) (Invitrogen, Carlsbad, California, USA). The PureLink® RNA Mini Kit (Cat. No. 12183018A) (Invitrogen, Carlsbad, California, USA), was then used for the isolation of high-quality total RNA. RNA quality and concentration in tissue samples was measured with Invitrogen Nano Drop spectrophotometer (NanoDrop 8000, Thermofisher Scientific, Waltham, Massachusetts, USA). Next, the cDNA synthesis was performed with QuantiTect® Reverse Transcription Kit (Cat. No. 205313) (Qiagen, Hilden, Germany). Primers for CCK (forward: 5'- CAGGCTCGAAAAGCACCTTC -3', reverse: 5'- GCGGGGTCTTCTAGGAGGTA -3', 157 bp), GCG (5'- AGAACTCCGCCGCAGACA -3', reverse: 5'- TAAAGTCTCGGGTGGCAAGATT -3', 65 bp) and GAPDH (forward: 5'- TGGTGAAGGTCGGAGTGAAC -3', reverse: 5'- GAAGGGGTCATTGATGGCGA -3', 104 bp) used in this study have previously been published by Tian et al., (2019) and da Silva et al., (2014). The reaction volume (10.05 ul) for the real-time PCR contained the following: 5 µl of SYBR Green master mix solution, 3 µl of RNAs free water, 1 µl of cDNA sample, 0.5 µl forward and reverse PCR primers and 0.05 µl of ROX reference dye solution. The PCR program was set for denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s using QuantStudioTM 6, Thermofisher Scientific (Waltham, Massachusetts, USA). All samples were measured in triplicates. GAPDH was used as reference gene for the relative calculations of gene expression levels following the Pfaffl method (Pfaffl, 2001).

4.1.6. Statistical analysis

CCK and GLP-1 secretion and mRNA expression data across intestinal segments were analysed using a two-way ANOVA considering "tissue" as fixed effect and "pig" as random effect and their interaction, followed by a Tukey post hoc test. Two-way ANOVA was run with "AA" as fixed factor and "pig" as random factor to assess the effect of individual AA on CCK secretion. Within each pig, each of the 20 AA was independently tested on three tissue samples (biological replicates). Data are presented as the mean ± SEM of absolute amounts or percentage of control. Results were considered statistically significant when the $p \leq 0.05$ and a statistical trend when $p \geq 0.05$ and $p \leq 0.1$. The number of samples (n) in this experiment refers to the number of pigs.

4.2. Phase 2: Porcine blood satiety hormonal profiles in response to amino acids

4.2.1. Aim:

To assess the blood-kinetics of satiety hormones (CCK and GLP-1) released in pigs administered highly anorexigenic amino acids by oral gavage.

4.2.2. Animals, housing, and diet

Eight male pigs (Landrace x Large White; BW = 18.23 ± 1.06 kg) were sourced from SunPork farm (Westbrook, Queensland), and moved into a room with environmental automatic control and plastic

fully slatted floor pens at HMRC (Herston Campus, The University of Queensland). Animals were individually housed. Pigs had ad libitum access to feed and water throughout the experiment, unless otherwise stated. The experimental diet contained no amino acidic supplements but, a higher content of crude protein (25%) to cover all amino acid requirements (Table 4.2). The reason for the utilization of this diet has been stated in the previous phase. At the end of the experiment, all animals were euthanized with an intravenous injection of Lethobarb (162.5 mg/kg).

4.2.3. Anaesthesia and surgery

Similar surgical procedures to those described by Pluschke and colleagues (2016) were followed. In short, pigs were initially sedated with intramuscular injections of Ketamine (5 mg/kg) and Xylazil (0.35 mg/kg). Animals were then moved into the surgery room, where complete sedation was achieved by the administration of isoflurane (4%) through an anaesthetic facemask. Pigs were then restrained on an operating table (in the dorsal recumbency position) and a laryngeal mask was inserted into their airway (5.5 mm endotracheal tube) and connected to an anaesthetic machine for the continuous administration of isoflurane. Pigs were maintained on 1.5-3% Isoflurane during the procedure. Animals were constantly monitored while anaesthetised for heart rate, breathing rate and blood oxygen levels.

After confirming complete anaesthesia induction, the neck was shaved and scrubbed. A small linear incision (approx. 5 cm) was made lateral to the lateral sternomandibularis muscle and blunt dissection was used to expose the external jugular vein (Figure 4.2, A). Two ligatures were then placed on the cranial and caudal side of the exposed section of the external jugular vein to keep the vessel in place for the insertion of a central venous catheter (Figure 4.2, B). A ligature with monosyn 3/0 was next placed around the jugular vein, to secure the catheter (Figure 4.2, C). Patency of the catheter was checked after the ligatures were secured. A second superficial incision (2 cm approx.) was subsequently made on the right side of the neck to allow for a secure and straight passage of the catheter through the subcutaneous layer of the neck to the nape (Figure 4.2, D). Incisions were closed with monosyn suture, cover with sterile surgical dressing and the catheter fix to the skin with the same suture (Figure 4.2, E). To secure the catheter placement externally, sterile adhesive tape (Elastoplast) was used around the neck couple with superglue (Figure 4.2, F).

A single dose of anti-inflammatory and analgesic (Meloxicam 0.3 mg/kg; Butorphanol 0.2 mg/kg) was administered intramuscularly, and a local anaesthetic (Lidocaine, 20mg/L) was infused in the incision site to reduce the discomfort of the animals following surgery. A single dose of tulathromycin (Draxxin, 2.5 mg/kg) was also intramuscularly injected to provide some antibiotic cover. Following the intervention, pigs were left with an oxygen masks, wrapped in blankets and closely monitored until signs of recovery appear. Pigs were then transferred back to their pens and monitored until fully recover from the anaesthesia.

Table 4.2. Composition of experimental diet (as fed basis).

Item		Item	
Ingredients	%	**Ingredients**	%
Wheat	60.8	*Analysed Composition*	
Soya bean full	16.0	Crude protein	24.65
Blood meal	3.0	Moisture	8.2
Meat meal	6.55	Ash	5.27
Fish meal	4.25	Crude fibre	2.56
Chocolate milk powder	5.0	Ether extract	6.95
Single cell protein	2.5	Lysine	**1.32**
Vegetable oil	1.5	Methionine	0.41
Salt	0.15	Threonine	0.93
Choline chloride 60%	0.0375	Tryptophan	0.29
Vitamin and mineral premix[1]	0.2	Glycine	1.31
Calculated Nutrient Content		Histidine	0.73
Crude protein	24.97	Arginine	1.50
Digestible energy (MJ/kg)	15.25	Alanine	1.41
Calcium	1.18	Tyrosine	0.69
Phosphorus	0.86	Valine	1.19
Lysine	1.40	Serine	1.11
Methionine	0.44	Phenylalanine	1.25
Threonine	0.94	Isoleucine	0.87
Tryptophan	0.28	Leucine	1.83
Met/Lys	0.31	Glutamic acid	4.45
(Met + Cys)/Lys	0.60	Proline	1.67
Trp/Lys	0.20	Hydroxyproline	0.27
Thr/Lys	0.67	Aspartic acid	2.05

[1] Provided the following: vitamin A (10,000 IU/kg), vitamin D3 (1,800 IU/kg), vitamin E (100 mg/kg), vitamin K3 (5 mg/kg), vitamin B1 (3 mg/kg), vitamin B2 (6 mg/kg), niacin (30 mg/kg), pantothenic acid (30 mg/kg), pyridoxine (4 mg/kg), biotin (0.3 mg/kg), folic acid (2.5 mg/kg), vitamin B12 (0.04 mg/kg), iron (100 mg/kg), iodine (0.7 mg/kg), manganese (45 mg/kg), selenium (0.3 mg/kg), zinc (120 mg/kg), cobalt (0.3 mg/kg), copper (10 mg/kg).

The pigs were closely monitored and left for 48 hrs with ad libitum access to feed and water to ensure full recovery from the surgical procedure. The recovery time of 48 hrs was decided based on post-surgery feed intake data acquire from a pilot study performed by our group (unpublished data) as well as that from Pluschke et al., 2018. During these 2 days, patency of catheters was checked once a day by the administration of 3 ml saline solution and 3 ml heparinised saline solution (100 IU).

4.2.4. Oral gavage procedure

Animals were fasted overnight (13 hrs) following which, an oral gavage with one of seven treatments (water, D-glucose, Lys, Glu, Ile, Leu or Phe) at a dose of 3 mmol.kg-1 was administered (partly as a suspension) under light anaesthesia with isoflurane by mask ventilation. When signs of the anaesthesia started to appear (Figure 4.3, A), the pigs were placed on a table with their heads lifted as to facilitate the administration of the gavage via the use of a 60 ml syringe and a 25 cm long plastic extension tube with soft edges (Figure 4.3, B). Pigs remained under observation for 2 min after the procedure to check for any abnormalities. Finally, animals were returned to their pens for the subsequent blood collection.

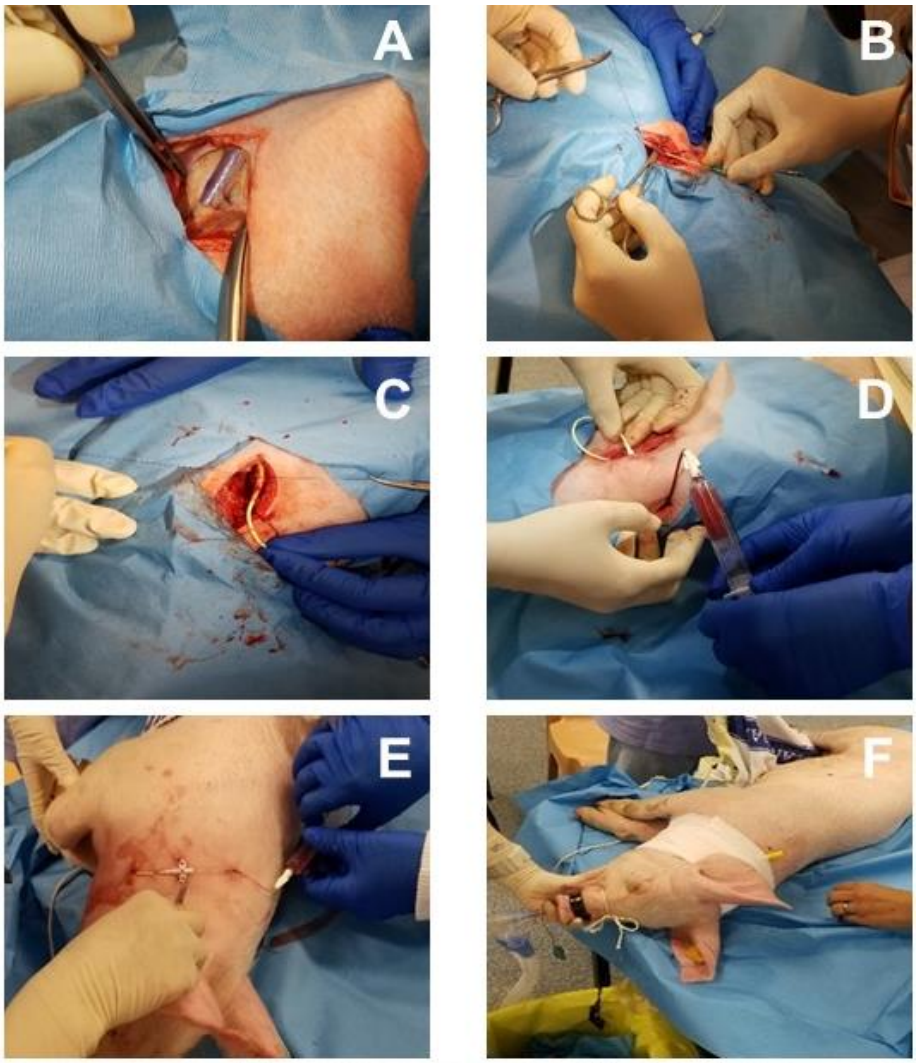

Figure 4.2. Photographs depicting the surgical catheterization procedure: exposure of the external jugular vein (A), penetration of jugular vein with sterile introducer needle (B), securement of catheter with ligature (C), internal passage of catheter to the nape (D), closure of incisions and check of catheter patency (E), external securing of the catheter with sterile adhesive tape (F).

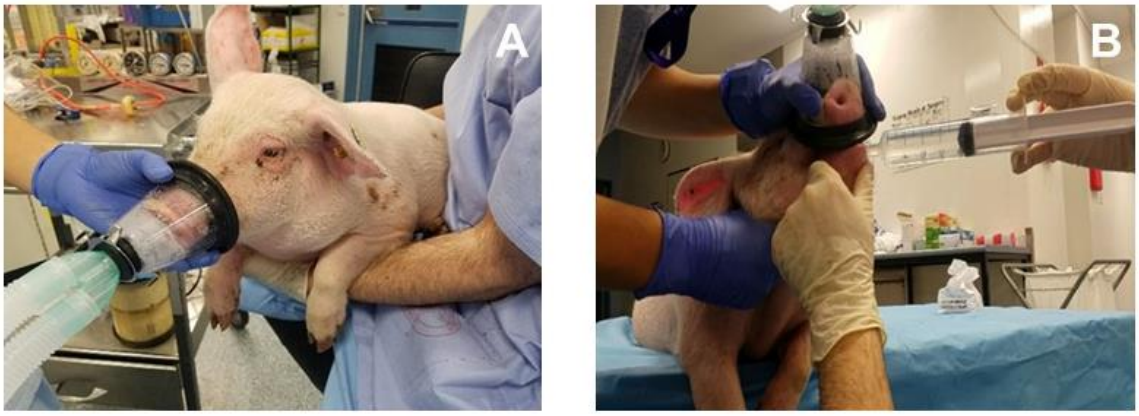

Figure 4.3. Photographs illustrating the oral gavage procedure: application of the light anaesthesia with isoflurane (A) and the administration of the treatment solution (B).

4.2.5. Blood collection

The blood sampling procedure is summarized in Figure 4.4. Samples were collected 5 min before the gavage (baseline values) and at 5-, 15-, 30-, 60- and 90-min post gavage. Before each sampling, 1 ml of blood was discarded to avoid heparin contamination. Two ml of blood were collected using a 3 ml syringe and immediately transferred into pre-chilled a vacutainer P800 (containing proteases inhibitors). Following every blood sampling, 3 ml of saline solution (0.9%) followed by 1 ml of heparinised saline solution (4 IU) were administered to the pigs to provide fluid restoration and ensure patency of the catheter. Three ml of saline solution followed by 1 ml of heparinised saline solution (100 IU) were administered to the pigs after the last sampling to ensure patency of the catheters until the next day. Feeders were then placed back in each pen and animals had access to food until 6:30 pm (9 to 9.5 hours of feed access approx.), after which, feed was retrieve again to allow for an overnight fasting before the next sampling day. The procedure was repeated for five consecutive days.

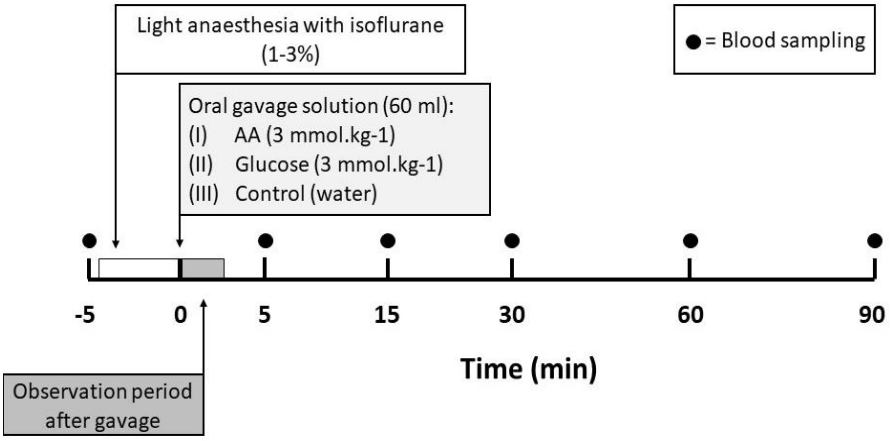

Figure 4.4. Blood collection protocol. At t = -5 min, baseline blood samples were collected. Immediately after the first blood sampling light anaesthesia with isoflurane was applied by mask ventilation. At t = 0, oral gavages with control (water) or 3 mmol.kg-1 of glucose (GLP-1 positive control), L-Glu, L-Ile, L-Leu, L-Lys or L-Phe were administered with a 25 cm plastic extension tube. Following a 2-3 min period of observation after the gavage, animals were placed back into their corresponding pen. For the next 90 min after the gavage, blood samples were collected at t = 5, 15, 30, 60 and 90 min. At t = 90, pigs had "ad libitum" access to food again.

4.2.6. Analytical procedures

Blood was centrifuged at 3000 rpm (4°C) for 10 min within 1 hour of collection and the plasma aliquoted in Eppendorf tubes before being frozen at -80°C for hormone analysis. Plasma samples were analysed for total CCK using a Porcine Cholecystokinin ELISA kit (MBS264395) from MyBioSource (San Diego, California, USA). Inter-assay coefficient of variation for the CCK kit was 10.1% and intra-assay coefficient of variation was 7.9%. GLP-1 levels in plasma samples were measured using a Glucagon-Like Peptide-1 (Total) ELISA kit (EZGLP1T36K) from Merck Millipore (Burlington, Massachusetts, USA). Inter-assay coefficient of variation for the GLP-1 kit was 12.5% and intra-assay coefficient of variation was 3.2%. CCK and GLP-1 sample levels were measured according to the manufacturer's instruction. The microplate reader equipment used for optical density recordings was BMG FLUOstar OPTIMA (BMG Labtech, Mornington, Victoria, Australia).

4.2.7. Statistical analysis

CCK and GLP-1 plasma data were analysed using the repeated measures ANOVA considering "treatment", "time" and their interaction as fixed effects and "pig" and "day" as random effects. The area under the curve (AUC) was analysed using a mixed model considering the fixed effect "treatment" and the random effects "pig" and "day". When significance was found for plasma or AUC data, Dunnett's *post hoc* test was performed for the comparison of individual treatments to the control group. AUC was calculated for each subject/treatment using the trapezoidal rule. Missing data for the calculation of the AUC were estimated using a Best Linear Unbiased Estimator (BLUE) in RStudio. Correlation analysis between plasma CCK and GLP-1 AUC was performed by Pearson correlation. Data are presented as the mean ± SEM. Results were considered statistically significant when $p \leq 0.05$ and tendencies when $0.05 \leq p \leq 0.1$.

4.3. Phase 3: Short-term feeding behaviour in pigs in response to AA

4.3.1 Aim

To assess the impact of orally administered anorexigenic AA on feeding behaviour (feed intake and meal pattern) in pigs.

4.3.2. Animals, housing, and diet

Twelve 6-week-old male pigs (Landrace x Large White, BW = 16.10 ± 2.69 kg) were used in the present study. Animals were transported from SunPork Farms, Westbrook, to Herston Medical Research Centre, Herston Campus, The University of Queensland. Pigs were housed individually in slatted floor pens (1.7m x 1.2m) and temperature maintain at 23-24°C during the complete duration of the experiment. Pigs were exposed to 12 hours of light (programmed from 7.00h to 19.00h) and light intensity was controlled and maintained between 40 – 60 lux. Pigs had ad libitum access to both feed and water throughout the experiment, unless otherwise stated. The experimental diet (Table 4.3) was formulated to contain a high crude protein (25%) to cover all AA requirements without synthetic/free AA supplements. The experimental diet was chosen based on the criteria explained in the first phase. At the end of the experiment, all animals were humanely euthanized (Lethobarb 162.5 mg/kg) before sample collection.

Table 4.3. Composition of the experimental diet (as fed basis).

Item	
Ingredients	**%**
Wheat	60.8
Soya bean full	16.0
Blood meal	3.0
Meat meal	6.55
Fish meal	4.25
Chocolate milk powder	5.0
Single cell protein	2.5
Vegetable oil	1.5
Salt	0.15
Choline chloride 60%	0.0375
Vitamin and mineral premix[1]	0.2
Calculated Nutrient Content	
Crude protein	24.97
Digestible energy (MJ/kg)	15.25
Calcium	1.18
Phosphorus	0.86
Lysine	1.40
Methionine	0.44
Threonine	0.94
Tryptophan	0.28
Met/Lys	0.31
(Met + Cys)/Lys	0.60
Trp/Lys	0.20
Thr/Lys	0.67

Item	
Ingredients	**%**
Analysed Composition	
Crude protein	24.17
Moisture	8.21
Ash	5.23
Crude fibre	2.44
Ether extract	6.95
Lysine	**1.31**
Methionine	0.40
Threonine	0.91
Tryptophan	0.29
Glycine	1.28
Histidine	0.72
Arginine	1.48
Alanine	1.37
Tyrosine	0.68
Valine	1.17
Serine	1.11
Phenylalanine	1.22
Isoleucine	0.86
Leucine	1.78
Glutamic acid	4.44
Proline	1.66
Hydroxyproline	0.27
Aspartic acid	2.01

[1] Provided the following: vitamin A (10,000 IU/kg), vitamin D3 (1,800 IU/kg), vitamin E (100 mg/kg), vitamin K3 (5 mg/kg), vitamin B1 (3 mg/kg), vitamin B2 (6 mg/kg), niacin (30 mg/kg), pantothenic acid (30 mg/kg), pyridoxine (4 mg/kg), biotin (0.3 mg/kg), folic acid (2.5 mg/kg), vitamin B12 (0.04 mg/kg), iron (100 mg/kg), iodine (0.7 mg/kg), manganese (45 mg/kg), selenium (0.3 mg/kg), zinc (120 mg/kg), cobalt (0.3 mg/kg), copper (10 mg/kg).

4.3.3. Feed intake and meal pattern recording

Video cameras (ShenZhen Foscam Intelligent Technology Co., Ltd., Guangdong, China) were installed on top of each pen to record individual feeding behaviour. Video cameras were turned on 10 min before the administration of an oral gavage (procedure previously described in phase 2) and left on for the following 240 min post gavage to account for the meal microstructure during that time including latency to first meal (LFM), first meal duration (FMD), second meal duration (SMD), inter-meal interval (IMI) and number of meals (NM) (within the first 2 hours post gavage). All video images were analysed by the same observer using the software BORIS (Behavioural Observation Research Interactive Software, version 7.4.7, Turín, Italy). Feed intake was measured manually by weighing food containers every 30 min for the first 4 hours post-gavage and then the next morning (24 hours post gavage).

4.3.4. Meal criteria

Treatment effects on meal pattern and structure were predicted following the meal criteria proposed by Bigelow and Houpt (1988). This meal assessment was chosen based on the similarity of the current experimental design, including body weight and age as well as housing conditions. In brief, the analysis considered any eating pause shorter than 10 min an inner pause within a meal, and the initiation of an eating episode following a pause higher than 10 min a new meal.

4.3.5. Statistical analysis

Statistical analysis was performed using RStudio, Inc., Boston, Massachusetts, USA. Data are expressed as absolute amounts or percentage of control. All data are presented as the mean ± SEM. Feed intake and meal pattern data were analysed using a mixed model considering "treatment" as fixed effect and "pig" and "day" as random effects. When significance for "treatment" was found, Dunnett's post hoc tests were performed. Data above or below three standard deviations of the mean were removed from the statistical analysis. The number of samples (n) refers to the number of pigs used. Results were considered statistically significant when $p \leq 0.05$ and a tendency when $0.05 < p \leq 0.1$.

4.4. Phase 4: Testing the effect of dietary Lys excess on growth performance

4.4.1 Aim:

To evaluate the effect of dietary Lys excess on the performance of post weaning piglets tested in-group or individually.

4.4.2. Animals, housing, and diet

Five hundred and sixty 3-week-old pigs (Landrace x Large White; BW = 6.42 ± 0.2 kg) were weaned and allocated into 40 pens (14 pigs/pen) based on sex and BW at SunPork Farms weaner-pig research facility, Westbrook, Queensland. Pens were classified into four groups according to their BW and gender. One of four experimental diets were provided to each pen: standard starter diet (1.20 Lys SID; T0) formulated to meet 100% of the Lys requirements or one of three diets supplemented with Lys in order to reach 120% (T1), 150% (T2) or 200% (T3) of SID Lys requirements (NRC, 2012). To maintain the same nitrogenous concentrations among dietary treatments, L-alanine was supplemented to T0, T1 and T2. The composition of experimental diets is shown in Table 4.5. Feed intake and body weight were recorded once a week (day 7, 14, 21 and 28 of experiment). Pigs had ad libitum access to both feed and water throughout the 4 weeks of experiment.

4.4.3. Primary intestinal cell culture

The intestinal samples were collected from post weaning pigs (Landrace x Large White; BW = 6.81 ± 0.12 kg) housed at Herston Medical Research Centre (HMRC, Herston Campus, The University of Queensland), and used in a primary intestinal culture model as previously described in phase 1. In brief, intestinal segments were cleaned of debris, cut open longitudinally and stripped of their muscle layer before equally sized samples were excised using a biopsy punch (Acuderm inc., Fort Lauderdale, Florida, USA) and placed in 24-well plates Thermofisher scientific, Waltham, Massachusetts, USA) filled with ice-cold KRB-HEPES buffer. After a 30 min period at room temperature, plates were moved into a humidified incubator for a period of 1 hour at 37 °C and 5 % v/v CO_2, after which the content of each well was replaced with a new solution of KRB/HEPES buffer containing Lys at 0, 10, 20 or 30 mM. Plates were placed back in the incubator for 1h followed by the collection of the supernatant. The samples were stored in Eppendorf tubes at -80°C for later hormone analysis. Tissue samples were placed in RNAlater at room temperature for a period of 24 hours before frozen at -80 °C for later mRNA analysis. The viability of the intestinal culture was assessed by measuring Lactate dehydrogenase (LDH) activity in tissue samples relative to a positive control incubated with 1% Triton-X 100.

4.4.4. Hormone analysis

Supernatant samples were analysed for total CCK using a Porcine Cholecystokinin ELISA kit from MyBioSource (MBS264395, San Diego, California, USA). Intra- and inter-assay coefficient of variation for the CCK kit was 9.3%, respectively. Supernatant GLP-1 levels were measured using a Glucagon-Like Peptide-1 (Total) ELISA kit from Merck Millipore (EZGLPT1-36K, Burlington, Massachusetts, USA). Intra- and inter-assay coefficient of variation for the GLP-1 kit was 1.9% and 2.2%, respectively. Optical density recordings of the ELISA plates were performed in BMG LABTECH FLUOstar OPTIMA (BMG Labtech, Mornington, Victoria, Australia).

4.4.5. Statistical Analysis

Statistical analysis was performed using RStudio Version 1.2.5033, Inc., Boston, Massachusetts, USA. All data were expressed as the mean ± SEM. The performance data was analysed using a linear mixed model considering "Lys dose", "week", "sex" and their interaction as fixed effect and "pen" and "batch" as random effect. The number of pigs per pen were accounted as well within the model. Gut hormone secretion data (ex-vivo) was analysed with a linear mixed model taking into account "Lys dose" as fixed effect and "pig" as random effect, followed by a Dunnett post hoc test. The number of samples (n) refers to the number of pens or pigs used in the commercial and ex-vivo trial, respectively. Results were considered statistically significant at $p \leq 0.05$ and trends when $0.05 \leq p \leq 0.1$.

Table 4.4. Composition of experimental diets (as-fed basis) (Westbrook trial).

	Dietary Treatments[1]			
Ingredients (%)	T0	T1	T2	T3
Wheat	50.26	50.17	50.04	50.59
Biscuit meal sweet	5.0	5.0	5.0	5.0
Soybean meal	8.4	8.4	8.4	8.4
Wilpromil	3.95	3.95	3.95	3.95
F/f. soyabean	1.6	1.6	1.6	1.6
Blood meal	2.0	2.0	2.0	2.0
Meat meal 51.0	3.0	3.0	3.0	3.0
Fish meal 60.0 skf/stp	5.0	5.0	5.0	5.0
Whey powder 11.5 % sweet	12.5	12.5	12.5	12.5
Hilyses	2.0	2.0	2.0	2.0
Canola oil	3.0	3.0	3.0	3.0
Salt	0.2	0.2	0.2	0.2
Zinco plus	0.1	0.1	0.1	0.1
Betaine (Betafin)	0.1	0.1	0.1	0.1
Dl methionine	0.125	0.125	0.125	0.125
Lysine hcl	0.4	0.79	1.38	1.59
L-alanine	1.53	1.22	0.76	0.0
L-threonine	0.1	0.1	0.1	0.1
L-tryptophan	0.035	0.035	0.035	0.035
Rovabio excel 10%	0.05	0.05	0.05	0.05
Hi-phos starter diets	0.0075	0.0075	0.0075	0.0075
Activo	0.02	0.02	0.02	0.02
Formi	0.4	0.4	0.4	0.4
Luctarom sweet apple flavour	0.03	0.03	0.03	0.03
Sunpork starter premix	0.2	0.2	0.2	0.2
Calculated Nutrient Content (%)				
Dry matter	91.18	91.18	91.18	91.18
Protein	22.77	22.77	22.77	22.77
Crude fibre	2.13	2.13	2.13	2.13
Non-digestible fibre	6.85	6.85	6.85	6.85
Digestible energy (mj/kg)	14.85	14.85	14.85	14.85
Isoleucine	0.86	0.86	0.86	0.86
Lysine	1.53	1.83	2.29	3.05
Methionine	0.48	0.48	0.48	0.48
Threonine	0.96	0.96	0.96	0.96
Tryptophan	0.29	0.29	0.29	0.29
Methionine + cysteine	0.84	0.84	0.84	0.84
Sid Lys	1.32	1.32	1.32	1.32
Calcium	0.86	0.86	0.86	0.86
Phosphorus	0.69	0.69	0.69	0.69
Available phosphorus	0.56	0.56	0.56	0.56
Cal/pho	1.26	1.26	1.26	1.26
Sodium	0.27	0.27	0.27	0.27
Potassium	0.75	0.75	0.75	0.75
Chloride	0.34	0.42	0.53	0.57
Choline (g/kg)	1.98	1.98	1.98	1.98
Sid Ly/de (%/mj)	0.09	0.11	0.14	0.18

[1]T0, 100% Lys group; T1, 120% Lys group; T2, 150% Lys group; T3, 200% Lys group.

5. Results

5.1. Gut hormone secretion and mRNA expression along the GI tract

Figure 5.1-A shows the concentration of CCK release from duodenum, jejunum, and ileum in post-weaning piglets. CCK secretion was higher in duodenum when compared to proximal jejunum ($p<0.05$) and ileum ($p<0.01$). A similar pattern was observed with the gene expression of CCK (Figure 5.1-B) where CCK mRNA abundance was greater in duodenum ($p\leq0.05$) than in proximal jejunum or ileum.

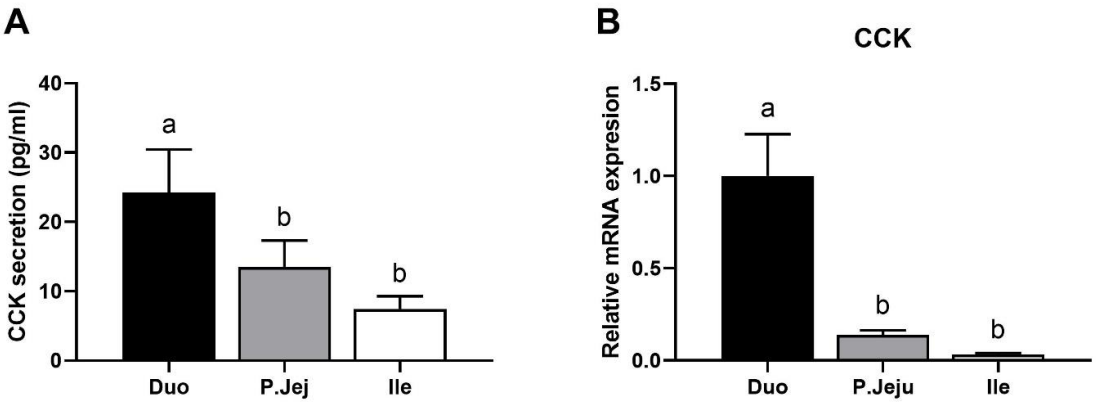

Figure 5.1. Tissue hormone levels (measured in supernatant) (A) and mRNA expression (B) of CCK in primary cultures of duodenum (Duo), proximal jejunum (P. Jeju) and ileum (Ile) of post-weaning pigs (n=5). Data are expressed as the mean + SEM. Different letters (a, b, c) indicate significant differences ($p\leq0.05$).

The concentration of GLP-1 and the pro-glucagon (GCG) gene expression levels obtained from duodenum, proximal jejunum, and ileum of post weaning pigs are shown in Figures 5.2-A and B. GLP-1 concentrations and GCG expression were significantly ($p\leq0.001$) higher in ileum when compared to jejunum or duodenum. Our results suggest that the ileum of post weaning pigs is the main intestinal segment for GLP-1 synthesis and secretion.

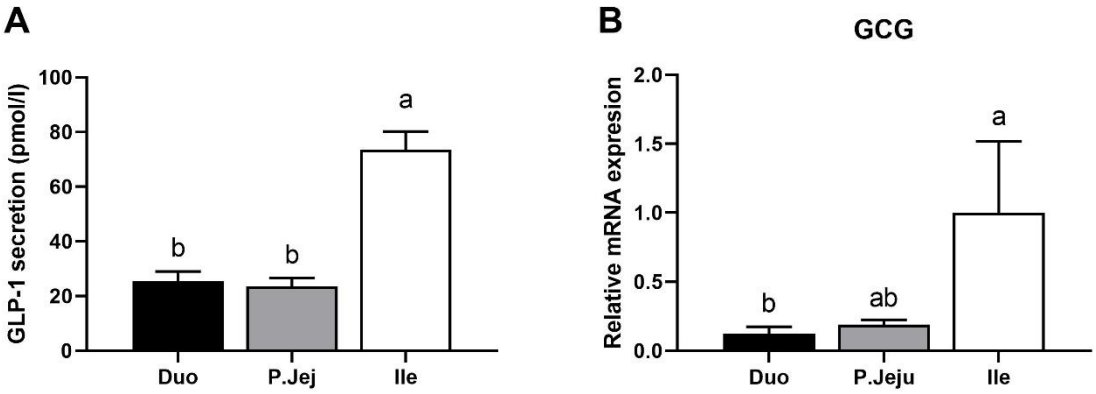

Figure 5.2. GLP-1 secretion and pro-glucagon (GCG) expression in primary cultures of duodenum (Duo), proximal jejunum (P. Jeju) or ileum (Ile) of post-weaning pigs (n=5); A) hormone levels of GLP-1 measured in the supernatant of the primary cultures; B) mRNA abundance of pro-glucagon (GCG) in the three tissues analysed (Duo, P.Jeju or Ile). Data are expressed as the mean + SEM. Different letters (a, b, c) indicate $p\leq0.05$.

5.2 AA effect on CCK and GLP-1 secretion

5.2. CCK and GLP-1 secretion from duodenum and ileum exposed to free AA solutions

Based on the previous results, duodenum and ileum were selected to study the impact of free AA on CCK and GLP-1, respectively. Figures 5.3 and 5.4 show the effect of 9 EAA and 11 NEAA on the release of CCK from the duodenum in post-weaning pigs, respectively. Phe (33.26 ± 3.44 pg/ml) was the only treatment that significantly ($p \leq 0.05$) increased CCK levels when compare to the control (20.11 ± 9.69 pg/ml). In addition, Leu (31.68 ± 3.57 pg/ml), His (29.84 ± 4.11 pg/ml), Trp (25.98 ± 2.59 pg/ml) and Thr (25.90 ± 2.80 pg/ml) tended to increase ($p \leq 0.1$) CCK levels compared to the control group. In contrast, Lys, Met, and Ile had no effect on CCK secretion at the concentration tested (10 mM) ($p > 0.1$).

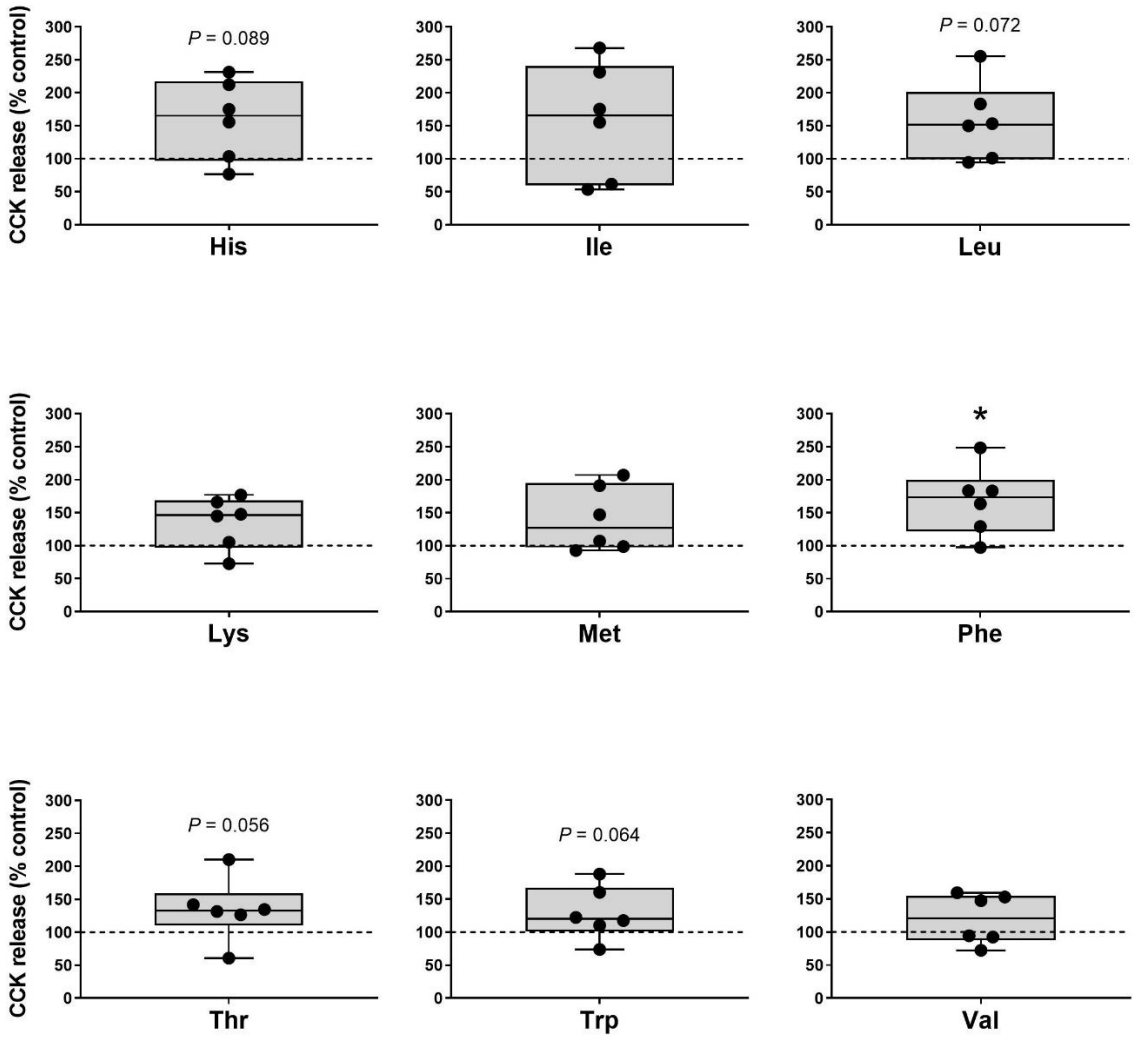

Figure 5.3. CCK secretion from porcine duodenum cultures following a 1-hour incubation with EAA (His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val) at 10 mM. Data are expressed as percentage of control (KRB buffer alone = dotted line). n=6, each data point within boxplots represents the average of three biological replicates within each pig. * = $p<0.05$, ** = $p<0.01$.

Amongst the 11 NEAA, Asn (31.46 ± 4.47 pg/ml) and Gln (30.71 ± 3.35 pg/ml) significantly stimulated ($p < 0.05$) CCK secretion whereas, Gly (27.08 ± 3.62 pg/ml), Ser (29.11 ± 4.22 pg/ml) and Cys (28.27± 4.34 pg/ml), tended ($p \leq 0.1$) to do so when compared to control samples (20.11 ± 9.69 pg/ml). Ala, Arg, Asp, Glu, Pro and Tyr at concentrations of 10 mM, had no effect on the release of the satiety hormone from porcine duodenum ($p > 0.1$).

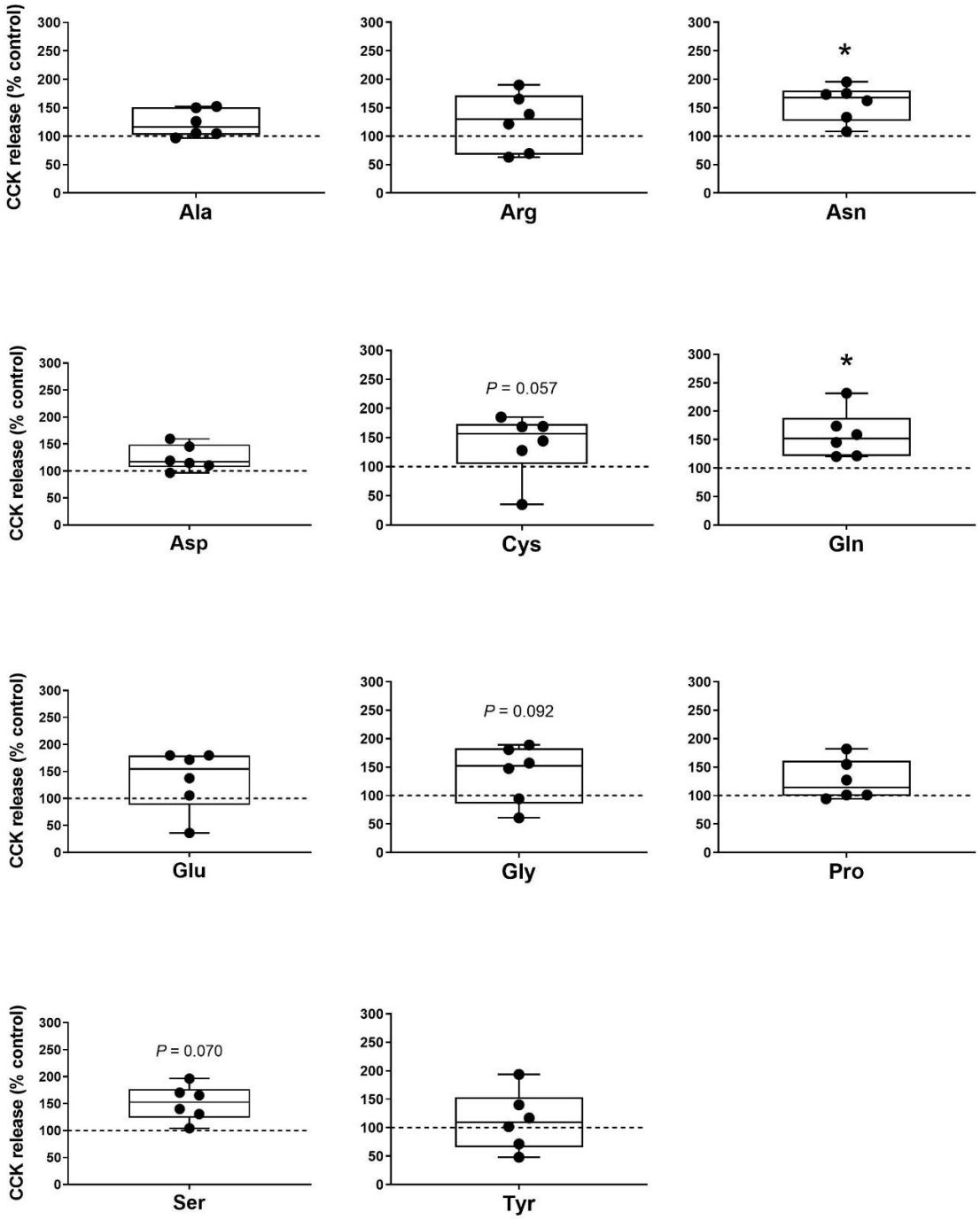

Figure 5.4. CCK secretion from porcine duodenum cultures following a 1-hour incubation with NEAA (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr) at 10 mM. Data are expressed as percentage of control (KRB buffer alone = dotted line). n=6, each data point within boxplots represents the average of three biological replicates within each pig. * = $p<0.05$, ** = $p<0.01$.

Figures 5.5 and 5.6 show the effect of EAA and NEAA on GLP-1 secretion from porcine ileum, respectively. GLP-1 secretion in ileum was significantly triggered by Ile (310.68 ± 36.33 pmol/l), Leu (297.44 ± 34.42 pmol/l), Met (265.35 ± 29.33 pmol/l) and Trp (287.04 ± 37.75 pmol/l) compared to the control (203.54 ± 29.46 pmol/l) ($p \leq 0.05$). In addition, a trend ($p \leq 0.1$) to increase GLP-1 levels was observed for Val (259.26 ± 28.69 pmol/l). On the other hand, His, Lys, Phe and Thr did not significantly ($p > 0.1$) affect GLP-1 secretion (Figure 5.5). Regarding NEAA, Arg (277.99 ± 36.49 pmol/l) and Glu (298.80 ± 31.07 pmol/l) significantly ($p \leq 0.05$) stimulated the release of GLP-1 when compared to control samples (203.54 ± 29.46 pmol/l). Furthermore, Asp (254.80 ± 27.11 pmol/l), Cys (244.91 ± 23.19 pmol/l), Gln (266.19 ± 32.59 pmol/l), Gly (264.10 ± 28.72 pmol/l) and Ser (260.48 ± 26.95 pmol/l) tended ($p \leq 0.1$) to increase GLP-1 released from ileum. In contrast, Asn, Pro, Tyr and Ala had no effect ($p \geq 0.1$) on the release of the peptide (Figure 5.6).

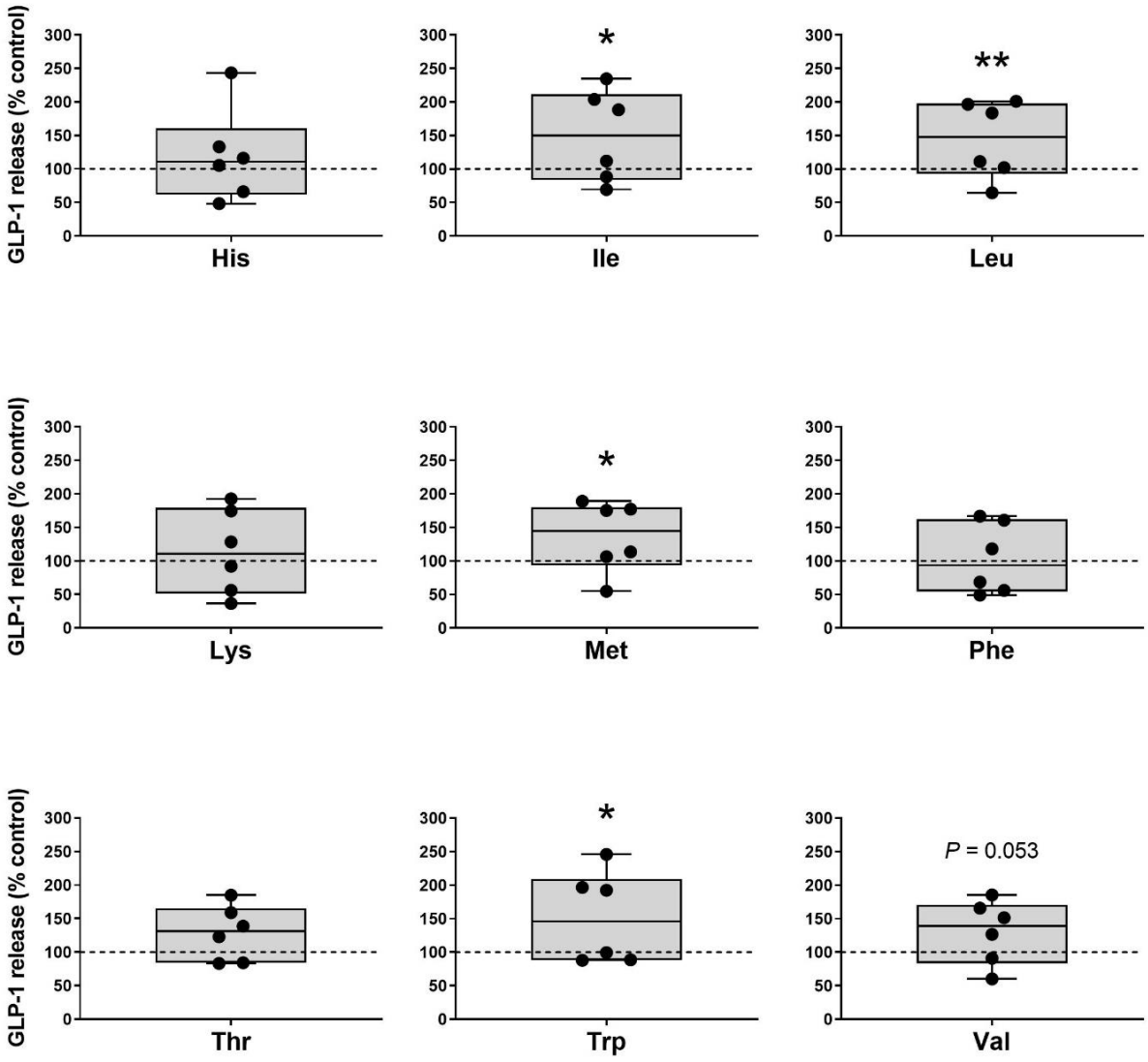

Figure 5.5. GLP-1 secretion from porcine ileum cultures following a 1-hour incubation with essential AA (His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val) at 10 mM. Data are expressed as percentage of control (KRB buffer alone = dotted line). n=6, each data point within boxplots represents the average of three biological replicates within each pig. * = $p<0.05$, ** = $p<0.01$.

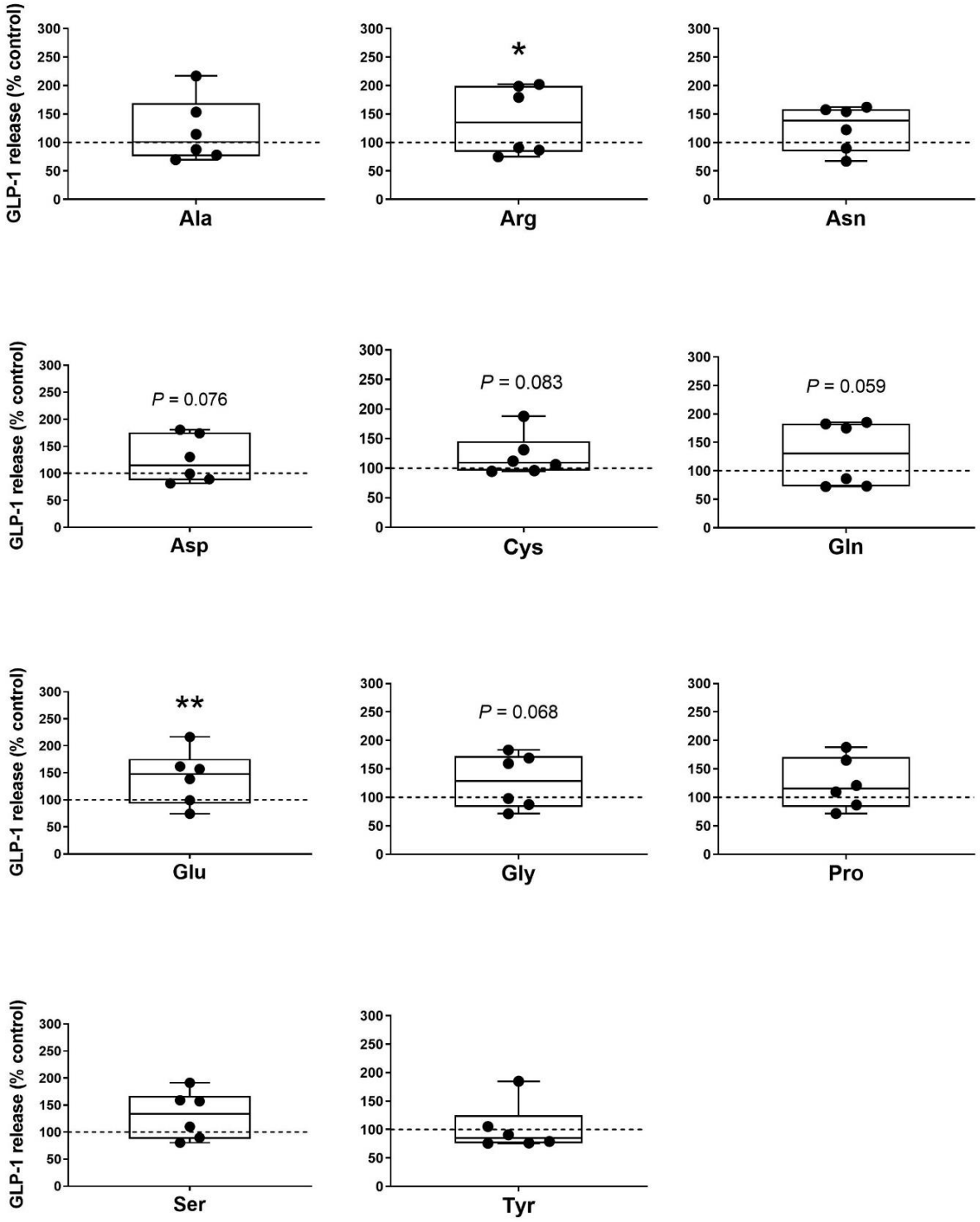

Figure 5.6. GLP-1 secretion from porcine ileum cultures following a 1-hour incubation with non-essential AA (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, Tyr) at 10 mM. Data are expressed as percentage of control (KRB buffer alone = dotted line). n=6, each data point within boxplots represents the average of three biological replicates within each pig. * = $p<0.05$, ** = $p<0.01$.

5.3. Blood kinetic of CCK and GLP-1 following oral gavages of free AA solutions

Circulating levels of plasma CCK before (baseline represented as time 0 in graphs) and 5, 15, 30, 60 and 90 min after administering the oral gavages of water (control) or 3 mmol.kg^{-1} solutions of glucose (positive control) or Leu, Ile, Lys, Glu, or Phe were analysed and are shown in Figure 5.7 (A-F). The baseline plasma CCK concentrations did not differ between treatments. It was observed that CCK concentrations peaked at 15 min post gavage in all the AA tested except Glu when the highest concentrations were observed at the 60 min mark. CCK AUC (0-90 min) for all treatments are shown embedded in Figure 5.7 (A-F). Plasma CCK AUC for Leu (1236.16 ± 150.26) was significantly ($p \leq 0.05$) higher than the control group (735.63 ± 156.81). Lys (1235.42 ± 129.59) tended ($p \leq 0.1$) to increase CCK AUC. No other treatment had a significant impact on CCK AUC. Non-significant differences were observed on CCK measurements at any individual time point of blood collection.

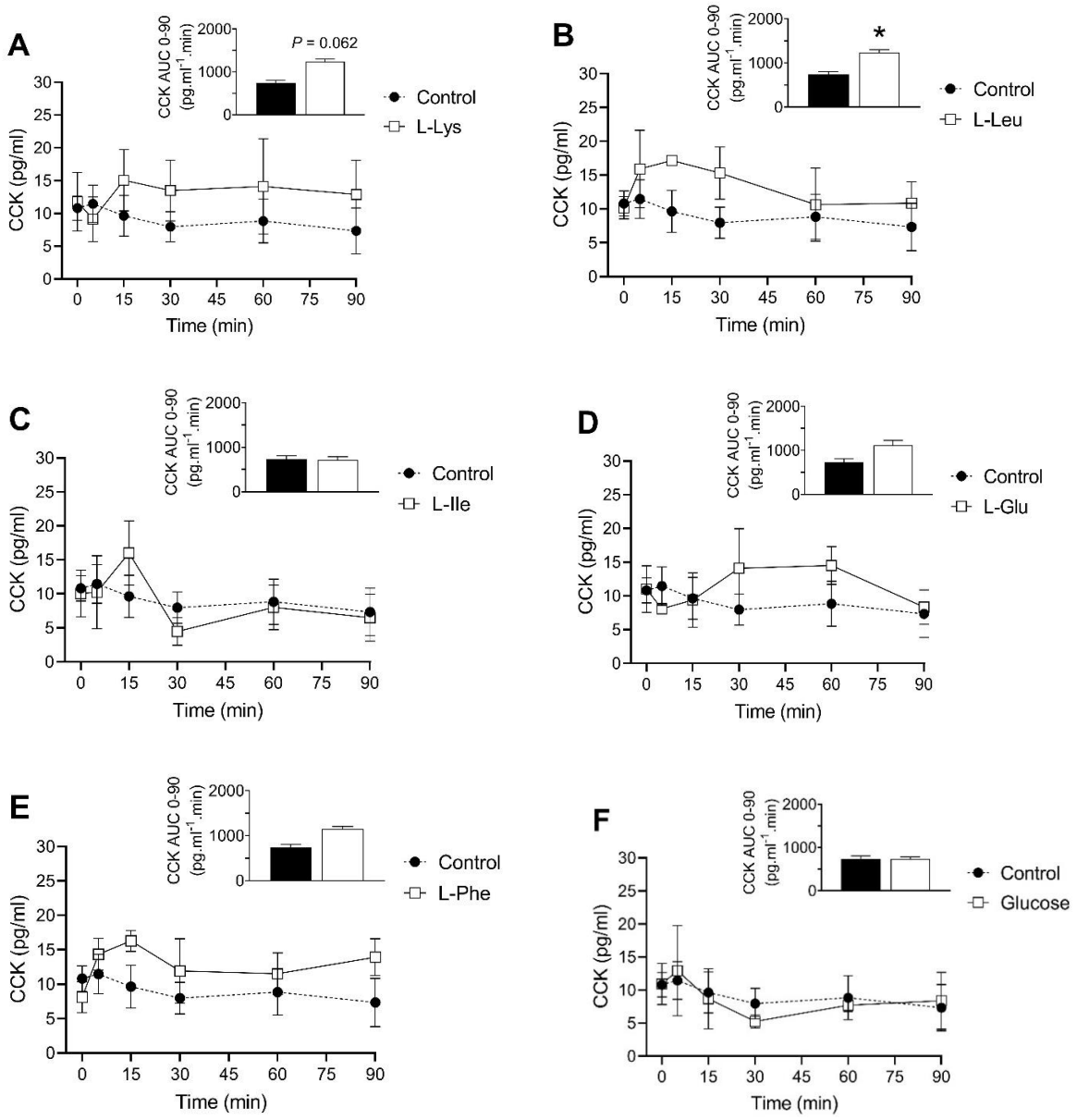

Figure 5.7. CCK plasma levels and AUC (0-90 min) in young pigs after an oral gavage with L-Lys (A), L-Leu (B), L-Ile (C), L-Glu (D), L-Phe (E) or glucose (D) at 3 mmol.kg^{-1} vs. control (water). AUC derived using BLUE of

missing data. Baseline values are represented as time 0. Data are expressed as the mean + SEM (n=4-5). * = $p \leq 0.05$ vs. control.

GLP-1 plasma concentrations before (baseline represented as time 0 in graphs) and 5, 15, 30, 60 and 90 min after administering the oral gavages of water (control) or 3mm.kg^{-1} solutions of glucose (positive control), Leu, Ile, Lys, Glu, or Phe are shown in Figure 5.8 (A-F). Baseline plasma GLP-1 concentrations did not differ between treatments. Glucose tended ($p \leq 0.1$) to rise GLP-1 levels at the 5 min mark (37.13 ± 6.22 pmol/l) when compared to the baseline (24.29 ± 5.12 pmol/l). Phe significantly increased circulating levels of GLP-1 relative to the baseline (18.43 ± 4.01 pmol/l) at 30-, 60- and 90-minutes post-gavage (25.30 ± 4.97 pmol/l, $p<0.05$; 31.25 ± 5.47 pmol/l, $p \leq 0.001$; and 32.75 ± 5.75 pmol/l, $p \leq 0.001$). Most AA triggered a bi-phasic release of GLP-1, with an early increase 5 min post administration followed by a second increase between 30- and 90-min post gavage. Lys was the only AA that triggered a single peak of GLP-1 plasma levels at the 15 min mark.

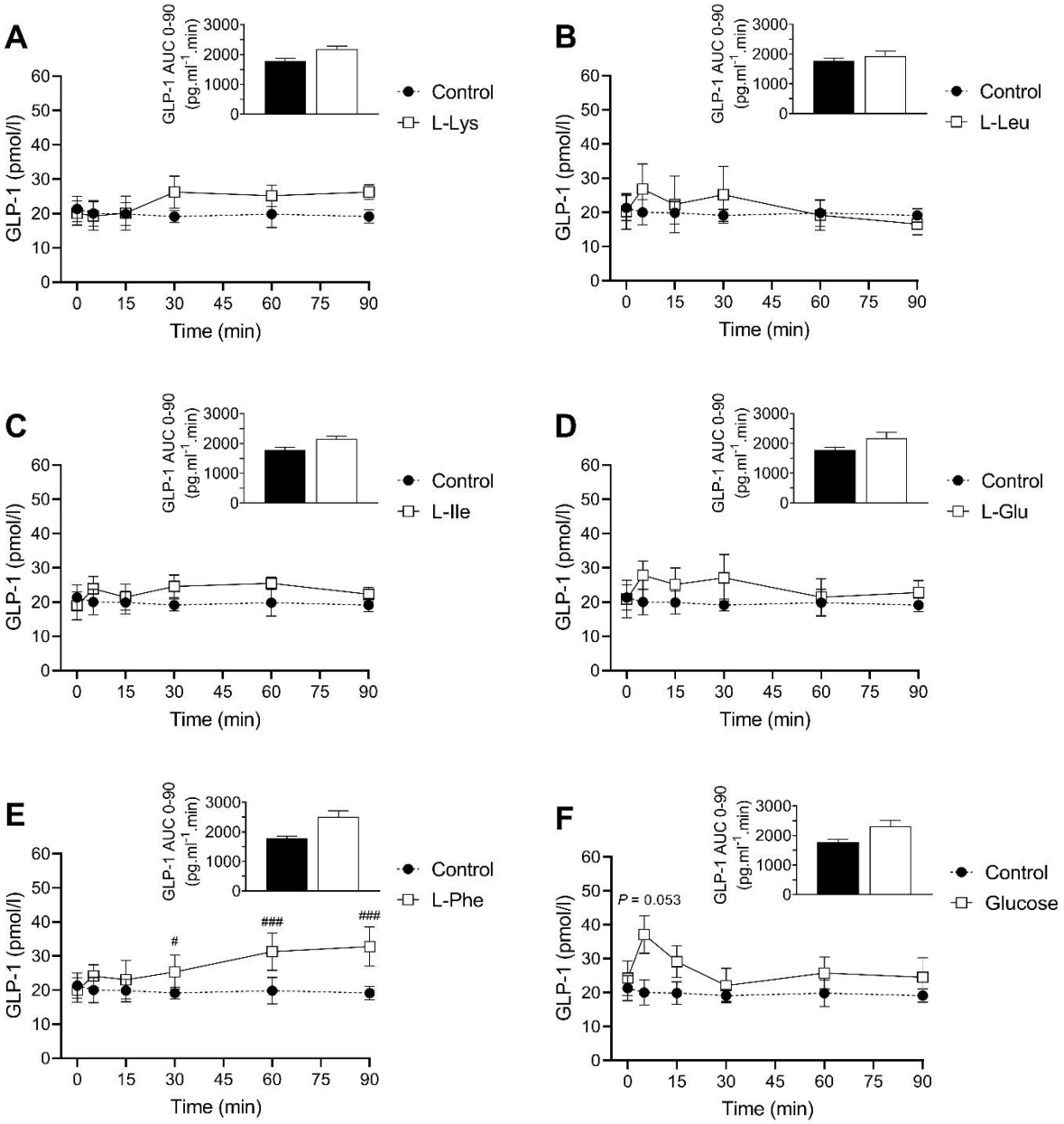

Figure 5.8. GLP-1 plasma levels and AUC (0-90 min) in young pigs after an oral gavage with L-Lys (A), L-Leu (B), L-Ile (C), L-Glu (D), L-Phe (E) or glucose (F) at 3 mmol.kg^{-1} vs. control (water). AUC derived using BLUE of

missing data. Baseline values are represented as time 0. Data are expressed as the mean + SEM (n=4-5). # = $p \leq 0.05$, ## = $p \leq 0.01$, ## = $p \leq 0.001$ vs. baseline. * = $p \leq 0.05$ vs. control.

Plasma levels of CCK and GLP-1 were positively correlated during the 60-90 min post gavage interval (r=0.34, p<0.05) but not during other intervals or the overall (Figure 5.9 A to D). Phe showed a highly significant positive correlation between CCK and GLP-1 (r=0.89, p<0.05) as highlighted in Figure 5.9 D.

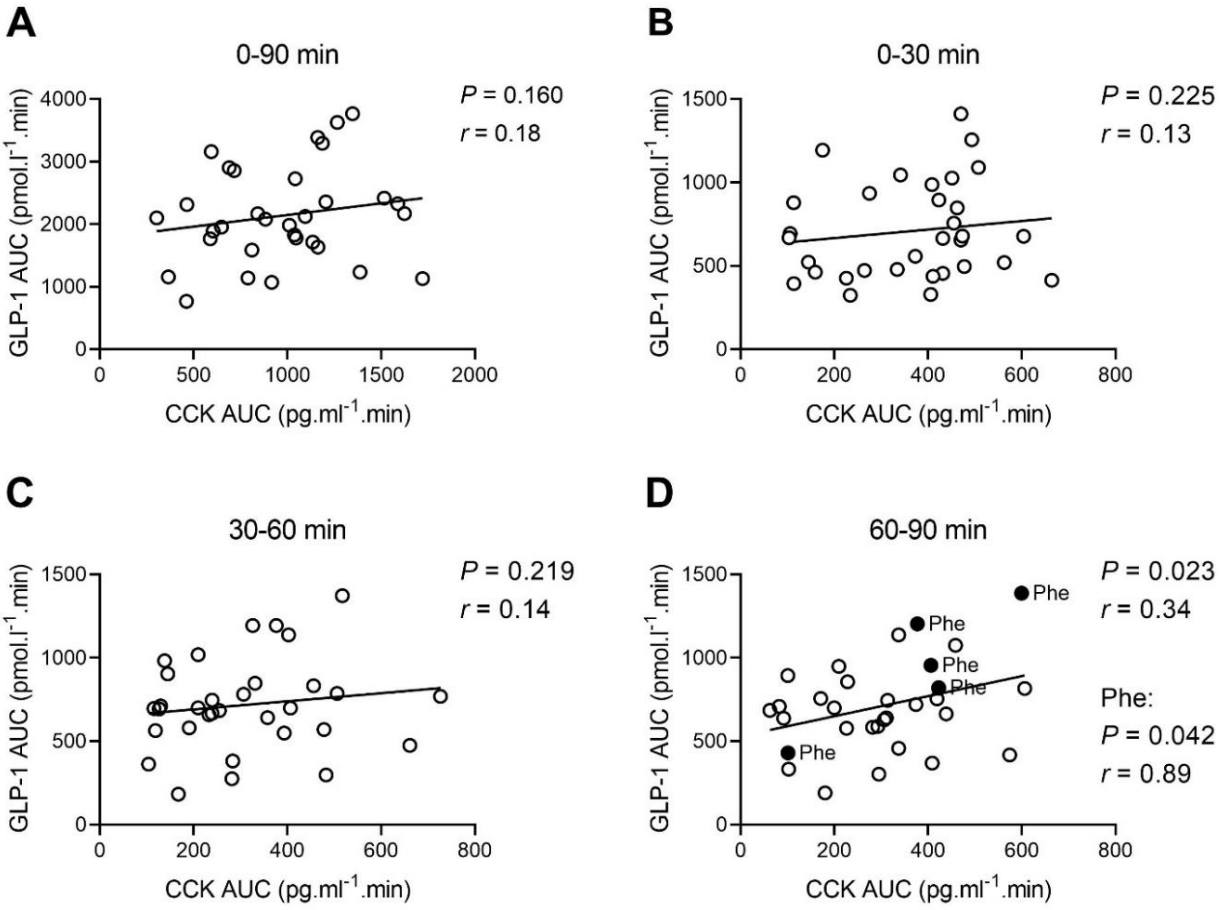

Figure 5.9. Relationship between CCK and GLP-1 plasma levels (expressed as AUC) at 0-90 min (A), 0-30 min (B), 30-60 min (C), 60-90 min (D) following an oral gavage with 3 mmol.kg^{-1} of L-Lys, L-Leu, L-Ile, L-Glu, L-Phe and glucose vs. control (water). Correlations between CCK and GLP-1 data was performed by Pearson correlation analysis. $p \leq 0.05$ was considered statistically significant.

5.4. Feeding behaviour following oral gavages of free AA solutions in piglets

The changes in feed intake and feeding behaviour including meal patterns, following an oral gavage of with 3 mmol.kg-1 of L-Lys, L-Leu, L-Ile, L-Glu, L-Phe and glucose were studied as part of phases 2 and 4. The impact of the oral dose of each AA selected and glucose on feed intake is shown in Figures 5.10 (A-F) and 5.11 (A-F). Lys ($p \leq 0.01$) or Leu ($p < 0.05$) decreased feed intake during the first hour post gavage by 28.6% and 16.5% compared to the control, respectively. Similarly, Lys or Leu significantly ($p \leq 0.05$) reduced the cumulative feed intake up to 3 hours post gavage when compared to the control. In addition, a highly significant ($p < 0.01$) effect associated with Ile was observed on cumulative feed intake between 2 and 2.5 hours after the gavage. In contrast, Phe, Glu and glucose did not significantly alter any of the feeding behaviour parameters tested.

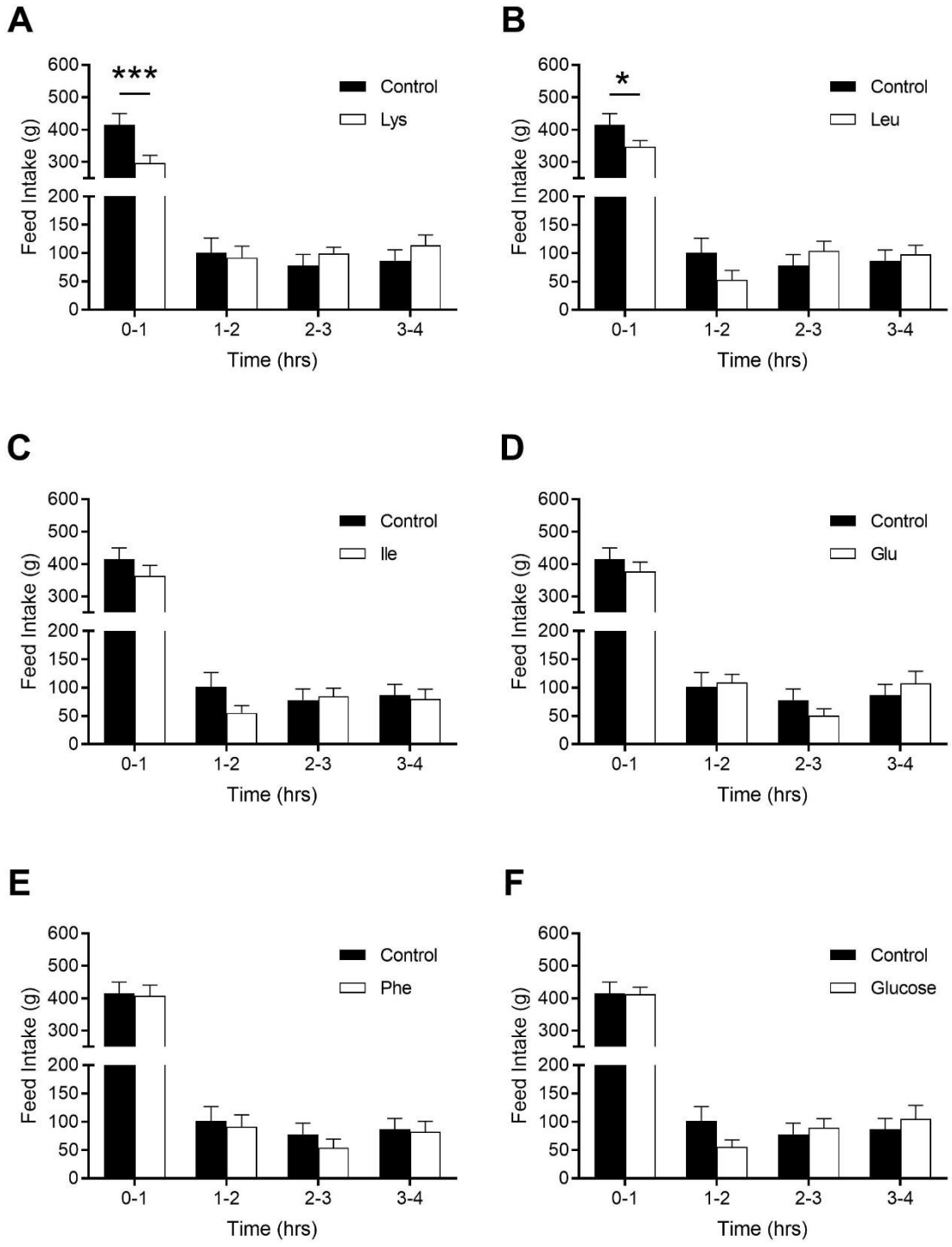

Figure 5.10. Feed intake in young pigs after an oral gavage with water or 3 mmol.kg^{-1} of L-Lys, L-Leu, L-Ile, L-Glu, L-Phe or glucose. Data are expressed as the mean + SEM (n=12). ** = $p \leq 0.01$, * = $p \leq 0.05$ vs. control.

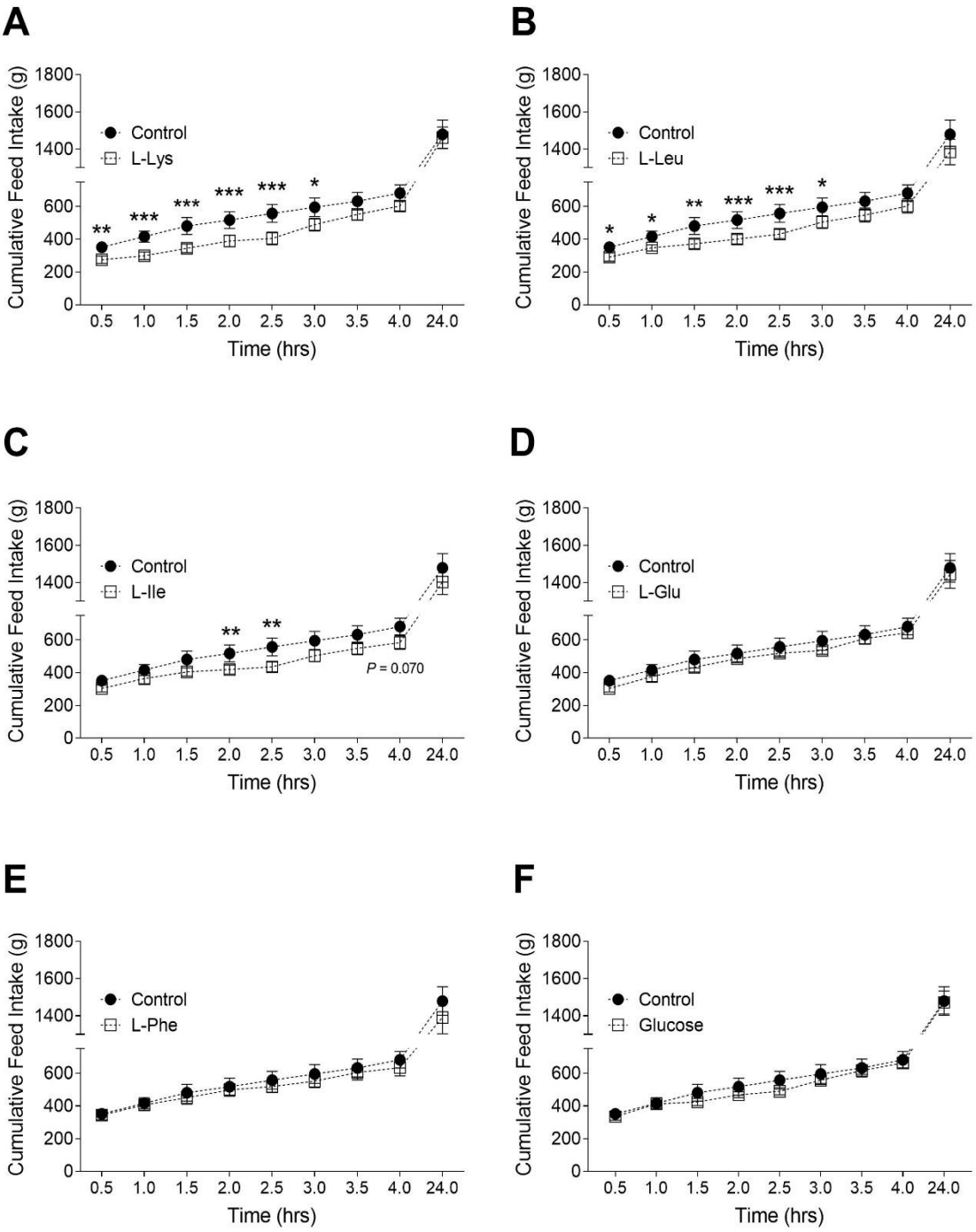

Figure 5.11. Cumulative feed intake in young pigs after an oral gavage with water or 3 mmol.kg^{-1} of L-Lys, L-Leu, L-Ile, L-Glu, L-Phe or glucose. Data are expressed as the mean + SEM (n=12). *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$ vs. control.

The study of the effect of the oral gavages of the selected AA and glucose on feeding behaviour parameters included latency to first meal (LFM), first meal duration (FMD), second meal duration (SMD), inter-meal interval (IMI) and number of meals (NM) and are illustrated in Figure 5.12 (A-F). The FMD (shown as time spent on feeder) was significantly ($p \leq 0.05$) reduced in Lys treated pigs when compared with the control group. In addition, Lys pigs tended ($p \leq 0.1$) to increase the IMI (by 1.9-fold). Furthermore, Leu showed a significant impact increasing IMI by 2.5-fold ($p \leq 0.05$) and decreasing

(p≤0.05) the NM in the first 2 hours post-gavage. None of the other treatments tested (Ile, Glu, Phe and glucose) showed significant impacts on meal pattern.

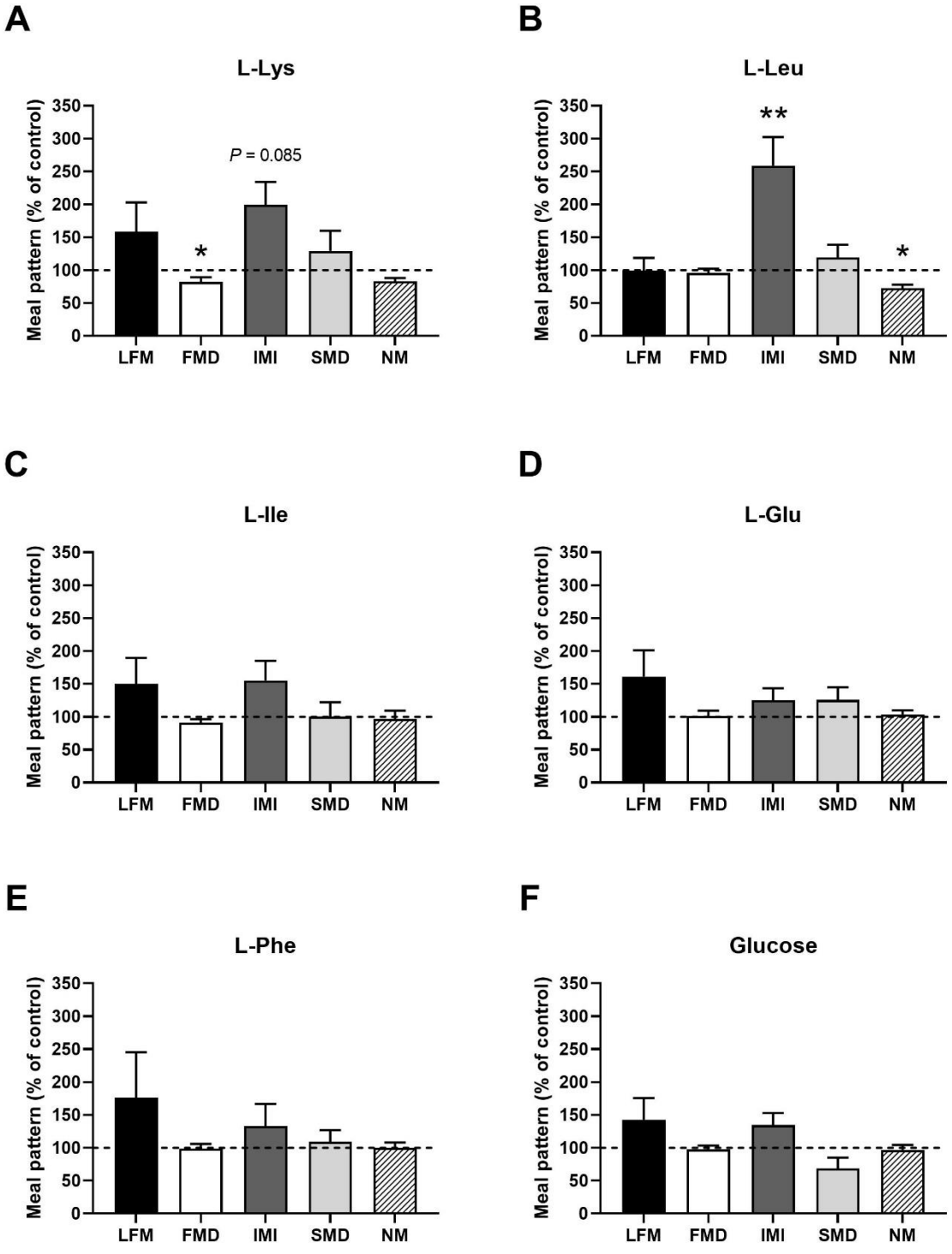

Figure 5.12. Meal pattern analysis. Latency to first meal (LFM), first meal duration (FMD), inter-meal interval (IMI), second meal duration (SMD) and number of meals (NM) in young pigs after an oral gavage with L-Lys, L-Leu, L-Ile, L-Glu, L-Phe, glucose at 3 mmol.kg^{-1} vs. control (water). Data are expressed as percentage of control (dashed line) (n=12). **= p ≤ 0.01, * = p ≤ 0.05.

5.5. Performance of post weaning pigs exposed to excess dietary Lys

In phase 4, a dose-response study was performed to assess dietary Lys levels at NRC recommended levels (T0) or 25% (T1), 50% (T2) or 100% (T3) excess. The results (Table 5.1) showed that ADFI was linearly reduced ($p<0.01$) with the increase of dietary Lys during the third- and fourth-week post weaning. The decrease in ADFI accounted for approximately 6.3 and 7.2 g/p for each 1% of added Lys above the level of the control diet for weeks 3 and 4, respectively. When looking at the overall (0 to 28 days post weaning) the Lys effect ($p<0.05$) was a reduction in feed intake by approximately 4.1 g/p for each 1% of added dietary Lys above the level of the control diet. A trend ($p<0.1$) and a significant ($p<0.05$) negative linear response was observed for ADG with increased dietary Lys content during weeks 2 and 3. Similarly, a tendency ($p<0.1$) to reduce the ADG of animals exposed to dietary Lys excesses was observed for the overall period of 4 weeks. No significant effects of the Lys doses were observed on G:F ratio.

Table 5.1 Performance parameter of post weaning pigs fed one of four dietary treatments covering 100% (T0), 120% (T1), 150% (T2) or 200% (T3) of lysine requirements.

	Dietary Treatment					
Amino acid	T0	T1	T2	T3	SEM	*p* value[1]
ADFI (kg/day)						
Week 1	2.03	1.88	1.85	1.90	0.0022	0.7002
Week 2	5.09	5.08	5.23	4.83	0.0022	0.3287
Week 3	8.35	8.32	7.90	7.74	0.0022	0.0049
Week 4	10.77	10.64	10.34	10.03	0.0022	0.0012
Overall	6.56	6.48	6.33	6.12	0.0017	0.0158
ADG (kg/day)						
Week 1	0.94	0.78	0.91	0.88	0.0027	0.9386
Week 2	4.46	4.49	4.21	3.94	0.0028	0.0614
Week 3	6.13	5.98	5.94	5.41	0.0028	0.0155
Week 4	7.69	7.64	7.71	7.55	0.0028	0.7378
Overall	4.81	4.72	4.69	4.45	0.0017	0.0717
G:F ratio						
Week 1	0.44	0.41	0.49	0.46	0.0005	0.4548
Week 2	0.88	0.88	0.81	0.80	0.0005	0.0703
Week 3	0.74	0.72	0.75	0.70	0.0005	0.5503
Week 4	0.71	0.72	0.75	0.75	0.0005	0.3903
Overall	0.69	0.68	0.70	0.68	0.0002	0.6867

[1] *Linear contrast on the effect of Lys dietary content on ADFI, ADG and G:F ratio. n=10 (number of pens).*

5.6. Gut hormone secretion following intestinal stimulation with different doses of Lys

In addition, in the phase 4 experiment, CCK and GLP-1 secretion following Lys exposure were measured and the results are presented in Figure 5.13. CCK secretion was enhanced in proximal jejunum tissues incubated with 20 mM ($p<0.05$) of Lys compared to the control (KRB buffer free of Lys). In contrast, GLP-1 levels did not significantly ($p>0.05$) change following the exposure to 10-, 20- or 30-mM Lys solutions using ileum as the ex-vivo model.

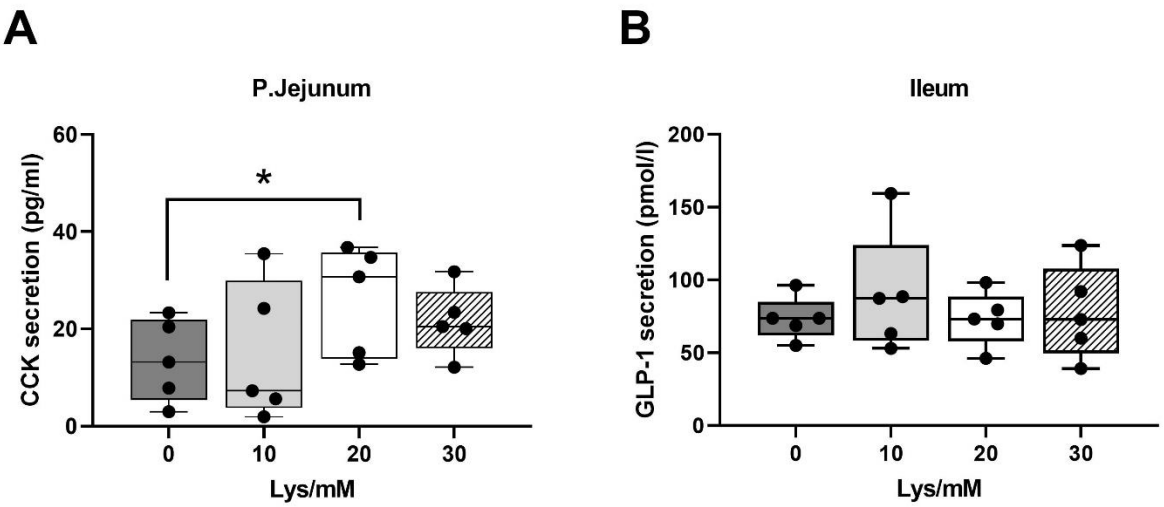

Figure 5.13. CCK and GLP-1 secretion in proximal jejunum and ileum, respectively, of young pigs following 1-hour incubation with Lys at 0, 10, 20 and 30 mM. Data are presented as the mean + SEM of three biological replicates per pig. n=5, refers to the number of pigs used. *= $p \leq 0.05$, tendency = $0.05 \leq p \leq 0.1$.

6. Discussion

In pig production, diets are frequently formulated to exceed the minimum requirements in limiting essential amino acids (EAA) to compensate for variations in feed production and individual growth rates (Presto Åkerfeldt et al., 2019). In addition, non-essential AA (NEAA) are not regarded in terms of feed formulation indicating that their dietary content is irrelevant to most pig nutritionists (Wang and Fuller, 1989). However, these nutritional approaches may not be ideal considering that excess of dietary EAA and potentially NEAA have previously been shown to depress feed intake and growth in pigs (Edmonds and Baker, 1987; Edmonds et al., 1987). One of the main mechanisms through which AA may modulate feed intake is through the stimulation of gut hormone secretion from enteroendocrine cells (EEC) (Reiman et al., 2004; Wang et al., 2011, Alamshah et al., 2016). Some of these gut peptides, such as cholecystokinin (CCK) and glucagon like-peptide 1 (GLP-1), can interact with the central nervous system (CNS) through paracrine (vagal afferents stimulation) or endocrine actions (blood) to mediate the onset of satiation and satiety in pigs (Steinert et al., 2013). BCAA, aromatic AA and AA with basic side chain properties have been associated with CCK release in pigs (Wang et al., 2018; Zhao et al., 2018; Feng et al., 2019; Tian et al., 2019). However, still much remains unknown on this topic. A better understanding of the role of individual AA on appetite modulation could help improve feed formulation and, in consequence, improve performance at critical stages of pig production such as in the post-weaning period.

The aim of this research project was to study the anorexigenic effect of dietary free/synthetic AA and the underlying physiological mechanisms in pigs. Thus, 3 main objectives were pursued: (1) to evaluate the effect of individual AA on the release of CCK and GLP-1; (2) to assess the impact of anorexigenic AA (based on CCK and/or GLP-1 secretion) on feeding behaviour; and (3) to quantify the impact of dietary excesses of anorexigenic AA on feed intake and performance in post-weaning pigs.

The regulation of appetite occurs mainly through neural and hormonal signals triggered by the presence of nutrients in the gastrointestinal tract (GIT). CCK and GLP-1 are the two most studied anorexigenic hormones secreted by enteroendocrine cells (EEC) (Steinert et al., 2013; 2017). While CCK is primarily released by enteroendocrine I-cells located in the proximal small intestine, GLP-1 is mainly secreted by L-cells present in the ileum and colon (Bacarese-Hamilton, 1984; Liddle, 1997; Holst, 2007; Voortman et al., 2012). Our results confirm previous reports showing that CCK and GLP-1 (including the precursor pro-glucagon GCG), were primarily expressed in duodenum and ileum of post-weaning pigs, respectively. The lineal distribution along the small intestine (CCK more proximal while GLP-1 more distal) indicates that the two hormones could play complementary roles in appetite modulation. In our study and others, we observed that some dietary free AA are strong inducers of CCK and GLP-1 secretion from EEC (Wang et al., 2011; Mace et al., 2012; Daly et al., 2013; Oya et al., 2013). Considering the relevance of CCK and GLP-1 on appetite modulation, they were included in our study as key biomarkers of the anorexigenic effect of AA in pigs.

Recent studies in young pigs showed a significant effect of Phe, Trp, Arg, Leu and Ile on CCK secretion (Wang et al., 2018; Zhao et al., 2018; Feng et al., 2019; Tian et al., 2019). Consistent with these studies, our data supports the role of Leu and Phe on CCK secretion, but also uncovered potential roles for Asn, Gln and Lys in stimulating anorexigenic peptide secretion in pigs using primary porcine intestinal culture (ex-vivo) model. Much less seems to be known regarding the impact of AA on GLP-1 release in pigs. The results reported demonstrate that Arg, Met, Trp, Leu, Ile and Glu are potent secretagogues of GLP-1 in porcine ileum at physiological relevant doses. GLP-1 has been described as both an incretin and an anorexigenic hormone (Flint et al., 1998; Gromada et al., 1998; Holst, 2007). Thus, our results suggest that Arg, Met, Trp, Leu, Ile and Glu play an important role in glucose homeostasis and feed

intake in pigs. Collectively, these data indicate that gut hormone secretion is partly dependent on the nature/characteristics of the AA content in diets. Our results regarding anorexigenic AA are consistent with previous literature in mice and human indicating that BCAA and aromatic AA are strong gut peptide secretagogues (Chen and Reimer, 2009; Wang et al., 2011; Mace et al., 2012). It seems reasonable to speculate that similar mechanisms/receptors involved in AA-induced CCK and GLP-1 secretion may have evolved and are currently common to mammalian species.

Leu stimulated the release of both CCK and GLP-1 suggesting that the AA could have long lasting effect on feed intake related to the diphasic release of satiety hormones (first CCK in duodenum followed by GLP-1 in ileum). On another aspect, our observations indicate that ECC are sensitive to the intestinal concentrations of Glu, Gln and Asn, which are major metabolic fuels for enterocytes (Posho et al., 1994; Wu et al., 1995; Burrin and Stoll, 2009; Wang et al., 2015). We speculate that CCK and GLP-1 secretion may contribute to gut health and function in pigs by stimulating pancreatic secretion and inhibiting gastric emptying and intestinal motility, thus, promoting protein digestion and facilitating the detection and absorption of Asn, Glu and Gln by enterocytes (Liddle, 1995; Schirra and Goke, 2005). In addition, our data corroborates the concept that NEAA do not only participate in protein synthesis but also in the regulation of key metabolic processes, such as hormone secretion and appetite control (Wu et al., 2009). Thus, the concentrations of NEAA in pigs' diet should not be disregarded.

CCK and GLP-1 enter the circulatory system to reach the hypothalamus and brainstem the main organ in the control of feed intake (Cummings and Overduin, 2007; Chaudhri et al., 2008). Thus, we evaluated the effect of an oral gavage of AA on blood kinetics of CCK and GLP-1 as well as their impact on feeding behaviour (feed intake and meal pattern) in pigs. Our results showed that Lys and the two BCAA Leu and Ile significantly decreased feed intake. Lys and BCAA are of particular interest to nutritionists due to their relevance in pig production. On the one hand, Lys is the most limiting dietary EAA resulting in that all pig cereal-based diets will require synthetic Lys supplementation to meet minimum requirements for optimal growth and development (Hou et al., 2015; Liao et al., 2015). On the other hand, BCAA have pivotal roles in muscle development and gut function in pigs (Escobar et al., 2005; 2010; Suryawan et al., 2008; Zhang et al., 2013; Sun et al., 2015). In addition, BCAA may also be limiting in the diet requiring synthetic supplementation in some formulations particularly in low crude protein feeds (Figueroa et al., 2013; Cemin et al., 2019).

Leu and Lys resulted in significant reductions in feed intake together with alterations in feeding behaviours including meal pattern (i.e., lower meal duration, increased inter-meal interval and decreased number of meals) reflecting an effect on both satiation (events leading to the termination of an ongoing eating episode) and satiety (feeling of fullness between eating episodes). These results are consistent with previous studies showing that dietary excess of Lys reduced feed intake and growth in pigs (Edmonds and Baker, 1985; Edmonds et al., 1987). Similarly, Leu dietary excesses have been linked with poorer growth performance (Wiltafsky et al., 2010; Millet et al., 2015; Kwon et al., 2019). In addition, Leu and Lys oral gavages were associated with an increase in CCK plasma levels short after the intervention, indicating that this peptide may mediate the anorexigenic effect of the AA. Previous studies have shown associations between dietary Lys and Leu and CCK in post-weaning pigs (Yin et al., 2017; Tian et al., 2019). One of the potential commercial implications of these findings is that the use of non-protein bound/synthetic Lys and/or BCAA have the undesirable potential to increase CCK and satiation secretion early in the post-prandial phase of feed intake in pigs. Finally, on this, it might be relevant to note that Lys and Leu showed an almost identical influence on CCK, feed intake and meal patterns that points at a common anorexigenic mechanism.

An important novel aspect of our findings involves a potential long-term effect of CCK in pigs. CCK was originally described as a satiation signal due to the relative short life span of its circulating forms that limited the peptide to altering meal size and duration only (Kissileff et al., 1981; Lieverse et al., 1995; Bowen et al., 2006). Evidence of a CCK short-term effect on feed intake has also been presented in pigs following the intravenous administration of the peptide (Anika et al., 1981; Houpt, 1983). However, to the best of our knowledge, no long-term effects of CCK have been describe in pigs to date. Recent studies in rodents suggested an effect on satiety as well, as circulating forms of the hormone with longer half-life (e.g., CCK-58) seemed to extend the interval between meals (Overduin et al., 2014; Sayegh et al., 2014). Our results are consistent with the rodent data in that CCK showed a long-term effect by reducing feed intake up to three hours associated with increased CCK plasma levels in Lys and Leu treated pigs. Thus, our data would suggest that in pigs, such as in mice, there might be circulating forms of CCK with longer lifespan (i.e., CCK-33 and/or CCK-58 like hormones). A gap in our study is that other appetite related peptides such as ghrelin, PYY, insulin or leptin, were not studied in the context of this project. These hormones have the potential to contribute to changes in meal pattern and to alter feeding behaviour in pigs (Barb et al., 1998; Salfen et al., 2004; Newman et al., 2013). Thus, their role on Lys and Leu anorexigenic effect cannot be discarded and would require further investigations.

Interestingly, Glu, Leu and Ile, which were identified as strong secretagogues of GLP-1 in porcine ileum, did not significantly stimulated the release of the peptide in live pigs following an acute oral gavage dose. Primary intestinal cultures have been used to evaluate the effect of nutrients on gut hormone secretion due to their high throughput capabilities (Voortman et al., 2012; Ripken and Hendriks, 2015). Although these models more closely replicate in-vivo conditions compare to in-vitro techniques, they still lack many of the neural and humoral signals and interactions with other organs (i.e., hypothalamus, liver…) that occur in live animals (Goldspink et al., 2018; Cummings and Overduin, 2007). Hence, factors such as the high absorption rate and catabolism of Glu and BCAA in the small intestine and/or the activation of intestinal feedback mechanisms to slow the transit of nutrients, could have contributed to the reduced activation of L-cell in the distal GIT and the lower GLP-1 response obtained under in-vivo conditions (Janeczko et al., 2007; Chen et al., 2009; Wu et al., 2015).

Phe raised GLP-1 plasma levels following the oral gavage in our pig study. GLP-1 inhibits hunger by delaying gastric emptying and intestinal motility as well as by directly interacting with appetite related structures in the brain in rodents and pigs (Turton et al., 1996; Schirra et al., 2002; Shah and Vella, 2014; Ribel, 2022). In addition, GLP-1 is considered an incretin hormone, stimulating insulin, a long-term satiety hormone secreted from β-cells in the pancreas (Obici et al., 2002; Plum et al., 2005; Woods et al., 2006). A significant reduction in feed intake and body weight were observed following the long-term administration of GLP-1 analogues in pigs (Raun et al., 2007; Renner et al., 2018). Therefore, it was expected that AA-driven increases in GLP-1 secretion would stimulate satiation and satiety in pigs. However, our results were unable to associate a rise in GLP-1 plasma levels with changes in feeding behaviour, indicating that GLP-1 may play primarily a role on glycaemic control and nutrient digestion, rather than appetite modulation in pigs. Our results indicate a different impact of endogenous GLP-1 may defer from GLP-1 analogues at least regarding the impact on short- and long-term regulation of feed intake in pigs.

When considering the behavioural and hormonal data together, Lys was identified as the strongest and most consistent anorexigenic AA in pigs. Thus, the effect of dietary excess of Lys on feed intake and growth was studied in post weaning pigs. Data from the Westbrook experiments showed a negative linear dose-response effect of Lys on ADFI and ADG. Our results are consistent with Edmonds and Baker study (1985) in which a linear depression of growth and feed intake was observed

with increased dietary content of Lys. Moreover, the negative impact of dietary Lys excesses on ADG may be related to the increased catabolism of Lys (nitrogen clearance from the system) which could have reduced the energy available to maximise growth (Chen et al., 1999). Considering the results from previous phases, Lys anorexigenic is associated with the secretion of CCK. However, a direct interaction of Lys with the CNS cannot be discarded either, as blood levels of AA may act as satiety related signals by relaying information to the brain on metabolic status (Mellinkoff et al., 1956; Harper et al., 1970). Lys has been shown to modulate the activity of appetite related structures in the CNS via interaction with tynacytes in rodents (Lazutkaite et al., 2017). On a practical aspect of the research presented, it is important to conclude that excess dietary AA have the potential to reduce feed intake (and growth) by approximately 4.1 g/p for each 1% of excess dietary Lys above the level of the control diet in post-weaning pigs (a 4 -week post-weaning period).

Collectively, the results from this project strongly support the role of dietary non-protein bound AA, particularly Lys and BCAA, modulating feed intake and growth in pigs. The research highlighted that gut hormones, mainly CCK, are key mediators in the appetite regulation of key dietary free AA in pigs. In addition, changes in plasma levels of EAA and NEAA following dietary AA intake may contribute to the control of hunger. It appears that he secretion of CCK and a decreased appetite triggered by dietary excesses of Lys (and Leu) is a mechanism to avoid severe AA imbalances. The take home message of the findings from an applied pig feed formulation principle is that extreme care should be applied to avoid potential dietary excess of key amino acids particularly Lys and BCAA.

7. Implications & Recommendations

Multiple dietary EAA and NEAA showed a significant impact on gut peptide secretion suggesting that in-feed excesses can be detrimental for the appetite of pigs. Current practice in feed formulation tend to deliver safe margins for the most limiting AA. This, in turn, may result in dietary excess of EAA, particularly Lys, Met, Trp, Thr and/or the BCAA.

Our studies resulted in that several EAA and NEAA triggered CCK (mainly Lys, Leu, Phe, Gln and Asn) or GLP-1 (mainly Met, Trp, Leu, Ile, Arg and Glu) secretion from duodenum and ileum, respectively. Importantly, Lys and Leu when administered in vivo, caused changes in the meal pattern, and lowered overall feed intake. The changes in meal pattern were indicators of early satiation (a consequence of activating the gut-brain axis) and early satiety (a response orchestrated by the CNS). One of the concluding remarks of this research is that dietary safety margins should be reviewed and narrowed, when possible, as to minimize any potential negative effects of excess Lys or other EAA, such as BCAA, on feed intake and growth in pigs.

A practical interpretation of the research presented, relates to quantifying the reduction on feed intake (and growth) by excess dietary AA. Excess dietary Lys decreased feed intake by 4.1 g/p for every 1% of excess in the feed above the recommended requirement levels (e.g., NRC) in post-weaning pigs (a 4 -week post-weaning period). For example, a 25% safety/excess margin in dietary Lys on average will result in 100 g smaller pigs at the end of the post-weaning period.

The present research shed new light on the impact of free dietary AA on appetite regulation in pigs. However, the work left new questions relevant to feed formulation practices worth considering in future studies. Some of the novel aspects uncovered by out project that would need to be addressed are:

- The project identified several AA with strong anorexigenic effects (mainly Lys and BCAA but also, Trp, Phe, Met, Arg, Gln, and Asn). However, due to the study limitations, only one performance trial was run as a final proof-of-concept selecting Lys. The quantification of the effect of other free AA in pig feeds would be a practical application worth developing.

- Our study focused in CCK and GLP-1 release to select the most anorexigenic AA candidates for in-vivo studies. However, other appetite modulatory hormones such as ghrelin, gastric leptin and PYY were not studied. Investigating these hormones will provide additional information regarding orexigenic or anorexigenic properties of dietary free AA, which, could be of great benefit for the pork industry.

- During the development of the study, it was noted that not only EAA had an impact on anorexic hormones. Some NEAA such as Arg, Gln, and Asn showed significant impacts on gut peptide release under *ex-vivo* conditions. Thus, the role of dietary NEAA on appetite and growth needs to be further investigated.

- Lys and Leu had a significant impact on cumulative feed intake up to 3 hours post-treatment in young pigs. The two AA were tested separately. Potential additive or synergistic effects between different EAA (particularly if/when added to a diet non-protein bound) should be further investigated.

8. Intellectual Property

Does not apply.

9. Technical Summary

A summary of information developed as a part of the research (eg. discoveries in methodology, equipment design etc) is listed below:

- Adaptation of the methods (ex-vivo) and acquisition of the know-how to accurately measure CCK and GLP-1 in pigs.

- An initial satiating profile for the 20 proteinogenic AA has been described based on their affinity to stimulate CCK and/or GLP-1 release in the small intestine of pigs.

- The project has helped develop an initial chart of the hormonal capacities of the porcine small intestine relevant to CCK and GLP-1 expression and secretions.

- In vivo gavage procedures were developed to analyse the effect of acute doses of nutrients on the blood kinetic of gut hormones and potentially other blood metabolites of interest as well relevant to nutrition interventions in pigs.

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11. Publications Arising

The following publications have been submitted:

Peer-reviewed conference paper:

Muller M, Tilbrook A, van Barneveld RJ, Roura E: Satiating responses to branched chain amino acids are mediated through ileal GLP-1 secretion in post-weaning pigs. Adv Anim Biosci. 2019; 10: s02.

Journal papers:

Additional manuscripts are currently in preparation. Approval from APL will be requested once the documents are closer to completion.

s02

Satiating responses to branched chain amino acids are mediated through ileal GLP-1 secretion in post-weaning pigs

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Application Avoiding excess dietary levels of branched chain amino acids (BCAA) may help decrease the secretion of satiety hormones and, therefore, increase feed intake in post-weaning pigs.

Introduction Previous studies have shown a negative effect of BCAA on feed intake and growth performance in young pigs when provided in high concentrations in starter diets (Wessels *et al.*, 2016). However, the mechanisms behind their satiating effect remain unclear. Similar to what has been described in other species, we hypothesized that the satiating effect of Valine (Val), Leucine (Leu) and Isoleucine (Ile) in post-weaning pigs is related to the secretion of the anorexigenic gut peptides Cholecystokinin (CCK) and Glucagon-like peptide 1 (GLP-1).

Material and methods Six 25 day-old male pigs (Landrace x Large White) with an average weight of 6.94 ±0.29 kg were housed in pairs in an environmentally controlled room. Animals had *ad-libitum* access to a high-protein (24%) diet in order to supply an adequate level of essential amino acids (AA) without adding synthetic AA. Pigs underwent an adaptation period of 3 days before being euthanized for collection of duodenum and ileum samples. Intestinal samples were stripped from their muscle layer and incubated in a 24-well plate for 1 h at 37°C in a humidified CO^2 incubator, with either glucose-free Krebs Ringer Bicarbonate (KRB) buffer containing no added AA (control) or with the addition of 10 mmol of Leu, Ile, or Val. Following incubation, the supernatant within each well was retrieved and used for hormone analysis. A mixed analysis of variance (ANOVA) model considering AA as a fixed effect, pigs as a random effect and interactions, was used to compare the effect of each AA individually with control on CCK and GLP-1 secretion.

Results The effect of BCAA on CCK release from duodenum (A) and GLP-1 release from ileum (B) of post weaning pigs are shown below (Figure 1). GLP-1 secretion from ileum samples was significantly ($P < 0.05$) increased after Ile and Leu exposure compared to the control (310.68 ±36.33, 297.44 ±34.42, and 203.54 ±29.46 pmol/L, respectively). However, only a tendency ($P > 0.1$), was observed for Val (259.26 ±28.69 pmol/L). Non-significant ($P > 0.05$) CCK values were observed for Ile and Val compared to control (30.36 ±4.77, 24.17 ±3.62, and 20.11 ±2.42 pg/mL, respectively). Nonetheless, Leu resulted in a tendency (31.68 ±3.57 pg/mL, P<0.1) with a 57% higher CCK release than control.

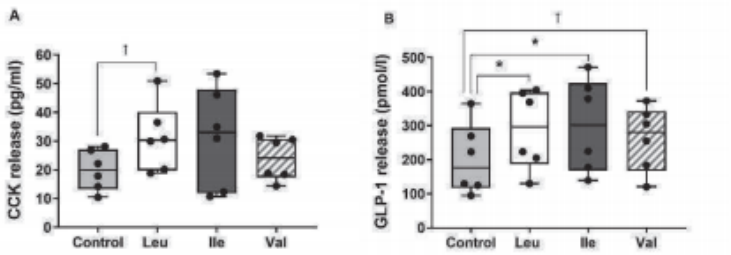

Figure 1. Duodenum CCK (A) and ileum GLP-1 (B) release following a 1-hour incubation with control (KRB buffer with no added AA), Ile, Leu or Val at 10 mmol. n=6, each data point represents the average of three biological replicates within each pig. * = $P < 0.05$, † = $P < 0.1$.

Conclusion The satiating effect of high dietary Ile and Leu might be mainly related to increase GLP-1 secretion from the ileum of young pigs. Excess Val did not significantly influence gut peptides from duodenum or ileum.

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References

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