# OPTIMISATION AND APPLICATION OF A METHOD DESIGNED TO ASSESS HOST DNA IN PIG AND POULTRY FAECES AS A POTENTIAL MARKER OF GUT CELL LOSS

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# ABSTRACT

Maintaining and replenishing the intestinal barrier is an energy demanding process. In farmed species the excessive energy utilised by this process could be better utilised in the production of proteins for lean muscle deposition, milk synthesis or egg production. Altering the diets of farmed animals can improve how efficiently feed energy is converted into useful product. Therefore, it would be useful to examine how any nutritional changes impact upon gut function, particularly gut cell turnover which utilises such a significant proportion of dietary energy. Currently all methods to assess gut turnover in farmed species are invasive to some extent and typically very expensive, therefore, there is scope to develop a novel non-invasive method that could potentially be used routinely in a farm setting. Sloughed epithelial cells are broken down in the lumen of the intestine by endonucleases, presumably leading to fragmented host DNA being present in the faeces. This thesis developed methodologies for analysing faecal host DNA from pig and chicken faeces and the developed protocols were then applied to feeding studies where differences in gut cell sloughing were expected.

Different faecal DNA extraction protocols were developed for the pig and chicken faeces. Pig faecal DNA was extracted using a modified phenol-chloroform based method. Chicken faecal DNA was extracted using a protocol that employed bead-beating with MagNA Lyser Green Beads (Roche) prior to using the QIAamp® DNA Stool Mini Kit (Qiagen). However, the same species-specific gene target was successfully used to amplify host DNA from the extracted faeces, which was *cytochrome b* (*CYTB*) from the mitochondrial genome. For each species, the appropriate developed methodology was applied to 2 feeding studies where faecal samples had been collected.

The first pig feeding study compared a control diet with a xylanase inclusion diet and found that the level of pig *CYTB* DNA present in the faeces of xylanase supplemented pigs was significantly (P=0.039) lower than the levels from control fed pigs. In the second pig feeding study, pigs were either given a

control diet or a treatment diet which was supplemented with yeast-enriched protein concentrate (YPC) at 1 of 3 possible inclusion levels (2.5%, 5.0% or 7.5%). Pigs fed YPC had significantly (P=0.008) reduced pig *CYTB* DNA in their faeces, as well as significantly improved feed conversion ratio, indicating that the YPC may have reduced gut cell losses which impacts upon pig performance. Equally in the YPC feeding study, faecal calprotectin, a marker of intestinal inflammation, was measured and this was found to only be affected by the age of the animal rather than the diet it received. Importantly, faecal pig *CYTB* DNA content positively correlated with feed conversion ratio, while faecal calprotectin did not. These results further illustrated that measuring host DNA content in faeces is measuring a completely different process to inflammation and may therefore be a measure of gut cell losses as anticipated.

In the first chicken feeding study, faeces were collected from control and treated birds from a phytase supplementation study. Birds were given a diet supplemented with 2500FTU of phytase, which was expected to decrease gut cell sloughing. However, there was no effect (P=0.493) of diet on the levels of chicken *CYTB* DNA present in the faeces. In a second feeding study, chickens were given either a control diet or a 20% wheat bran diet, which was expected to increase gut cell sloughing. Like the phytase study, there was no effect of a 20% wheat bran inclusion diet on chicken *CYTB* DNA levels in the faeces. Digesta from the duodenum, jejunum, ileum and colon were also collected on day of cull from birds receiving each diet. The levels of chicken *CYTB* DNA were highest at the duodenum and significantly (P<0.001) decreased down the gut, indicating that DNA is degraded down the gastrointestinal tract, or being diluted with other DNA sources, such as microbial DNA, as expected. In addition, the jejunum appeared to be the region where a high fibre diet had the greatest effect on gut cell loss as indicated by higher levels of chicken *CYTB* DNA from digesta of the wheat bran treated birds. This was in accordance with a study by Jin *et al.* (1994), who observed greater levels of DNA synthesis in the jejunum of pigs fed 10% wheat straw.

Taken together the results from this thesis indicate that measuring host DNA content in faeces may be a marker of gut cell loss, but further studies are required to validate this.

# PUBLICATIONS & CONFERENCE PRESENTATIONS

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# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

**Figure 3.4** The effect of DNA extraction method on [**A**] DNA Yield (ng/mg faecal material) and [**B**] DNA Quality (260nm/280nm) of DNA isolated from pig faeces. Labelled: Q – QIAamp DNA Stool Mini Kit, Q + M beads – QIAamp DNA Stool Mini Kit with prior bead-beating using MagNA Lyser Green Beads & Phenol – phenol-chloroform based DNA extraction method. n=1 for all groups.

**Figure 3.5** Relative [**A**] pig *CYTB* DNA and [**B**] bacterial *16S* DNA detected in pig faecal samples extracted using either a phenol-chloroform based method (Phenol) or a MagNA Lyser bead-beating method followed by QIAamp DNA Stool Mini Kit (Q + M Beads). Error bars are ± Standard Deviation, n=4 biological replicates for all groups. Two-way ANOVA P values: [**A**] diet = 0.0144, extraction method = 0.0005, interaction = 0.3062; [**B**] diet = 0.5968, extraction method = 0.0001, interaction = 0.6315.

**Figure 3.6** [**A**] The relationship between phenol extracted DNA conc. and the relative pig *CYTB* DNA in the pig faeces. [**B**] The relationship between Q + M beads extracted DNA conc. and the relative pig *CYTB* DNA levels in the faeces. [**C**] The relationship between phenol extracted DNA conc. and relative bacterial *16S* DNA in the pig faeces. [**D**] The relationship between Q + M beads extracted DNA conc. and the relative bacterial *16S* DNA in the pig faeces. Q + M beads = Qiagen DNA Stool Mini Kit with prior bead-beating using MagNA Lyser Green Beads. n=8 biological replicates in each graph. P and r values are displayed within each graph.

**Figure 3.7** The DNA purity profiles assessed by the Nanodrop™ 2000 (Thermo Scientific) for [**A**] chicken faecal DNA and [**B**] pig faecal DNA, extracted using the modified phenol-chloroform method.

**Figure 3.8** The effect of DNA extraction method employed for extracting DNA from chicken faeces on [**A**] DNA Yield (ng/mg faecal material) achieved and [**B**] the resulting DNA integrity as observed on a 1% agarose gel. $M^1$ - 1kb DNA ladder. n=3 experimental replicates per group, error bars are ± Standard Deviation. DNA and RNA bands on the agarose gel are indicated with arrows.

**Figure 3.9** The effect of chicken faecal DNA extraction method on chicken CYTB PCR amplification. Labelled: + = positive control (chicken gDNA from chicken calf muscle), Qiagen – QIAamp® DNA Stool Mini Kit, Zymo – ZR Fecal DNA MiniPrep™, Phenol – phenol-chloroform based extraction, $M^1$ - 25bp DNA ladder and $M^2$ - 100bp DNA ladder. Expected chicken CYTB amplicon – 160bp, indicated by the red arrow. Replicates are experimental replicates.

**Figure 3.10** The effect of 4 different DNA extraction methods from chicken faeces on [**A**] DNA Yield (ng/mg faecal material) and [**B**] DNA integrity achieved. Three methods involved bead-beating prior to the QIAamp DNA Stool Mini Kit (Qiagen). Beads used were: (M) – MagNA Lyser Green Beads (Roche), P – PowerBead (MO BIO), ZR – ZR Bashing Bead Lysis Tubes (Zymo Research). (Q) – QIAamp DNA Stool Mini Kit alone. DNA Yield: error bars are ± Standard Deviation, n=3 experimental replicates for each method and it is these replicates displayed on the agarose gel. M and P were significantly (P=0.003) different to ZR and Q as indicated by different letters on the graph. $M^1$ = 1kb DNA ladder.

**Figure 3.11** The effects of 4 different DNA extraction methodologies on the success of chicken cytochrome (CYTB) semi-quantitative PCR. $M^1$ - 100bp DNA ladder, $M^2$ - 25bp DNA ladder, N – negative control, M – Bead-beating using MagNA Lyser Green Bead (Roche) + QIAamp DNA Stool Mini Kit, P – Bead-beating using PowerBead (MO BIO) tubes + QIAamp DNA Stool Mini Kit, ZR – Bead-beating using ZR BashingBead Lysis tubes + QIAamp DNA Stool Mini Kit, Q – QIAamp DNA Stool Mini Kit alone with no prior bead-beating. Predicted chicken CYTB amplicon length – 160bp, as indicated by the red arrow. Replicates are experimental replicates.

**Figure 3.12** The effect of the species the faecal sample is collected from and whether the sample is extracted immediately or post 3 hours incubation on ice on the DNA yield achieved when samples are spiked with a known amount of calf thymus DNA. Mean values ± SEM, n=3 experimental replicates for all groups. P values for 2-way (species x incubation duration) ANOVA: interaction (P=0.008), species (P<0.001) and incubation duration (P=0.002).

**Figure 3.13** The assessment of 4 different *Taq* DNA Polymerases on the PCR amplification of pig actin (ACTB) from DNA extracted from pig's blood. The expected amplicon length was 165bp, as indicated by the red arrows. M1 = 100bp DNA ladder. Negative controls are labelled with the (-) symbol above the appropriate lane for each enzyme tested. Replicates are experimental replicates.

**Figure 3.14** Assessment of the detection limits of PCR from pig faecal DNA using primers for actin or cytochrome b. A = *actin*, C = *cytochrome b* (*CYTB*), Positive controls = DNA from pig's blood used in the PCR reaction.

**Figure 3.15** Alignment of pig *cytochrome b* PCR amplicon sequences identified using Sanger Sequencing. Labelled: cytb150 – predicted 150bp amplicon sequence, positive – amplicon produced from PCR using DNA from pig's blood, control – amplicon produced from pig faecal DNA from pigs on a control diet, xylanase – amplicon produced from pig faecal DNA from pigs on a xylanase supplemented diet. (*) indicates where sequences are the same.

**Figure 3.16** Alignment of chicken *cytochrome b* PCR amplicon sequences identified using Sanger Sequencing. Labelled: predicted – predicted 160bp amplicon sequence, sequenced – amplicon produced from chicken faecal DNA. (*) indicates where sequences are the same.

**Figure 3.17** Comparison of the PCR cycle numbers where a signal was first detected from pig faecal DNA using bacterial, pig *cytochrome b* and human *actin* primers.

**Figure 4.6** [**A**] The relationship between the pig *CYTB* DNA content in faeces at day 14 and recorded FCR (wean-day 14). [**B**] The relationship between the pig *CYTB* DNA content in faeces at day 28 with FCR (day 14-day 28). [**C**] The relationship between pig faecal calprotectin concentration (ng/ml) on day 14 and FCR (wean-day 14). [**D**] The relationship between pig faecal calprotectin (ng/ml) on day 28 and FCR (day 14-day 28). P values and r values are provided within each graph.

**Figure 5.1** The effect of a phytase (2500FTU) inclusion diet on [**A**] DNA yield (ng DNA/mg faeces) and [**B**] DNA quality (260nm/280nm) of the DNA extracted from chicken faeces from 3 replicate extraction procedures. Error bars are ± SEM. n=12 biological replicates for the control group. n=10 biological replicates for the phytase group, as 2 values were deemed inaccurate and removed due to negative values for 260nm/280nm.

**Figure 5.2** The effect of a phytase (2500FTU) inclusion diet on [**A**] Relative chicken *CYTB* DNA in the chicken faeces, [**B**] Relative bacterial *16S* DNA in the chicken faeces and [**C**] Relative chicken *CYTB* DNA normalised for bacterial *16S* DNA content in the faeces. There was a trend for an effect of diet on the relative bacterial *16S* DNA (P=0.077) content. Means ± SEM. n=12 biological replicates for all groups.

**Figure 5.3** The relationship between chicken *CYTB* DNA at in the faeces at day 42 and the corrected FCR between days 21 and 42. P and r value displayed on the graph.

**Figure 5.4** The chicken wheat bran study design and sample collection. Faecal samples were collected from known individual birds at each time point, by collection from a bucket. Two faecal samples were collected from each pen floor on days 18 and 25, when faecal samples were large enough for clear differentiation and separation from the bedding. and Labels: BW – body weight, DUO – duodenum, JEJ – jejunum, ILE – ileum and COL – colon. ‘n’ numbers displayed are biological replicates.

**Figure 5.5** The qPCR standard curves produced from a 1 in 4 dilution series of 5 different DNA pools when amplified with either [**A**] chicken *CYTB* DNA primers or [**B**] bacterial *16S* DNA primers. Only the linear range of the standard curves are displayed in the figures and the PCR efficiencies for this range are also displayed.

**Figure 5.6** Comparison of qPCR standard curves created from different DNA pools using either [**A**] chicken *CYTB* DNA primers or [**B**] bacterial *16S* primers. Slopes from the equation of the line are displayed in the graphs. A slope value of between -0.15 and 0.15 was accepted as the standard curves behaving the same way in the qPCR reaction independent of the DNA pool used. The duodenum standard curve was not comparable to any other standard curve assessed.

**Figure 5.7** The effect of a wheat bran inclusion diet on [**A**] the DNA yield and [**B**] the DNA quality from faecal samples collected from individual birds and [**C**] the DNA yield and [**D**] the DNA quality from faecal samples collected from the pen floor. DNA yield is expressed as ng DNA/mg faeces and DNA quality is displayed as the 260nm/280nm value. Means ± SEM. Inaccurate values removed from the analysis. [**A**&**B**] n= 14, 14, 13, 9, 5, 7, 14 and 13 respectively. [**C**&**D**] n= 15, 16, 16 and 18 respectively. n numbers = biological replicates.

**Figure 5.8** The effect of a wheat bran inclusion diet on digesta [**A**] DNA yield (ng DNA/mg digesta) and [**B**] DNA Quality (260nm/280nm). Means ± SEM, n=9 biological replicates for all samples. There was a significant ($P<0.001$) effect of gut section on both DNA yield and DNA quality, see **Table 5.5** for post hoc results, however there was no effect ($P>0.05$) of diet or an interaction.

**Figure 5.9** The effect of a wheat bran inclusion diet on [**A**] faecal chicken *CYTB* DNA content in individual bird faeces, [**B**] faecal chicken *CYTB* DNA content in samples collected from the pen floor, [**C**] bacterial *16S* DNA content in individual bird faeces, [**D**] bacterial *16S* DNA content in samples collected from the pen floor, [**E**] *CYTB* DNA normalised for *16S* DNA in individual bird faeces and [**F**] *CYTB* DNA normalised for *16S* DNA in samples collected from the pen floor. Means ± SEM. [**A**, **C** & **E**] n= 15, 16, 15, 12, 10, 13, 14 and 16 respectively. [**B**, **D** & **F**] n=18 for all groups. n numbers = biological replicates. Significant ANOVA results with Bonferroni analyses are illustrated in **Table 5.6**.

**Figure 5.10** The relationship between [**A**] chicken *CYTB* DNA in the faeces on day 11 compared to FCR (day 4 – day 11), [**B**] chicken *CYTB* DNA in the faeces on day 18 compared to FCR (day 11 – day 18), [**C**] chicken *CYTB* DNA in the faeces on day 25 compared to FCR (day 18 – day 25) and [**D**] chicken *CYTB* DNA in the faeces on day 32 compared to FCR (day 25 – day 32). Relationships were analysed by Pearson's correlation. R values and P values are provided below:

**Figure 5.11** The effect of a wheat bran inclusion diet on [**A**] Relative chicken *CYTB* DNA, [**B**] Relative bacterial *16S* DNA in chicken digesta and [**C**] Relative *CYTB* DNA normalised for *16S* DNA. Means ± SEM, n=9 biological replicates for all samples. There was a significant ($P<0.001$) effect of gut section on each DNA measurement, post hoc analyses are illustrated in **Table 5.7**. There was also a trend for the interaction ($P=0.054$) between diet and gut section on normalised *CYTB* DNA.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADF | Acid Detergent Fibre |
| ANOVA | Analysis of Variance |
| bp | Base pair |
| BW | Body Weight |
| BWG | Body Weight Gain |
| CALP | Calprotectin |
| COL | Colon |
| Con | Control |
| Cp | Crossing point |
| CV | Coefficient of Variation |
| CYTB | Cytochrome b |
| DFI | Daily Feed Intake |
| DLWG | Daily Live Weight Gain |
| DNA | Deoxyribonucleic Acid |
| DUO | Duodenum |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FCR | Feed Conversion Ratio |
| FI | Feed Intake |
| g | Gravitational force |
| gDNA | Genomic DNA |
| GIT | Gastrointestinal Tract |
| ILE | Ileum |

| | |
|---|---|
| JEJ | Jejunum |
| kbp | Kilobase pair |
| ME | Metabolisable Energy |
| MJ | Mega Joules |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| ml | Millilitres |
| NaAc | Sodium Acetate |
| NDF | Neutral Detergent Fibre |
| ng | Nanogram |
| nm | Nanometre |
| NSPs | Non-Starch Polysaccharides |
| NTC | No Template Controls |
| PCR | Polymerase Chain Reaction |
| qPCR | Quantitative Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |
| rpm | Revolutions per minute |
| SEM | Standard Error of the Mean |
| V | Volts |
| WB | Wheat Bran |
| YPC | Yeast-enriched Protein Concentrate |
| µM | Micromolar |
| µg | Microgram |

# CHAPTER 1. INTRODUCTION

Ensuring global food security is an essential responsibility of government policy makers considering the rapidly increasing human population, hence, there is also cause for significant levels of academic research in this area. One significant area of research being explored to assist with ensuring global food security is to improve the feed efficiency of livestock, both in ruminants and monogastric animals. However, one common area that is often ignored when measuring feed efficiency is the levels of energy expended in maintaining the gastrointestinal tract lining. Cells lining the gastrointestinal tract are naturally lost and replenished, however, the rate of these processes may be elevated in times of poor health status and poor diet quality. Measuring internal processes such as gastrointestinal lining replenishment is difficult and is currently only assessed using invasive techniques, therefore, the main aim of this thesis is to explore whether a non-invasive methodology could be developed and applied to feeding studies involving pigs and poultry. This introduction will cover global food security and feed efficiency, along with an explanation of how the process of gastrointestinal cellular replenishment impacts upon feed efficiency. Additionally, the theory behind using faecal DNA analysis as a potential novel, simple, non-invasive assessment of gut cell loss will be detailed.

## 1.1 GLOBAL FOOD SECURITY

It is estimated by the United Nations that the world's population will be approximately 9.77 billion by 2050, which is around a 32% increase compared with population records in 2015 (United Nations, 2017). This is a medium estimation, the United Nations predicts a low, medium and high estimation for population size. With the ever-growing human population there are increasing pressures to ensure global food security (Nabarro and Wannous, 2014). Food security was first defined at the World Food Summit 1996 which stated:

"*Food security exists 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, 1996).

In developing countries, as wealth increases, dietary preferences change to favour a more animal based diet including more meat, eggs and dairy (Godfray *et al.*, 2010; Smith, 2013). Pigs and poultry are monogastric species. Global monogastric meat production increased by 103% in the 20 year period from 1987 to 2007, with ruminant meat production only increasing by 28% in the same period (Steinfeld and Gerber, 2010). Monogastric animals are more efficient than ruminants at converting feed energy into product and advances in feeding strategies, genetics, health and reproduction management, and husbandry have continually improved this efficiency (Steinfeld and Gerber, 2010). However, they consume human edible materials such as wheat, maize and barley (Brameld and Parr, 2016). Approximately a third of global cereal production is fed to animals (Schader *et al.*, 2015), creating competition with humans for human edible grains, particularly in intensive monogastric production systems employed globally and intensive cattle ranch systems in large parts of the North and South American continents. Extensive ruminant production systems, such as those employed in the Welsh hills and Scottish Highlands, require little to no human edible grains and instead use effective grassland management to feed their animals. Additionally, it is estimated that global food production will need to increase by 70-100% by 2050 in order to meet the projected human population alone (Godfray *et al.*, 2010).

### 1.1.1 Land use and environmental impact

Land is a finite resource and as a result there is competition for land use between agriculture, other human activities and protecting natural areas for the maintenance of biodiversity (Godfray *et al.*, 2010). The very recent fires in the Amazon rainforest have further highlighted the conflict between deforesting land for agriculture and the stark warnings from scientists to reduce emissions contributing to climate change. Trees help to remove greenhouse

gases from the atmosphere, acting as carbon sinks (Godfray *et al.*, 2010), and therefore their removal to provide more land for livestock rearing or crop production negatively impacts upon the environment (Shukla *et al.*, 1990). Additionally, intensive livestock rearing results in greater environmental pollution onto land, into waterways and the atmosphere from ammonia present in faeces and urine, as well as methane produced mainly from ruminant production (de Vries and de Boer, 2010). It is believed that approximately 18% of global greenhouse gas emissions can be attributed to agriculture with the emissions resulting from deforestation, fossil fuel combustion, methane emission from manure, enteric fermentation by ruminants and from fertilisers applied to crops (de Vries and de Boer, 2010).

The rising global population, combined with the changing dietary preferences of people in developing countries to a more animal based diet (Brameld and Parr, 2016) and the limited land available for agricultural purposes (Godfray *et al.*, 2010), means there is a real need to invest in research to tackle the global issue of maintaining food security, whilst being mindful of the environmental impact of agriculture.

### 1.1.2 Feed Conversion Ratio

In order to reduce pressures on the land use required for agriculture, particularly of livestock systems, one key solution is to increase efficiency of animal production and this has already been taking place, particularly in monogastric animal production (Steinfeld and Gerber, 2010). Two common methods that have been employed to improve the feed efficiency of livestock species and poultry are 1) the feeding of feed additives to improve digestibility and therefore nutrient availability from poorly digestible feed materials and 2) the use of growth promoters (Brameld and Parr, 2016). Feed efficiency is commonly commercially measured as feed conversion ratio (FCR), which is defined as the total amount of feed ingested by an animal divided by the total amount of product produced, with the same units used for each term (Gill *et al.*, 2010). The lower the value, the more efficient the animal is at converting

feed to useful product and it is important to note the lowest theoretical value is 1.0 which would mean the animal is 100% efficient (Brameld and Parr, 2016). Furthermore, improvements in efficiency and utilisation of protein by an animal benefits the environment as there is less nitrogenous waste excreted (Sillence, 2004).

Feed conversion ratio is effected by the genetics of the animal, health status, nutritional quality of feed and housing conditions such as stocking density and the type of feeders used (Pierozan *et al.*, 2016). Therefore, measuring feed conversion ratio provides the farmer with a better understanding of their production systems and can indicate areas for improvement to ensure productivity and profitability. Feed conversion is relatively simple to measure on farm for the whole unit as a farmer knows how much feed the animals consume and for meat production animals will know the final carcass weight of his livestock. However, measuring individual animal efficiencies on a commercial unit is very difficult. Equally, even if a farmer finds a new diet improves the feed conversion ratio of livestock in the unit as a whole, it is impossible to know exactly how the feed ingredients are being utilised by the gut and therefore the mechanism resulting in the benefit to animal growth.

## 1.2 HOW THE GASTROINTESTINAL TRACT CONTRIBUTES TO FEED CONVERSION RATIO

### 1.2.1 Protein turnover and energy utilisation

Protein turnover involves simultaneous degradation and synthesis of protein and is a dynamic process (Waterlow, 1984). Energy is required for both the synthesis and degradation of protein, however, the former typically requires more energy, see **Figure 1.1**. Additionally, breakdown products such as amino acids from protein breakdown are utilised in metabolism to create new proteins (**Figure 1.1**). At the gut this particularly relates to the degradation of enzymes, which are secreted into the lumen of the tract to aid digestion, and their constituent amino acids reabsorbed by the gastrointestinal tract and used to synthesise new proteins which may be used in other processes within the

body (Lobley, 2003). Equally the replenishment of the epithelial lining of the intestinal barrier, which is a naturally occurring process, also requires protein synthesis and a lot of energy. However, the rate of protein turnover is affected by growth status, nutritional status, injury, inflammation and infection (Vincent *et al.*, 2015; Waterlow, 1984).

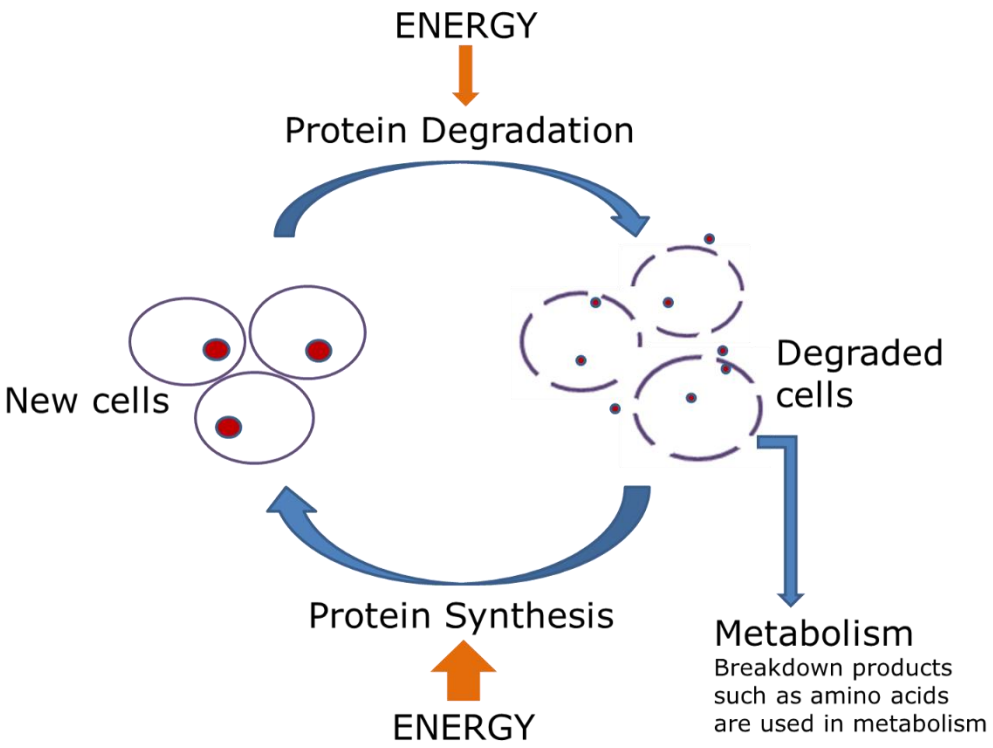


**Figure 1.1** Protein turnover. Both protein degradation and synthesis require energy, although the latter utilises more energy as indicated by a larger arrow in the diagram. Adapted from: (Baskin and Taegtmeyer, 2011).

Studies have assessed the protein synthetic rate of different organs within the body. One study injected newly weaned pigs with a flooding dose of stable isotope-labelled phenylalanine and collected various tissues at a number of time points post injection, ranging from 15 to 75 minutes post injection (Bregendahl *et al.*, 2004). Bregendahl *et al.* (2004) found that the fractional protein synthetic rate in the gut was extremely high at approximately 80%/day or more, whereas in the liver it was 40%/day, and even lower in muscle at <10%/day. As energy is required for protein synthesis, the magnitude of the synthetic rate in the gut is grossly inefficient and the ideal would be to direct

more of this energy to lean muscle for meat production, as well as decrease the amino acid utilisation at the gut for protein turnover. One method used to promote lean muscle production is to reduce protein degradation and basal metabolic rate. This can be achieved using growth promoters such as trenbolone, which is a structural analogue of testosterone (Sillence, 2004). Growth promoters are used across a number of countries globally, including the USA and Australia, but their use is banned within the EU (Sillence, 2004). Hence, much of the focus in improving livestock efficiency in this region over the last few decades has been to improve animal genetics and the utilisation of feed ingredients.

### 1.2.2 Monogastric gastrointestinal tract anatomy and function

Both the pig and chicken gastrointestinal tract comprise of the stomach, the small intestine, the caecum and the colon (**Figure 1.2**, **Figure 1.3**), however, it is important to note that in the chicken the stomach is divided into the proventriculus, or the glandular stomach, and the gizzard (**Figure 1.3**). The proventriculus acts in the same way as the stomach in the pig and is often referred to as the 'true stomach'. The stomach is where the majority of protein digestion takes place. The stomach contains mucus-secreting neck cells, pepsinogen-secreting chief cells and hydrochloric acid secreting parietal cells (Kararli, 1995). Pepsinogen is an inactive enzyme and is activated in the presence of hydrochloric acid into the protease enzyme pepsin, which digests proteins. The gizzard in the chicken has a thick muscular wall and is thought of as the 'mechanical stomach' as it is here where feed particles are further ground together and broken down. (**Figure 1.3**). In both species the small intestine can be further divided into 3 constituent parts: the duodenum, jejunum and the ileum (**Figure 1.2** and **Figure 1.3**). It is at the small intestine where further digestion takes place utilising enzymes which are produced by intestinal cells, the liver and the pancreas. The small intestine is also where the majority of nutrient absorption takes place. The small intestine is lined with villi (see **Figure 1.2**), which are small finger shaped projections which increase the surface area of the intestine to enable greater absorption of nutrients across

the intestinal barrier into the blood stream. In addition, an increased villus height increases the surface area even more, further increasing nutrient absorption (Caspary, 1992). The caecum and colon form the large intestine in the pig (**Figure 1.2**), whereas in the chicken, the caeca and the colon are more separated (**Figure 1.3**). It is in this latter region where microbial fermentation takes place, as well as the absorption of water and minerals such as sodium, and the production and absorption of volatile fatty acids which act as an energy source (Kararli, 1995). The chicken digestive tract has an interesting and distinctive difference to the pig in that chicken have a cloaca (**Figure 1.3**). It is at the cloaca where the faeces are mixed with urine prior to being excreted out of the bird. In pigs the urinary tract and the digestive tract are separated, the same as humans. It is this key anatomical difference that results in observable differences in pig and chicken faeces. Pig faeces tends to be very consistent in appearance and consistency between defecations. On the other hand, due to varying levels of urine and faeces mixed together in the cloaca and then excreted, chicken faeces can vary largely in both texture, colour, and consistency between defecations.

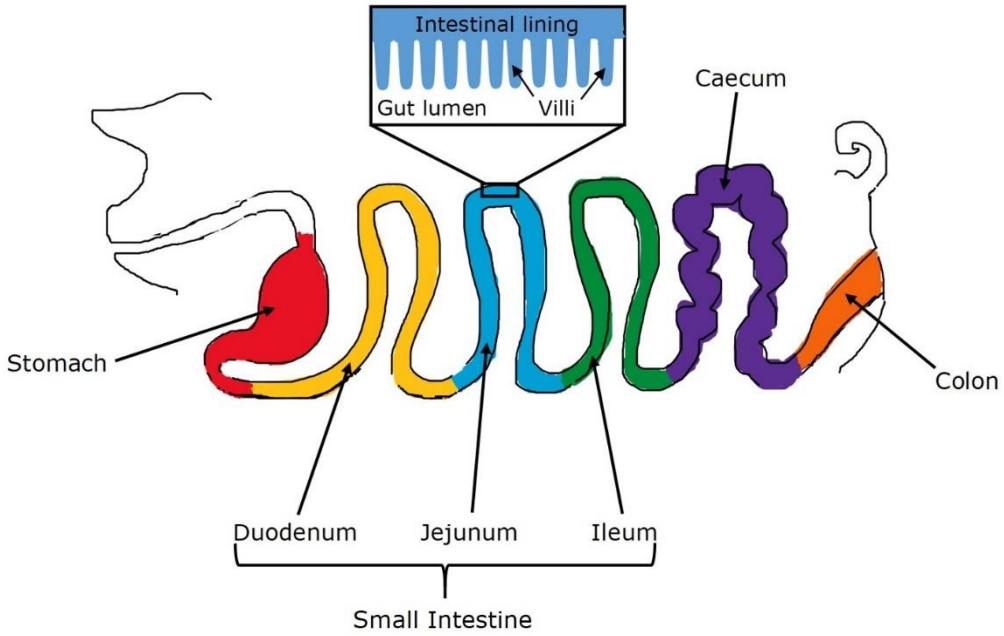


Figure 1.2 Pig gastrointestinal tract anatomy.

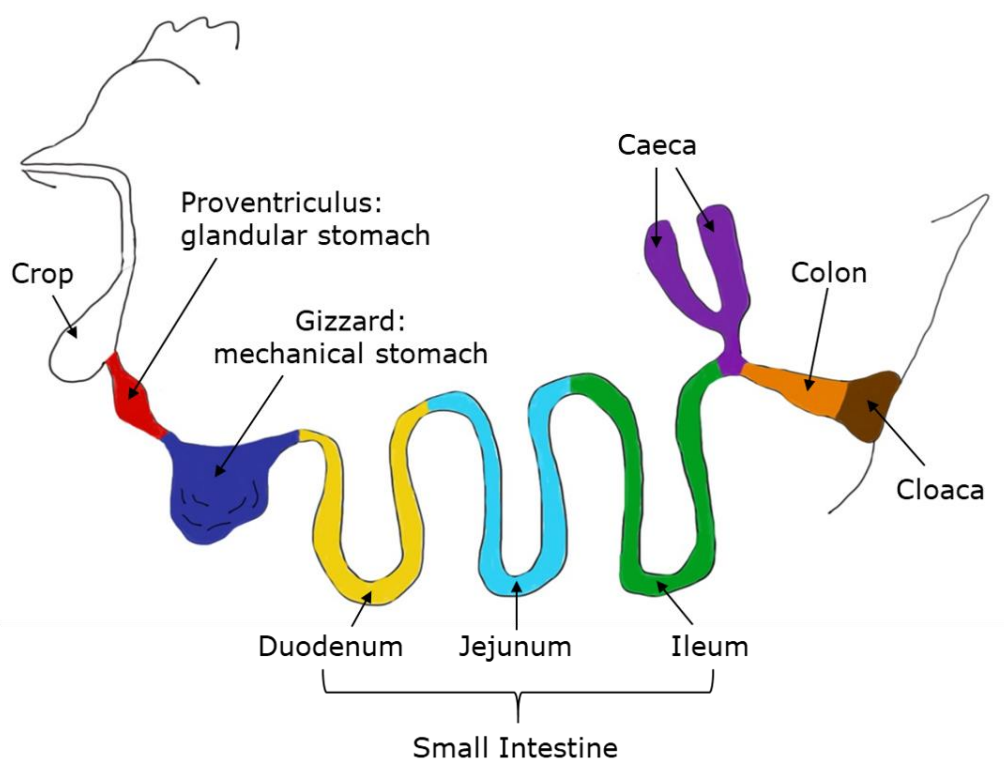


Figure 1.3 Chicken gastrointestinal tract anatomy.

### 1.2.3 Epithelial cell turnover

Intestinal epithelial cells are a terminally differentiated cell type which originate from stem cells found near the base of the intestinal crypts, see **Figure 1.4** (Park *et al.*, 2016). They coat the villi, which are the projections that increase the surface area of the intestinal barrier. As the stem cells undergo differentiation into progenitor cells, and eventually epithelial cells, they migrate towards the villus tip, see **Figure 1.4** (Park *et al.*, 2016). It is believed that it is the proliferation of cells in the crypt that is the driving force of cellular migration (Parker *et al.*, 2017). Once at the villus tip the cells undergo apoptosis, programmed cell death, and are shed into the intestinal lumen where they are degraded by enzymes present in the luminal space (**Figure 1.4**) (Park *et al.*, 2016). Epithelial cells are continuously being produced by the differentiation of stem cells, but this is balanced by the continual loss of cells once they reach the villus tip. Therefore, the rate of turnover of epithelial cells is very high. As indicated (in 1.2.1), a higher level of protein synthesis takes place at the gut compared with other organs. A large portion of this protein synthesis can be attributed to maintaining the epithelial barrier due to the high rate of turnover of the epithelial cells. Equally, a high rate of protein synthesis means a large amount of energy is utilised by this process. Paneth cells are also a terminally differentiated cell type, but unlike the epithelial cells they migrate downwards towards the base of the intestinal crypts (**Figure 1.4**) (Park *et al.*, 2016).

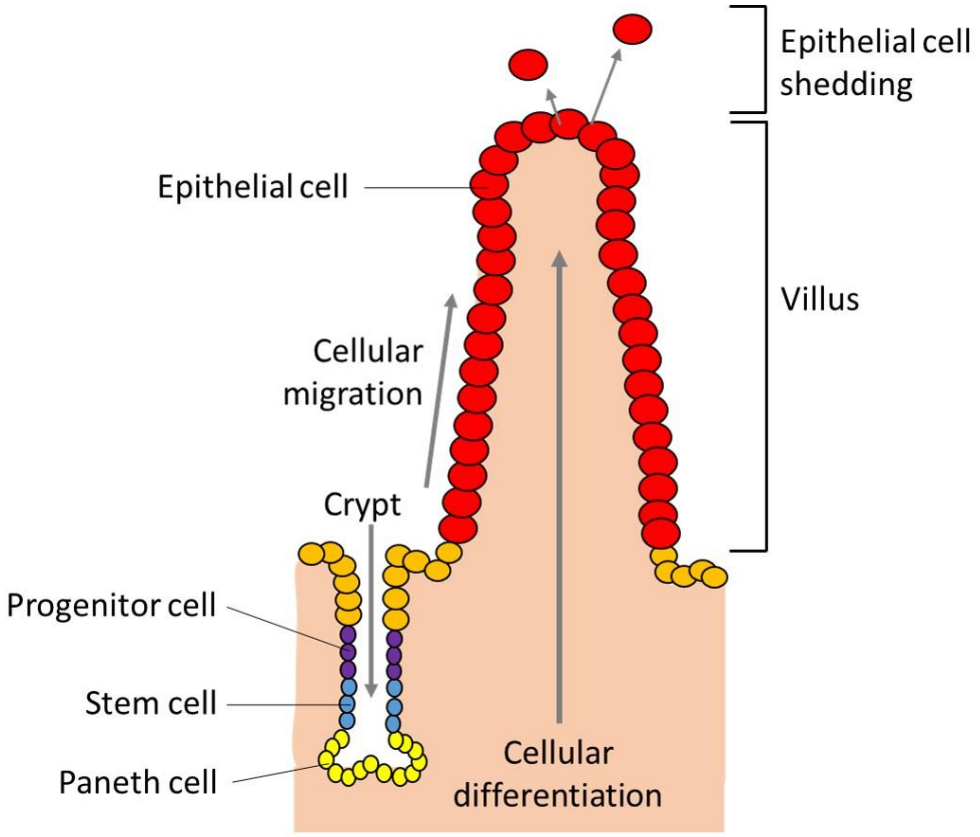


Figure 1.4 The process of epithelial cell proliferation, differentiation and migration toward the villus tip where they are shed into the intestinal lumen. Re-drawn from (Ashida *et al.*, 2012).

### 1.2.4 Factors affecting epithelial cell turnover

Epithelial cell turnover is an important process as terminal cells are shed into the intestinal lumen, therefore, in order to maintain the intestinal barrier the proliferation, differentiation, and migration of cells to the villus tip must be a tightly controlled process (Parker *et al.*, 2017). Without proper control, inflammation or tumour growth can result (Parker *et al.*, 2017). The rate of turnover can be altered, either increased or decreased, due to a number of different factors. In the presence of infectious agents or inflammation there is a greater level of damaged or dead epithelial cells shed into the intestinal lumen (Vincent *et al.*, 2015). Nutrition and the ingestion of food also affects epithelial cell turnover (Eastwood, 1977). A study found that in starved mice there was a 50% reduction in cellular proliferation, impaired cellular differentiation and a decrease in the migration of cells to the villus tip,

compared to control animals which were not starved (Brown *et al.*, 1963). Feed ingredients have also been shown to effect epithelial cell turnover processes. A study in broilers observed that birds given diets supplemented with xylanase had reduced crypt cell proliferation compared to control birds (Silva and Smithard, 2002). Local gastrointestinal hormones such as gastrin, as well as neurotransmitters such as serotonin, also impact upon epithelial cell renewal but effects may be tissue or cell specific as well as dose dependant (Eastwood, 1977). More recently there has been greater focus on the gastrointestinal microbiome. Commensal bacteria have been shown to promote intestinal epithelial cell turnover (Park *et al.*, 2016). Germ-free mice and specific pathogen-free mice had attenuated levels of crypt cell proliferation and cellular migration (Park *et al.*, 2016). Commensal bacteria produce short chain fatty acids such as acetate, propionate and butyrate via fermentation. It is believed that the short chain fatty acids produced by the bacteria are what results in increased epithelial cell turnover (Park *et al.*, 2016). However, it should be noted that studies have suggested that short chain fatty acids may target hypo-proliferative mucosa and therefore increases in proliferation may in fact be a return to the normal situation (Key *et al.*, 1996).

## 1.3 NOVEL DNA METHOD

As intestinal cellular turnover utilises a significant amount of dietary energy to replenish the intestinal barrier, and nutrition impacts upon the rate of turnover, it is important to have some measure of the process. Currently all methods to assess gut cell turnover are invasive involving stable isotope or radioactively labelled amino acids (Buttery, 1981), or the more recent method involving deuterium labelled water (Gasier *et al.*, 2010). Measuring gut cell loss non-invasively may give greater insight into *in vivo* gut function without the need for costly invasive experiments, which ensures animal welfare and may enhance farmer profitability in the long run. A method which provides insight into how novel feed ingredients, which may benefit feed conversion ratio, are impacting on the gastrointestinal tract would be useful for research and development into future ingredients.

### 1.3.1 Current invasive measures of gut turnover

To measure gut turnover using labelled amino acids the fractional synthetic rate method is used. This involves administering one large dose or a continuous infusion of a labelled amino acid, measuring activity of the protein pool and incorporation of the tracer into proteins at several interval points typically over a 30-minute period (Bregendahl *et al.*, 2004; Buttery, 1981). Due to the rapid rate of turnover at the gut, it is likely that several preliminary experiments are required to determine an appropriate dosing and sampling protocol. Home Office licenced experimental facilities are required to carry out labelled amino acid experiments in animals, of any species. Equally, the experiment would have to be carried out under a valid Home Office project licence requiring licenced animal technicians to carry out the regulated procedures of dosing animals and invasive sample collection. Hence, the cost of such experiments can be very high and depend on the number of animals being used in the study.

The deuterium labelled water method is less invasive than amino acid labelling but is still considered an invasive process. The deuterium labelled water can be used to measure protein synthesis as the water is ingested and the amino acid alanine, present in the plasma, incorporates deuterium increasing its' molecular weight (Xiao *et al.*, 2008). The enrichment of alanine into a protein of interest can then be measured using gas chromatography or mass spectrometry (Xiao *et al.*, 2008). The deuterium labelled water method is a cheaper alternative to stable isotope or radioactively labelled amino acids as the labelled water is ingested rather than requiring more controlled dosing of the animal, however, the methodology still requires licenced procedures to be carried out by licenced personnel.

### 1.3.2 DNA in faeces - a non-invasive marker of gut cell loss?

As previously described, cells are formed at the base of a single villus lining the small intestine and these cells differentiate and migrate towards the tip of the villus (Creamer *et al.*, 1961) (**Figure 1.5**). Once at the tip of a single

villus the epithelial cells are sloughed into the small intestine lumen (Creamer *et al.*, 1961), where they are degraded by enzymes secreted into the lumen (Loretz *et al.*, 2006) (**Figure 1.5**). Cellular degradation leads to the release of DNA which was once encapsulated in the cell nucleus, or mitochondria, and the DNA is then digested by endonuclease enzymes (Loretz *et al.*, 2006), leading to fragmented DNA being present in expelled faeces (Vincent *et al.*, 2015) (**Figure 1.5**). It is important to recognise that not only host cells will be degraded in the gastrointestinal tract lumen, but also bacterial cells, as well as cellular fractions from feed ingredients (**Figure 1.5**). In addition, some epithelial cells, bacteria, and feed present in faeces will remain undigested (Varela *et al.*, 2009; Vincent *et al.*, 2015). As the host DNA in faeces presumably originates from sloughed epithelial cells, measuring the relative quantity of host DNA present in faecal samples may provide a novel, simple method for the non-invasive assessment of gut cell sloughing and in turn gut function and animal intestinal health status. This methodology has already been utilised in human studies with the aim of looking at disease and the disruption to intestinal health by increases in inflammation (Vincent *et al.*, 2015), as well as radiation induced mucosal damage (Varela *et al.*, 2009). However, to the best of our knowledge it has not been attempted in livestock species as a non-invasive measure of gut cell sloughing which influences animal feed efficiency.

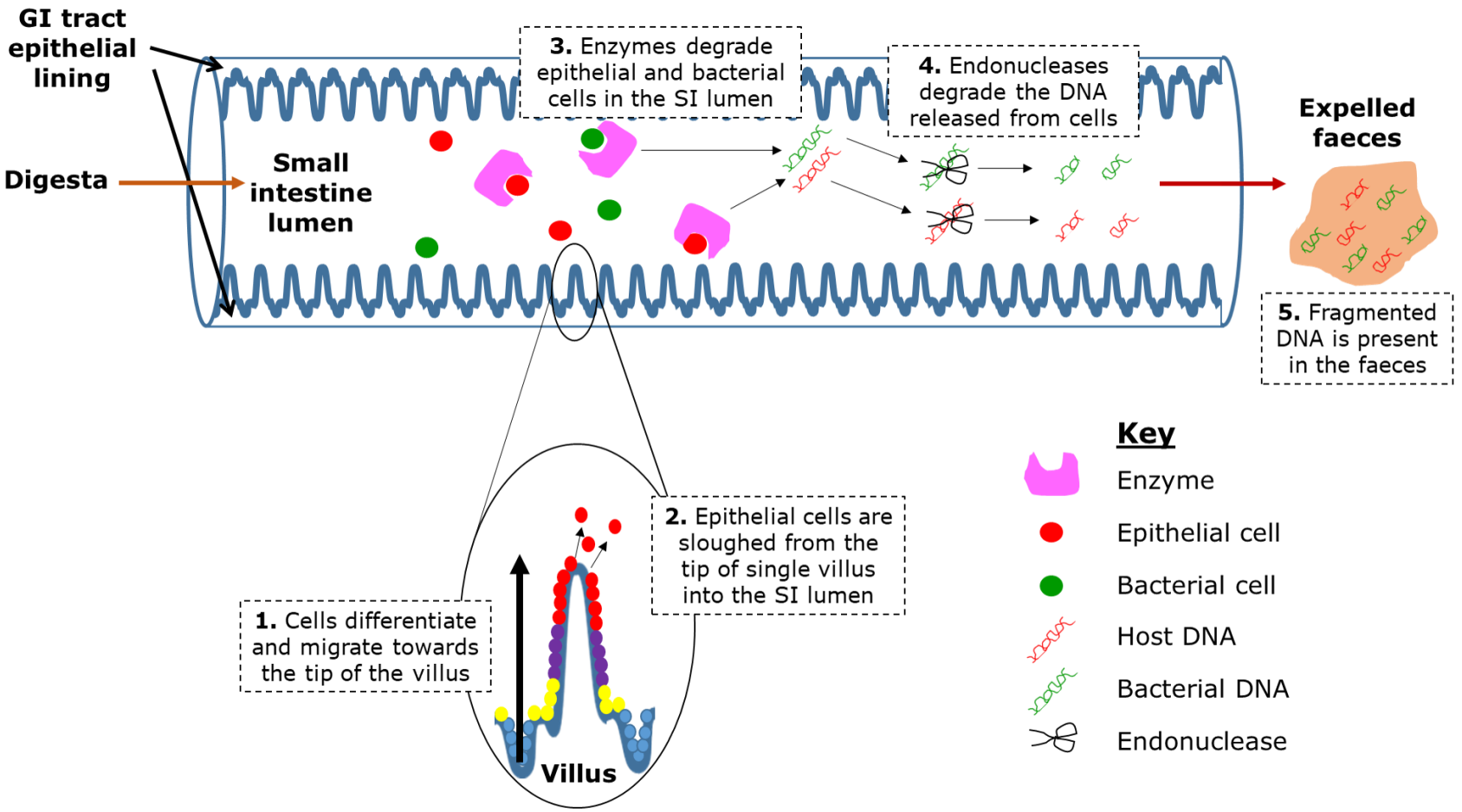


Figure 1.5 Diagram to illustrate how fragmented host DNA present in faeces may be a non-invasive marker of intestinal epithelial cell loss.

The concept behind measuring host DNA in faeces as a non-invasive marker of gut cell loss is depicted in **Figure 1.6A**. Changes in animal and human health and physiological state, as well as nutritional status, can influence the rate of intestinal epithelial cell replacement and therefore gut cell sloughing (Vincent *et al.*, 2015). For example, an abrasive feed can increase the level of gut cell sloughing compared to animals receiving a better quality feed (**Figure 1.6B**). When there is an increase in gut cell sloughing, it can be expected that there will be an increased level of fragmented host DNA in the faeces that can be extracted using DNA extraction protocols, see **Figure 1.6B**. A chosen host gene of interest can then be targeted and amplified using polymerase chain reaction methodology (PCR) in order to provide a relative quantity of host DNA in the faeces and in turn a measure of the level of gut cell loss, see **Figure 1.6**.

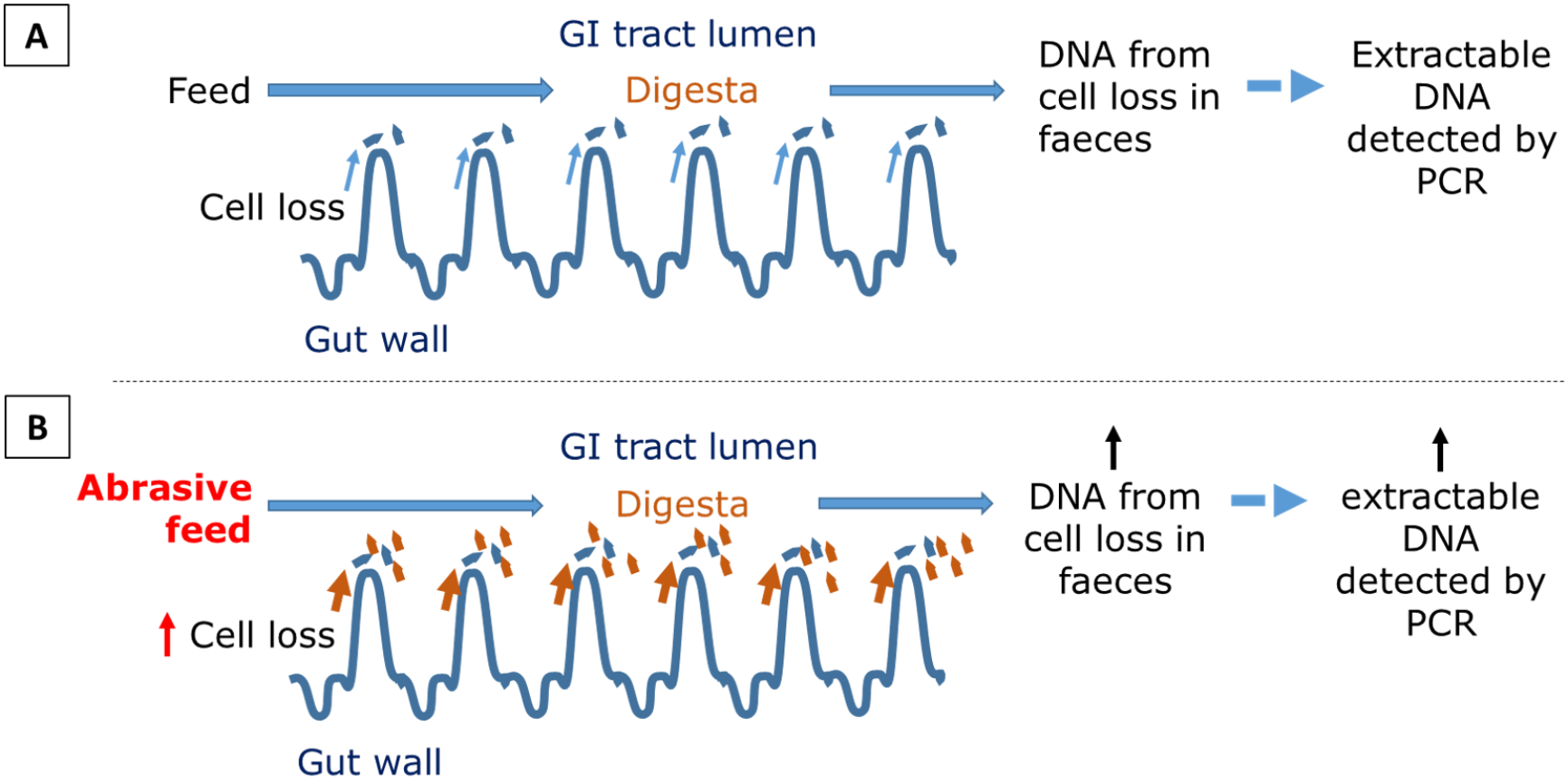


Figure 1.6 Schematic of gut cell sloughing and how an abrasive or poor quality feed may increase gut cell sloughing which should be measurable by the DNA-based assay.

### 1.3.3 DNA assay development

Due to the high levels of bacteria in faeces, the relative quantity of host DNA in faeces is low in comparison (Vincent *et al.*, 2015). Equally the quality of DNA is reduced due to degradation by endonucleases (Dimsoski, 2017). Extracting DNA from faeces provides greater challenges due to faeces being a heterogeneous matrix containing a number of products which negatively impact upon DNA extraction and in some cases downstream PCR (McOrist *et al.*, 2002). These products include bile salts, haemoglobin degradation products, complex polysaccharides, as well as DNA from a range of sources (McOrist *et al.*, 2002). As a result, optimisation is required to develop a method for the extraction of DNA from faeces which is representative. To illustrate the extent of research which has been carried out in the area of DNA extraction from faeces, **Appendix 1** contains a non-exhaustive list of methodologies that have been attempted by researchers across the globe in order to improve resulting DNA yields, quality and PCR ability. Methods attempted include commercial kits as well as traditional, chemical, non-commercial methods (**Appendix 1**). Often commercial methods have to be altered in some way to meet the particular requirements of the researcher. Commercial kits typically cost a few hundred pounds (£) for 50 samples, making the cost per sample average approximately £5.50 when considering additional ethanol that may need to be added to wash buffers and the cost of tubes and pipette tips. Phenol-chloroform DNA extraction is a traditional and effective method of extraction, and is significantly cheaper than modern commercial kits (Zhang *et al.*, 2006). A 100ml bottle of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) costs approximately £85, and typically 1ml may be used per sample, making the cost of phenol-chloroform extraction approximately £1 per sample when taking into account additional solutions also required for the method, such as 100% ethanol and sodium acetate, as well as tubes and pipette tips. However, phenol is a toxic chemical and is difficult to handle, which has driven the interest in commercial DNA extraction kits which are believed to be safer to use (Tang *et al.*, 2008).

As already highlighted the amount of host DNA present in the faeces will be low. As a result, a high copy number target gene is preferable to design host-specific PCR primers. Mitochondrial genes are commonly used as species-specific gene targets due to their high copy number and slight differences between species enables specificity (Hopwood *et al.*, 1996). In order to develop a DNA assay for the analysis of host DNA in livestock faeces, first a reproducible DNA extraction protocol will need to be identified, followed by an appropriate target gene for the amplification of host DNA using PCR. PCR is also relatively cheap, for example SYBR Green PCR Mastermix (Roche Diagnostics) can cost several hundred pounds (£) for 5ml, but it is important to note much lower volumes (microlitres vs. millilitres) are required per sample. Additionally, hundreds of samples can be analysed on a single quantitative PCR plate, reducing the processing time and manual labour cost.

## 1.4 SUMMARY

The increasing demands for meat due to a rising global population, increasing wealth and need to ensure global food security, as well as the drive to reduce land use for agricultural production systems, mean that improvements in livestock feed efficiency are needed. One area of energy utilisation by livestock that is often ignored, or the magnitude not fully appreciated, is gut cell turnover. A large amount of dietary energy is required by livestock to maintain and replenish the intestinal barrier. The intestinal epithelial cells are naturally lost and replenished, but levels may be elevated in times of poor health status or poor diet quality, leading to increased energy requirements to maintain the intestinal barrier function, which negatively impacts upon feed efficiency. Currently all methods to analyse gut function in livestock require invasive techniques. Invasive techniques are costly as they must be carried out in Home Office licenced establishments and by licenced animal technicians, furthermore, the methodologies for analysing the samples are also complex and as a result expensive. Additionally, invasive techniques require extensive periods of time and increased complexity as Home Office approval is required which takes time to gain, running the study may require

preliminary experiments to define a protocol and once samples are collected the methods for analysing them are complicated. Comparatively, a non-invasive method utilising faecal samples which can be collected on farm and analysed by any trained scientist in a laboratory using simple molecular methods, is significantly less costly. Equally, in a single day several dozen manual faecal DNA extractions can be carried out, and hundreds of PCR reactions can be run. Therefore, a faecal DNA-based method may provide a cheaper, simpler and inherently non-invasive alternative. Extensive research has been carried out into the suitability of various DNA extraction protocols for the analysis of DNA in faeces of many different species, and for different purposes such as microbiome analysis and forensics. The overall aim of this thesis is to develop, optimise, and evaluate a novel non-invasive DNA-based method using faecal samples to assess gut cell losses, and apply the developed methodology to pig and poultry feeding trials.

## 1.5 HYPOTHESIS

The overall hypothesis of this thesis is that host DNA can be extracted from faeces and quantified as a potential marker of gut cell loss (**Figure 1.6A**). When the methodology is applied to animal feeding studies, designed to impact upon gut function, a difference in animal DNA content in the faeces is expected and this should be measurable by the DNA assay (see **Figure 1.6B**). As gut cell losses influence animal performance, particularly feed conversion ratio, there will be a correlation between faecal host DNA content and animal feed efficiency.

## 1.6 OBJECTIVES

1. Optimise DNA extraction from pig and poultry faeces by examining a number of different DNA extraction protocols.
2. Identify a target gene for analysing host DNA in pig and poultry faeces that can be reproducibly amplified using PCR.
3. Apply developed methodologies to pig and poultry feeding studies designed to assess the effects of novel ingredients or differing diet qualities on gut function and animal performance.

4. Determine whether any effects on gut function or differences in animal performance can be measured using the DNA-based assays.
5. Identify the region of the intestinal tract where the host DNA likely originates from by examining the levels in digesta.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 ANIMAL TRIALS

As part of this thesis faecal samples from 4 animal trials and gut digesta from 1 of the 4 trials have been analysed to assess the hypothesis. Samples have been collected from 2 pig trials and 2 chicken trials. The first pig trial was a xylanase inclusion trial, carried out at the University of Nottingham by Katherine May as part of her doctoral studies. The second pig trial was a yeast-enriched protein concentrate (YPC) inclusion trial carried out at Harper Adams University for AB Agri Ltd. The first chicken trial was a phytase inclusion trial carried out at ADAS, an external contract research facility, for AB Vista. The second chicken trial was a wheat bran inclusion trial carried out at the University of Nottingham as part of my PhD. The animal trial details will be included in the appropriate thesis chapters. Faecal samples collected from each study were frozen at -20°C post collection. In the instance where trials were not carried out at the University of Nottingham, samples were transported frozen with ice packs and delivered to Kimberley Slinger as soon as possible. Due to the distance between external research facilities and the University of Nottingham, samples may have been out of the freezer for up to 2 hours before being stored at -20°C again once delivered. Equally, due to some samples being analysed from previous research studies, such as samples collected during Katherine May's doctoral studies, there was a difference in the length of time samples were frozen for prior to DNA extraction procedures.

## 2.2 DNA EXTRACTION OF POSITIVE CONTROLS

Positive controls were required for PCR experiments, specifically for the detection of host DNA in animal faeces. No positive control was used for bacterial DNA as the primers used were from the literature and had already been validated. The positive control used for pig DNA detection was kindly provided by Sarah Jones. The positive control was genomic DNA extracted from pig's blood, from a previous study, using the Wizard® Genomic DNA Purification Kit (Product number: A1120, Promega UK, Southampton, UK). The positive

control used for chicken DNA detection was genomic DNA extracted from chicken calf muscle DNA. The DNA extraction protocol followed for the extraction of genomic DNA from chicken calf muscle was the purification of total DNA from animal tissues protocol included with the DNeasy Blood and Tissue Kit (Product number: 69504, QIAGEN, Hilden, Germany). All buffers used were provided within the kit, and names given are those provided by the manufacturer (QIAGEN). The chicken calf muscle tissue was crushed using a pestle and mortar with the aid of liquid nitrogen and 25mg of frozen tissue was weighed into a 1.5ml microcentrifuge tube. To lyse the tissue, 180µl of Buffer ATL was added followed by 20µl of proteinase K and the sample was mixed by vortexing. The sample was then incubated at 56°C in a heat block for 90 minutes with occasional vortexing at 30-minute intervals. After incubation, the sample was vortexed for 15 seconds and then 200µl of Buffer AL was added to the sample, which was then mixed by vortexing. Then 200µl of 100% (v/v) ethanol was added to the sample which was then mixed again thoroughly by vortexing. The mixture (including any precipitate) was then pipetted onto the DNeasy Mini spin column placed in a 2ml collection tube. The spin column was then centrifuged at 8,000xg for 1 minute. The flow-through and the collection tube were then discarded. The DNeasy spin column was then placed into a new 2 ml collection tube and 500µl of Buffer AW1 was added to the spin column, which was then centrifuged for 1 minute at 8,000xg. The flow-through and collection tube were discarded. The DNeasy spin column was then placed into another new 2ml collection tube and 500µl of Buffer AW2 was added to the spin column, which was then centrifuged for 3 minutes at 14,000xg to dry the DNeasy membrane within the spin column. The flow-through and collection tube were discarded. The DNeasy Mini spin column was then placed into a clean 1.5ml microcentrifuge tube. To elute the DNA, 200µl of Buffer AE was applied directly onto the DNeasy membrane and this was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 8,000xg. The DNA was then assessed; see Materials and Methods 2.5 and 2.6. The DNA was stored at -20°C until utilised as a positive control in the PCR reactions.

## 2.3 DNA EXTRACTION METHOD FOR PIG FAECES

Different methodologies were required for the extraction of DNA from pig and chicken faeces, identified by optimisation experiments outlined in Chapter 3. Pig faecal samples were stored at -20˚C prior to DNA extraction and samples were not dried prior to processing. Genomic DNA was extracted from pig faeces using a modified phenol-chloroform extraction method, followed by ethanol precipitation. All centrifugation steps were at 16,000xg and at room temperature. Thirty milligrams of frozen pig faeces were weighed in a 1.5ml tube and 600µl of Nuclei Lysis Solution (Product number: A7941, Promega UK, Southampton, UK) added. The sample was homogenised using a handheld homogeniser and then centrifuged for 2 minutes. The supernatant was transferred to a clean 1.5ml tube and the pellet discarded. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Product number: P3803, Sigma-Aldrich Company Ltd, Dorset, UK) was added to the supernatant and the sample was then vortexed for 10 seconds followed by gentle mixing by inversion for 1 minute. The sample was then centrifuged for 2 minutes, creating 3 layers; an upper aqueous phase containing the DNA, a middle layer containing the conjugated proteins and the lower organic phase containing the phenol and unwanted contaminants such as carbohydrate. The upper aqueous phase was transferred into a clean 1.5ml tube, taking care to not disrupt the middle layer in the process. Another equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added to the new starting volume of the aqueous phase. The sample was vortexed for 10 seconds, followed by gentle mixing by inversion for 1 minute. The sample was then centrifuged for 2 minutes and the upper aqueous phase added to a clean 1.5ml tube. To the final volume of aqueous solution, 0.1 x volume of sample of 3M sodium acetate (NaAc, pH 5.2) and 2.5 x volume (of sample + NaAc) of 100% (v/v) ethanol were added. The sample was incubated at -20˚C overnight.

Following overnight incubation, the sample was centrifuged for 15 minutes. The supernatant was discarded, and pellet retained. To the pellet, 500µl of 70% (v/v) ethanol was added to wash the DNA pellet. The sample was

then centrifuged for 2 minutes. The supernatant was discarded, and the pellet was then air dried by inverting the tube and blotting onto paper towel for 10 minutes. The pellet was then re-suspended in 100µl of water (DNase and RNase free) and vortexed for 10 seconds to ensure the DNA was completely re-suspended. The DNA concentration, quality and integrity were then measured (see Materials and Methods 2.5 and 2.6). The DNA was then stored at -20˚C.

### 2.3.1 Removal of RNA contaminants from extracted DNA

After assessing the integrity of DNA extracted from pig faeces of the YPC trial it was evident that the samples were heavily contaminated with RNA. As a result, the samples were cleaned up using RNase A Solution (Product number: A7973, Promega UK, Southampton, UK). All centrifugation steps were at 16,000xg and at room temperature. A 25µl portion of the eluted DNA was incubated with 0.5µl of RNase A solution (Promega) at 37˚C for 15 minutes. After incubation, 200µl of RNase free water was added to the sample to increase the starting volume. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added to the sample and then vortexed for 10 seconds and gently mixed by inversion for 1 minute. The sample was then centrifuged for 2 minutes, separating the sample into 3 layers (upper aqueous phase which contains the DNA, a middle layer containing conjugated proteins and the lower solvent phase containing the phenol-chloroform). The upper aqueous phase was removed and added to a clean 1.5ml tube. Another equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added to the new volume of supernatant and the sample was then vortexed again for 10 seconds and mixed gently by inversion for a further minute. The sample was then centrifuged for 2 minutes and the upper aqueous phase removed and added to a clean 1.5ml tube. To the final volume of aqueous solution, 0.1 x volume of sample of 3M sodium acetate (NaAc, pH 5.2) and 2.5 x volume (of sample + NaAc) of 100% (v/v) ethanol were added. The sample was incubated at -20˚C overnight and then centrifuged for 15 minutes to pellet the DNA. The supernatant was discarded, and the pellet was retained. To wash the DNA pellet, 200µl of 70% (v/v) ethanol was added and the sample was centrifuged

for 2 minutes. Following centrifugation, the supernatant was discarded, and the pellet was air dried at room temperature by inverting the tube and blotting against paper towel for 10 minutes. The DNA pellet was then re-suspended in 10µl of RNase free water and gently vortexed for 10 seconds to ensure the DNA was re-suspended. DNA concentration, quality and integrity were then measured (see Materials and Methods 2.5 and 2.6).

## 2.4 DNA EXTRACTION METHOD FOR CHICKEN FAECES

Different methodologies were required for the extraction of DNA from pig and chicken faeces, identified by optimisation experiments outlined in Chapter 3. Chicken faecal samples were stored at -20˚C prior to DNA extraction and samples were not dried prior to processing. A phenol-based extraction method was not suitable for the extraction of DNA from chicken faeces, see Chapter 3, therefore a modified kit-based methodology was used and will be outlined here in full. Genomic DNA was extracted from chicken faeces using an initial bead-beating step which involved mechanically and chemically lysing cells in the faeces followed by an adapted protocol for the QIAamp® DNA Stool Mini Kit (Product number: 51504 - product is no longer manufactured, QIAGEN, Hilden, Germany). Buffers from the QIAamp® DNA Stool Mini Kit (QIAGEN) were prepared according to the manufacturer's instructions by adding the appropriate volumes of 100% (v/v) ethanol. The specific protocol will now be outlined, all centrifuge steps were carried out at room temperature.

Buffer ASL and Buffer AL (QIAGEN, Hilden, Germany) were pre-incubated at 70˚C to dissolve any formed precipitate in the solutions. Two hundred milligrams of frozen chicken faeces were added to a 2ml MagNA Lyser Green Beads tube (Roche Diagnostics, Burgess Hill, UK) which were prefilled with 1.4mm ceramic beads to aid mechanical lysis. Eight hundred microliters of Buffer ASL (QIAGEN) (pre-incubated at 70˚C) was added to the MagNA Lyser Green Beads tube containing the chicken faeces and the tube was then secured in a MagNA Lyser Instrument (Roche Diagnostics, Burgess Hill, UK) and processed at 6,000rpm for 30 seconds. The tube was then centrifuged at 14,000xg for 1 minute in a benchtop centrifuge and the supernatant

(approximately 500µl) was transferred to a clean 2ml tube. A second volume of 800µl of Buffer ASL was added to the MagNA Lyser Green Beads tube containing the remaining chicken faecal sample and then the tube was secured in the MagNA Lyser Instrument (Roche) and processed for a further 30 seconds at 6,000rpm. The tube was then centrifuged at 14,000xg for 1 minute in a benchtop centrifuge and the supernatant (approximately 700µl) was added to the 2ml tube which already contained supernatant from the previous centrifugation step. A final centrifugation of the MagNA Lyser Green Beads tube at 14,000xg for 1 minute was carried out to remove any remaining supernatant (approximately 200µl) and this was also added to the 2ml tube already containing supernatant. The remainder of the protocol followed the manufacturer's protocol for the isolation of DNA from stool for human DNA analysis using the QIAamp® DNA Stool Mini Kit (QIAGEN), with some adaptions required when extracting DNA from the chicken wheat bran trial samples due to difficulties with extracting sufficient DNA from the samples (see 2.4.1).

To the ~1.4ml of supernatant, 1 InhibitEX tablet (QIAGEN) was added and the sample was then vortexed immediately and continuously for 1 minute to allow inhibitors to adsorb to the InhibitEX matrix. The sample was then centrifuged at 14,000xg for 6 minutes to pellet stool particles and inhibitors bound to InhibitEX matrix. Immediately after centrifugation, the supernatant was transferred into a clean 1.5ml tube and the pellet discarded. The supernatant was centrifuged at 14,000xg for 3 minutes. While the sample was being centrifuged, 25µl of Proteinase K (QIAGEN) was pipetted into a clean 2ml tube. Once the centrifuge had stopped, 600µl of the supernatant was pipetted into the 2ml tube containing Proteinase K (QIAGEN) and 600µl of Buffer AL was also added and the sample was vortexed for 15 seconds. The sample was incubated at 70˚C for 10 minutes. After incubation, 600µl of 100% (v/v) ethanol was added to the lysate and vortexed for 20 seconds. The QIAamp® DNA Stool Mini Kit contained QIAamp spin columns which utilise a silica membrane to bind nucleic acids and allow contaminants to pass through, resulting in purified DNA being retained in the column until re-suspension. To a QIAamp spin column

inside a 2ml collection tube, 600µl of the supernatant/ethanol mix was added and centrifuged at 14,000xg for 1 minute. The eluted liquid was discarded, and the spin column retained. This process was repeated until all the lysate had been processed through the same spin column, a total of 1.8ml. To the QIAamp spin column, 500µl of Buffer AW1 was added to wash the DNA retained in the membrane. The sample was centrifuged at 14,000xg for 1 minute and the liquid in the collection tube discarded. To the same spin column 500µl of Buffer AW2, another wash buffer, was added and the sample centrifuged at 14,000xg for 3 minutes. The eluted liquid was discarded, and the spin column placed into a clean 2ml collection tube and centrifuged at 14,000xg for 1 minute to eliminate the chance of possible wash buffer carryover. Finally, the QIAamp spin column was transferred to a clean 1.5ml tube and 200µl of Buffer AE was pipetted directly onto the membrane and incubated for 1 minute at room temperature, followed by centrifugation at 14,000xg for 1 minute to elute the DNA. The spin column was then discarded and the eluted DNA was assessed, see Materials and Methods 2.5 and 2.6. The DNA was then stored at -20˚C.

### 2.4.1 Explanation of the adaptions to QIAamp® DNA Stool Mini Kit

DNA extracted from chicken faeces from the wheat bran feeding study using the above methodology was of a low concentration and in some instances the concentration and quality were below the limits of detection of the NanoDrop™ 2000 (Thermo Scientific) instrument. Therefore, some slight modifications were made to the QIAamp® DNA Stool Mini kit (Qiagen) protocol outlined in Materials and Methods 2.4 as follows. The first adaptation was that following incubation of the sample with Proteinase K, the sample was centrifuged for 1 minute to pellet any protein degradation products that may interfere with the silica membrane in the spin columns. The second and final adaptation was that the 600µl of 100% (v/v) ethanol was added to a clean 2ml microcentrifuge tube, with the supernatant from the previous centrifugation step being added directly to the alcohol and mixed via pipetting. The lysate was then added to the spin column with the same pipette tip.

## 2.5 ASSESSMENT OF DNA YIELD AND QUALITY

DNA concentration (ng/µl) and DNA quality (260nm/280nm) assessments were made by analysing 1.5µl of eluted DNA using the NanoDrop™ 2000 (Thermo Scientific) spectrophotometer. To obtain a value for DNA yield the DNA concentration (ng/µl) value was multiplied by the elution volume (µl) and divided by the starting faecal sample weight (mg), this was then expressed as nanograms of DNA per milligram of faeces. DNA yield for DNA extracted from the pig xylanase trial was expressed as micrograms of DNA per milligram of faeces due to the high DNA concentrations achieved.

## 2.6 NON-DENATURING GEL ELECTROPHORESIS FOR MEASUREMENTS OF DNA INTEGRITY

DNA integrity was assessed by running extracted DNA on a 1% (w/v) agarose 1xTAE (Tris-acetate-EDTA) gel in 1xTAE running buffer, with 1kbp DNA ladders for DNA sizing. Gels were run using the RunOne™ electrophoresis system (Embi Tec) at 100V, stained in diluted ethidium bromide and imaged using a UV Gel Doc imager (Bio-Rad).

## 2.7 PCR PRIMERS

Primers outlined in **Table 2.1** were used for final PCR analysis, both semi-quantitative and quantitative, of all 4 animal trial samples. Additional host DNA PCR primers were assessed (see Chapter 3) but the ones described in this chapter are those which were the most successful and therefore used for final analysis. Bacterial DNA in faeces and digesta was detected using published primers (Mieszkin *et al.*, 2009, see **Table 2.1**). Host DNA was detected using primers designed specifically for *cytochrome b* (*CYTB*) and the species of interest to ensure specificity, see Chapter 3 and **Table 2.1**.

Table 2.1 Primer details. F and R stand for forward and reverse primer, respectively.

| Primer name | Gene target | Accession number or reference | Primer position in the gene (5'-3') | Primer sequence (5'-3') | Annealing temp. in PCR (˚C) | Predicted amplicon length (bp) |
|---|---|---|---|---|---|---|
| 16SAllBact – F | Bacterial 16S ribosomal DNA | (Mieszkin *et al.*, 2009) | Unknown – universal primers | CGGTGAATACGTTCCCGG | 56.5 | 142 |
| 16SAllBact – R | Bacterial 16S ribosomal DNA | | Unknown – universal primers | TACGGCTACCTTGTTACGACTT | | |
| Chicken – CYTB - F | Gallus gallus CYTB | NP_006926 | 369-388 | ACTCATAGCCACCGCCTTTG | 61.2 | 160 |
| Chicken – CYTB - R | Gallus gallus CYTB | | 528-509 | AAGGGTTGGGTTGTCGACTG | | |
| Pig – CYTB_150 – F | Sus scrofa CYTB | AY920909.1 | 382-401 | TTCATAGGCTACGTCCTGCC | 60 | 150 |
| Pig – CYTB_150 – R | Sus scrofa CYTB | | 531-512 | TCGTGTGAGGGTTGCTTTGT | | |

## 2.8 SEMI-QUANTITATIVE PCR

### 2.8.1 Protocol for AmpliTaq Gold® DNA Polymerase

A number of different commercially available DNA Polymerase enzymes were assessed (see Chapter 3), but the one used for all future reactions was AmpliTaq Gold® DNA Polymerase (Applied Biosystems) as it gave the most reproducible results. The PCR amplification was carried out in a 25µl reaction volume in 200µl tubes and performed in a GeneAmp 9700 (Applied Biosystems) thermal cycler for 40 cycles for host DNA PCR amplification, and 30 cycles for bacterial *16S* DNA PCR amplification. The PCR constituent final concentrations are outlined in **Table 2.2** and the PCR cycling conditions outlined in **Table 2.3**.

Table 2.2 PCR Constituents.

| PCR Constituents | Final concentration in a 25µl reaction volume |
|---|---|
| 10xPCR Buffer II (Applied Biosystems) | 1x |
| $MgCl_2$ (25mM) | 1.5mM |
| dNTP mix (10mM) | 0.2mM |
| Forward Primer (10µM) | 0.36µM |
| Reverse Primer (10µM) | 0.36µM |
| AmpliTaq Gold® DNA Polymerase (Applied Biosystems) | 0.04units/µl |
| DNA template | 50ng DNA template |

$MgCl_2$ = Magnesium chloride.

Table 2.3 PCR protocol for *CYTB* and bacterial *16S* amplification.

| PCR Step | Temperature (°C) | Duration | Number of cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 10 mins | 1 |
| **Denaturation** | 95 | 15 secs | CYTB: 40 |
| | | | 16SAllBact: 30 |
| **Anneal** | See **Table 2.1** | 30 secs | CYTB: 40 |
| | | | 16SAllBact: 30 |
| **Extension** | 72 | 30 secs | CYTB: 40 |
| | | | 16SAllBact: 30 |
| **Final extension** | 72 | 5 mins | 1 |
| **Hold** | 4 | ∞ | N/A |

### 2.8.2 Non-denaturing gel electrophoresis of PCR amplicons

PCR products were visualised on 4% (w/v) MetaPhor® Agarose (Lonza Biologics, Slough, UK) 1xTAE gels which had been chilled in the fridge overnight. High resolution agarose was used to produce gels for electrophoresis to enable accurate sizing of PCR amplicons. Gels were run in chilled 1xTAE running buffer in Wide Mini-Sub® Cell GT running systems (Bio-Rad). Typically, 4µl of 6x blue/orange loading dye was added to tubes containing PCR product, mixed and 10µl of mixed PCR product was loaded into each well. Promega DNA ladders (25bp and 100bp DNA ladders) were used for accurate sizing of PCR product. Each gel was run for approximately 90 minutes at 100V, stained for 30 minutes in dilute ethidium bromide and imaged using a UV Gel Doc imager (Bio-Rad).

### 2.8.3 Assessment of band absorbance

To quantify differences in band absorbance, either to assess differences in DNA quantity or PCR amplicon generation, optical density was measured using Quantity One® (Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK) software.

## 2.9 PCR AMPLICON VERIFICATION

### 2.9.1 Excising amplicon bands from gels

When PCR products were to be verified following gel electrophoresis, particularly for bacterial PCR products where more than one amplicon band was visible on the gel, the PCR product was run on another 4% (w/v) MetaPhor® Agarose (Lonza Biologics, Slough, UK) gel with 1xSYBR® Green I nucleic acid gel stain (Product number: S9430, Sigma-Aldrich Company Ltd, Dorset, UK). The gel was then visualised using a Dark Reader™ transilluminator (Clare Chemical Research Inc, Dolores, Colorado, United States). The PCR amplicon bands were then excised using a scalpel blade, removing any excess agarose gel, and the piece of gel was put into a pre-weighed 2ml tube and the PCR amplicon purification kit used was QIAquick® Gel Extraction Kit, see Materials and Methods 2.9.2.

### 2.9.2 PCR amplicon purification

PCR products were purified using 1 of 2 methods, dependent on whether there was a clean single product when run on a 4% (w/v) MetaPhor® Agarose (Lonza) gel or there were multiple PCR amplicons produced. When a single clean amplicon was produced the QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) was employed to purify the PCR product so that the amplicon sequence could be identified using Sanger Sequencing. Following bacterial *16S* PCR there were always multiple PCR amplicons produced, likely due to the primers being universal and designed to amplify genes which contain a common conserved region. When numerous PCR amplicons were produced the method employed for purification was the QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany), after the amplicon of interest was excised from the gel (Materials and Methods 2.9.1). The protocols for each method are outlined as follows.

*QIAquick® PCR Purification Kit*

Buffers were prepared according to the manufacturer's instructions. All centrifugation steps were carried out at 13,000xg at room temperature. Five volumes of Buffer PB were added to one volume of the PCR sample and mixed by vortexing. Then a QIAquick spin column was placed into a 2ml collection tube provided with the kit. To bind the DNA to the column, the sample was applied to the QIAquick column and centrifuged for 1 minute, then the flow-through discarded and the column placed back into the same collection tube. The DNA was then washed by adding 750µl of Buffer PE to the column followed by centrifugation for 1 minute. The flow-through was discarded and the QIAquick column placed back into the 2ml tube. The column was then centrifuged for an additional 2 minutes to remove any residual buffer. The QIAquick column was then placed into a clean 1.5ml microcentrifuge tube. To elute the DNA, 50µl of Buffer EB was added to the centre of the QIAquick membrane and the column centrifuged for 1 minute. The eluted DNA concentration was then measured using the NanoDrop™ 2000 (Thermo Scientific) spectrophotometer, as outlined in Materials and Methods 2.5.

*QIAquick® Gel Extraction Kit*

Buffers were prepared according to the manufacturer's instructions. A heating block was set to 50˚C prior to the commencement of the protocol. All centrifugation steps were carried out at 13,000xg at room temperature. Six volumes of Buffer QC were added to one volume of gel as per the manufacturer's instructions, this was due to the percentage of agarose in the gel being >2%. The tube (containing the gel extract and Buffer QC) was then incubated at 50˚C for 10 minutes to dissolve the gel, mixing by vortexing the tube was carried out every 2 minutes during the incubation to aid the dissolving process. One gel volume of isopropanol was added to the sample and mixed by vortexing. A QIAquick spin column was then placed into a provided 2 ml collection tube. To bind the DNA, the sample was applied to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the QIAquick column placed back into the same collection tube and the process

repeated until the entire sample volume had been processed. The maximum volume capacity of the column was 800µl, therefore this process was always carried out more than once due to the large volumes of Buffer QC required to dissolve gel fragments with such high agarose percentage (4% gels used). To wash the DNA, 750µl of Buffer PE was added to the QIAquick column, followed by centrifugation for 1 minute. The flow-through was discarded and the QIAquick column centrifuged for an additional 1 minute to remove any residual ethanol from Buffer PE. The QIAquick column was then placed into a clean 1.5ml microcentrifuge tube. To elute the DNA, 50µl of RNase and DNase free water (Sigma) was added to the centre of the QIAquick membrane in the column and the sample was centrifuged for 1 minute. The eluted DNA concentration was then measured using the NanoDrop™ 2000 (Thermo Scientific) spectrophotometer, as outlined in Materials and Methods 2.5.

### 2.9.3 Sanger sequencing

Sanger sequencing was carried out by Source BioScience (Nottingham, UK) using the primers detailed in Materials and Methods 2.7. Samples were diluted to a concentration of 1ng/µl per 100 base pairs of amplicon. Therefore, if the PCR amplicon was 150 base pairs then the sample was diluted to 1.5ng/µl. The volume of sample required was 5µl per reaction; for a sample to be tested with both the forward and reverse primer then 10µl of the specified concentration was sent off for Sanger Sequencing. Source BioScience tested the samples on the day of dispatch and then the raw data and a sequence file was analysed. DNA chromatograms were analysed using Chromatogram Explorer Lite v4.0.0 (Heracle BioSoft, www.DnaBaser.com). These were analysed to assess the base peaks and therefore the purity of the sample. This was particularly useful when looking at the sequence files for bacterial DNA amplicon sequences where the sequencer had difficulty to call the bases. The sequence files were then compared against the expected sequence for host DNA amplicons using the Multiple Sequence Alignment tool on Clustal Omega (EMBL-EBI, Hinxton, UK). Sequences were then put through Nucleotide BLAST® (NCBI) to ensure specificity for the gene and species of interest. The bacterial

DNA amplicon sequences were only put through Nucleotide BLAST® (NCBI) as there is no expected amplicon sequence as the primers are universal primers. Bacterial DNA amplicon sequences were accepted as correct if the results through Nucleotide BLAST® (NCBI) came back as bacterial *16S* DNA sequences.

## 2.10 QUANTITATIVE PCR

Quantitative PCR (qPCR) was carried out on 384-well plates (Roche Diagnostics, Burgess Hill, UK) using SYBR Green® methodology and a LightCycler® 480 instrument (Roche Diagnostics, Burgess Hill, UK), to assess the relative content of host genomic DNA (gDNA) and bacterial *16S* DNA in samples. Standard curves were created using an appropriate dilution series of a pool of gDNA, the details of which are outlined in the appropriate results chapter. Each well contained 5µl of the appropriately diluted gDNA sample, PCR standard or negative control (RNase and DNase free water) and 10µl of mastermix which comprised of: 7.5µl of SYBR Green Mastermix (Roche Diagnostics, Burgess Hill, UK), 1.6µl of RNase and DNase free water and 0.45µl of both the forward and reverse primer (10µM each, final reaction concentration 0.3µM). Samples were pre-incubated at 95˚C for 5 minutes, followed by 45 amplification cycles (95˚C for 10 seconds, 60˚C for 15 seconds, and 72˚C for 15 seconds), a single melt cycle (95˚C for 5 seconds followed by 65˚C for 1 minute) and then a cooling cycle at 40˚C for 10 seconds. A melt cycle was carried out to confirm a single product was produced in the PCR reaction. Standard curves were then generated by displaying log DNA concentration on the x axis by the cycle number where fluorescence exceeded the threshold on the y axis of a line graph and then adding a trend line. The PCR efficiency was calculated by the following calculation [PCR efficiency = $10^{(-1/\text{gradient of the trend line})}$]. A PCR efficiency value of 2 is optimal as this indicates a doubling of DNA template per PCR cycle, which is what should theoretically happen. A PCR efficiency value below 2 indicates a problem with the PCR such as the presence of inhibitors, and a value greater than may indicate other issues such as contamination.

## 2.11 PIG CALPROTECTIN ELISA

A Pig Calprotectin (CALP) ELISA Kit (CUSABIO BIOTECH Co Ltd, China) was used to assess the levels of calprotectin in faeces of pigs from the yeast inclusion trial. Calprotectin is a marker of intestinal inflammation. The kit had a <8% intra-assay coefficient of variation (CV) and a <10% inter-assay CV. The kit was optimised for analysing calprotectin levels in faeces (see Chapter 3). The sample preparation protocol followed was the manufacturer's protocol for preparation of tissue homogenates as it was the closest to faeces when compared with the alternative protocols for serum and plasma. This involved weighing out 100mg of faeces and homogenising in 1ml of 1x PBS by vortexing for 1 minute and then storing at -20˚C overnight. After two freeze-thaw cycles, which were performed to break any remaining cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g and 4˚C. The supernatant was removed and stored overnight at -20˚C and assayed the following morning.

The samples were diluted 1 in 5 (see Chapter 3) onto a 96-well plate and then assayed immediately. Standards were made according to the manufacturer's instructions, whereby a 1 in 2 dilution series was created using the Standard provided in the kit. Standards and samples were assayed in duplicate over 2 ELISA plates, with a standard curve on each plate and an even balance of dietary treatment groups and time points on each plate. A blank well with no solution to be added was also assigned on each plate as per the manufacturer's instructions. All reagents were prepared in accordance with the manufacturer's instructions. 50µl of standard or sample were added per well, followed by 50µl of HRP-conjugate (1x). The plate was then covered with adhesive strip and incubated at 37˚C for 30 minutes. Following incubation, the reaction liquid was discarded and the wells washed by adding 200µl of Wash Buffer and allowing it to stand for 2 minutes and then discarding the liquid by blotting the plate on clean paper towel and ensuring all liquid was removed. The washing process was repeated 5 times in total per plate. 90µl of TMB (3,3',5,5'-tetramethylbenzidine) Substrate was then added to each well and the

plate was then incubated at 37˚C for 20 minutes in a closed incubator to ensure protection from light. To stop the reaction, 50µl of Stop Solution was added to each well and the plate was tapped gently to ensure thorough mixing. The optical density of each well was then measured within 5 minutes using a FLUOstar® Omega plate reader (BMG LABTECH Ltd, UK) at a wavelength of 450nm. Duplicate readings for each standard and sample were averaged and the average optical density of the blank subtracted. The standard curve was created by generating a four-parameter logistic (4-PL) curve-fit using online software ('elisaanalysis.com'). The results for the samples were then multiplied by the dilution factor (x5) and analysed using the appropriate statistical test (see Chapter 4).

## 2.12 DATA ANALYSIS

The appropriate statistical analysis was carried out on results and in-depth descriptions of the statistical software and tests used are outlined in the Materials and Methods section of each results chapter.

# CHAPTER 3. METHOD OPTIMISATION

## 3.1 INTRODUCTION

As explored in Chapter 1, in order to establish a non-invasive methodology for assessing gut cell losses utilising faecal samples, the first step is to optimise the DNA extraction protocol from faeces, and subsequently the polymerase chain reaction (PCR) procedure to enable successful amplification of animal and bacterial DNA from the extracted DNA sample. This is because faeces is a heterogeneous material containing substances that interfere with efficient extraction and effective amplification of gene targets through PCR, such as bile salts, haemoglobin degradation products and complex polysaccharides, in addition to DNA from a range of sources (McOrist *et al.*, 2002; Kolodziej *et al.*, 2013). It is believed that these inhibitors work by either interfering with cell lysis, interfering with the nucleic acids in the PCR, or by inactivating the thermostable DNA polymerase (Abu Al-Soud and Rådström, 2000). The aim of this chapter was to establish DNA extraction and PCR protocols for the assessment of host DNA in pig and poultry faeces, to enable the analysis of faecal host DNA levels from dietary manipulation studies. Additionally, a protocol for the assessment of faecal calprotectin, a marker of intestinal inflammation, using a commercial enzyme-linked immunosorbent assay (ELISA) was optimised for pig faecal analysis.

### 3.1.1 DNA extraction

Most literature regarding DNA extraction from faeces in humans and animals relates to i) determination of the microbial population in the gut (Ariefdjohan *et al.*, 2010); ii) use in human diagnostics of particular cancers (Roperch *et al.*, 2015); iii) species confirmation in wildlife research (Ramón-Laca *et al.*, 2015); or iv) use in forensics (Hopwood *et al.*, 1996). However, our main aim was to be able to extract host DNA from a single faecal sample, of the animal of interest, in order to identify a non-invasive marker of gut cell loss. A large number of faecal DNA extraction protocols have been tested in numerous studies previously, from traditional non-commercial methods to commercial

DNA extraction kits (see Chapter 1 and **Appendix 1**), but to the best of our knowledge none have been used for analysing host DNA as a potential marker of gut cell loss in animals. In this chapter a range of commercial kits and a phenol-chloroform based method were assessed for use in pig and chicken faecal DNA extraction. Extraction methodologies need to be optimised for each species of interest as a number of researchers have found that the species origin of faeces can vastly impact upon the decision as to which methodology is most suitable (Hart *et al.*, 2015; Knudsen *et al.*, 2016). Potentially total bacterial DNA content in faeces could also be utilised as a normalising marker when assessing host DNA content in faeces. However, the DNA extraction method can impact upon the profile of the isolated bacteria and procedures can preferentially extract certain types of bacteria (Mirsepasi *et al.*, 2014), therefore, the relationship between DNA yield achieved from extraction methods and total bacterial DNA content, measured using universal bacterial *16S* primers (Mieszkin *et al.*, 2009), was also examined.

Additionally, bead-beating modifications to extraction protocols were also analysed. This is because bead-beating has been shown to increase mechanical lysis of cells in the first step of a DNA extraction protocol and this may lead to increased yields of DNA from a particularly difficult matrix such as faeces (Li *et al.*, 2007; Salonen *et al.*, 2010; Smith *et al.*, 2011; Yu and Morrison, 2004). Increases in DNA yields may result in greater success in the amplification of specific gene targets using PCR methodology.

### 3.1.2 Polymerase Chain Reaction

PCR briefly involves i) the denaturation of a DNA helix to expose the 2 DNA strands; ii) primers annealing to the exposed DNA at specific points; iii) an enzyme called DNA polymerase elongating new DNA strands using free nucleotides, which leads to iv) the amplification of a gene sequence of interest from a DNA pool. In order to optimise PCR methodology for the amplification of particularly host DNA from a single faecal sample there are 2 key areas that can be analysed, the first being the DNA polymerase enzyme used due to

potential inhibition and the second being the target gene of interest due to DNA degradation.

*DNA Polymerase*

Not only do inhibitors present in faeces impact upon DNA extraction efficiency, but they can also negatively influence the DNA polymerase used in PCR (Maksimov *et al.*, 2017). There are thought to be 3 potential mechanisms by which inhibitors can impact upon DNA polymerases, i) inhibitor binding to the polymerase itself; ii) interaction of the inhibitor with the DNA and iii) interference with the polymerase during the extension phase of PCR (Opel *et al.*, 2010). It is believed that even an appropriate DNA extraction procedure that yields high quality DNA may not fully remove PCR inhibitors, therefore, it is preferred that both DNA extraction and PCR are optimised in parallel to find the best method combination (Maksimov *et al.*, 2017).

Commercially available DNA polymerase enzymes originate from isolated bacterium's which are able to withstand the high temperatures during the denaturing phase of PCR, the most commonly used being a *Taq* DNA polymerase which originates from the *Thermus aquaticus* bacterium (Abu Al-Soud and Rådström, 1998). The type of DNA polymerase used in PCR has also been shown to impact upon successful amplification, particularly in the presence of various inhibitors (Abu Al-Soud and Rådström, 1998). Abu Al-Soud and Rådström (1998) examined the ability of 9 thermostable DNA polymerase enzymes, from a range of bacterium origins, to amplify *Listeria*-specific *16S* RNA gene targets from purified *Listeria monocytogenes* in the presence of differing concentrations of ions and complex biological samples, including blood and homogenates of faeces, cheese and meat. The results illustrated that the effects of PCR-inhibiting components present in different sample types may be alleviated by choosing the appropriate DNA polymerase for the sample of interest (Abu Al-Soud and Rådström, 1998). We aimed to assess the suitability of 8 commercial DNA polymerase enzymes, which were from a range of bacterium origins, to amplify pig *actin* from genomic DNA extracted from pig's

blood. Nevertheless, following observations from initial testing further analysis was carried out on 4 of the enzymes which all happened to be *Taq* polymerases.

*Detection targets*

As stated in Chapter 1, the target gene for host DNA detection is also an important factor in creating a non-invasive method to assess gut turnover, as faeces is likely to contain very low levels of fragmented host DNA and significantly higher levels of bacterial DNA (Dimsoski, 2017). In this study the genes encoding two abundant proteins were used for designing host-specific primers to be used in the PCR reaction. The gene encoding *actin* was chosen due to its high copy number in the genome and constitutive expression in a number of porcine tissues (Nygard *et al.*, 2007), therefore, theoretically increasing the likelihood of detecting fragmented DNA isolated from pig faeces. Equally, the copy number of mitochondrial DNA molecules per cell are high (Andréasson *et al.*, 2002), including in sloughed epithelial cells, and DNA of mitochondrial origin may be better placed to survive degradation (Rodríguez *et al.*, 2004). Rodríguez *et al.* (2004) showed sterilisation of meat, from a number of sources, at 121˚C for 20 minutes still resulted in successful PCR amplification of species-specific ribosomal mitochondrial DNA amplicons. Hence, mitochondrial genes have been successfully used as species-specific targets in a range of studies relying on PCR methodology, including meat authentication (Shabani *et al.*, 2015), forensics (Andréasson *et al.*, 2002), the study of ancient DNA (Alonso *et al.*, 2004) and species confirmation when assessing water contamination (Martellini *et al.*, 2005; Caldwell and Levine, 2009). *Cytochrome b* (*CYTB*) originates from mitochondrial DNA and due the high number of copies per cell (Tanabe *et al.*, 2007) and the specificity of mitochondrial DNA (Caldwell *et al.*, 2007) was also tested as a target gene in this study.

### 3.1.3 Calprotectin Enzyme-Linked Immunosorbent Assay

Calprotectin is a zinc and calcium binding protein complex which has a molecular weight of 36 kilo Daltons (Varela *et al.*, 2009) and is predominantly found in neutrophils (Banerjee *et al.*, 2015). Mucosal neutrophils are shed into

the intestinal lumen and degraded, resulting in calprotectin being present in faeces (Banerjee *et al.*, 2015; Turvill *et al.*, 2016). Levels of calprotectin in faeces, otherwise termed faecal calprotectin, are measured by an enzyme-linked immunosorbent assay (ELISA) and extensive research into its diagnostic use in humans has been carried out (Turvill *et al.*, 2016). Intestinal inflammation is characterised by an increase in neutrophil infiltration, as a result, faecal calprotectin levels correlate well with intestinal inflammation (Banerjee *et al.*, 2015; Turvill *et al.*, 2016). Faecal calprotectin is used as a National Institute for Health and Care Excellence (NICE) approved diagnostic marker for inflammatory bowel disease and can be used to make an initial non-invasive assessment in cases of suspected bowel cancer (Turvill *et al.*, 2016). Additional research in humans has aimed to correlate faecal calprotectin levels with faecal DNA content and found a weak but significant relationship between the two measurements (Varela *et al.*, 2009). Studies have also attempted to provide reference values for faecal calprotectin in sows, piglets, and growing pigs, as use of a faecal calprotectin ELISA kit for the assessment of gut inflammation in swine was lacking (Lallès and Fagerhol, 2005). As faecal calprotectin may be linked to gut cell sloughing (Varela *et al.*, 2009), we aimed to measure calprotectin in pig faeces from a weaner pig feeding study and compare with measurements of faecal host DNA. However, the Pig Calprotectin (CALP) ELISA kit from CUSABIO BIOTECH Co Ltd (China) had not been optimised for use with faecal samples. The manufacturer only had protocols for tissue and serum or plasma. As a result, as advised in the manufacturer's instructions, we aimed to optimise the protocol by utilising pig faeces from pigs receiving 2 dietary extremes (a control diet and a 7.5% yeast-enriched protein concentrate supplemented diet). Pig faecal samples were diluted over a broad range using PBS buffer, with guidance from the Lallès and Fagerhol (2005) study, and tested with the Cusabio Pig CALP ELISA kit to determine the best dilution for use with weaner pig faeces from dietary manipulation studies.

## 3.2 HYPOTHESES

1. The faecal DNA extraction protocol needs to be optimised for the species of interest.
2. Each DNA polymerase tested would amplify the target gene sequence, but some may perform better than others due to the inhibitors present in faeces.
3. Both *actin* and *cytochrome b* will be successfully amplified from pig and chicken faeces as they are high copy number gene targets.

## 3.3 OBJECTIVES

1. Assess various methodologies for the extraction of DNA from pig and chicken faeces:
    a. Crude extraction
    b. Modified phenol-chloroform extraction
    c. 2 commercial extraction methods (ZR Fecal DNA MiniPrep™, QIAamp® DNA Stool Mini Kit)
    d. Bead-beating (using beads from 3 manufacturers) prior to the QIAamp® DNA Stool Mini Kit
2. Examine the extractability and stability of spiked DNA from the two faecal sources (pig vs. chicken).
3. Assess which DNA polymerase is best for the PCR of DNA from faeces.
4. Identify the best target gene for the amplification of host DNA from pig and chicken faeces.
5. Optimise a protocol for using a Pig Calprotectin ELISA (Cusabio, China) for the analysis of calprotectin levels in pig faecal samples.

## 3.4 MATERIALS AND METHODS

### 3.4.1 Animal trials

Faecal samples were obtained from one pig and one chicken dietary intervention study to optimise the faecal DNA extraction method and the PCR protocol for each species of interest. The pig trial was a xylanase supplementation trial and is outlined in full in Chapter 4 Pig Trials 4.1.4.1. Briefly 16 pigs were assigned to 1 of 2 dietary treatment groups, either a control group or a xylanase supplemented group, at weaning (approximately 4 weeks of age). Faecal samples were collected from every individual pig at the end of weeks 2, 4 and 6 of the 6-week study, providing a total of 48 pig faecal samples which were stored at -20˚C until processed. Additionally, a subset of pig faecal samples were collected from another dietary intervention study comparing a control diet with a diet supplemented with yeast-enriched protein concentrate (7.5% inclusion level, see Chapter 4 Pig Trials 4.2.4.1), and these samples were used to optimise the Pig Calprotectin ELISA protocol.

Chicken faecal samples (n=48) were kindly provided by Dr Emily Burton, Nottingham Trent University, and were collected from a dietary intervention study carried out at the institution. This chicken trial was not analysed as part of the current PhD thesis and therefore only brief details regarding the trial are indicated. The main purpose for using the chicken faecal samples was simply to optimise the protocol for use with chicken samples which would be generated from other studies, see Chapter 5. The dietary details remain unknown as the aim was to do the analysis blind and also the diet received by the bird should not influence the DNA extraction procedure if it is to be a reproducible and robust method. However, it is known that this was a study analysing the supplementation of 2 inclusion levels of xylanase compared with a control group. Briefly, chickens were subjected to one of 3 experimental diets (A, B or C) and faecal samples were collected from the pen floor, which included bedding, on the final day of the experiment (Day 42) and stored at -20˚C until processed.

### 3.4.2 DNA extraction methods

An initial crude extraction was conducted to gain an estimation of the levels of DNA present in faeces from pigs and chickens. Then, three DNA extraction methods were used to extract DNA from pig faeces. A modified phenol-chloroform based method was compared with the commercial QIAamp® DNA Stool Mini Kit (Qiagen, UK) and an adapted version of the Qiagen kit involving initial bead-beating with MagNA Lyser Green Beads (Roche). A total of 6 methods were employed to find the optimal method for extracting DNA from chicken faeces. The 6 methods were: phenol-chloroform based extraction, QIAamp® DNA Stool Mini Kit (Qiagen), ZR Fecal DNA MiniPrep™ (Zymo Research), and three methods which were adaptations to the Qiagen kit using 3 different types of beads for initial bead-beating. Each of the DNA extraction methods will now be explained in detail. DNA yield (ng/mg faecal material) and DNA quality (260/280) measures were used to assess the extraction methodologies and in some cases DNA integrity when run on a 1% agarose gel, as well as ability to PCR from the extracted DNA. It should be highlighted that some methodologies examined for pig faeces were deemed unsuitable for chicken faeces, based on DNA extraction and PCR results obtained. Therefore, more methodologies were assessed for chicken faeces as further optimisation experiments were required in order to identify a method which provided consistent, acceptable, results between replicates for the measures examined.

#### *3.4.2.1 Crude extraction*

A single pig faecal sample was extracted in triplicate, and a single chicken faecal sample was extracted in triplicate. Briefly, 50mg of frozen faeces (pig or chicken) was homogenised by vortexing in 1ml of 50mM Tris/HCl pH 7.5. The sample was then spun at 13,000xg for 10 minutes at room temperature and 600µl of the resulting supernatant was removed and transferred to a clean 2ml tube. To the supernatant, 1.2ml of 100% (v/v) ethanol was added followed by 60µl 3M sodium acetate, the sample was then incubated at 4˚C for 30 minutes to precipitate the DNA in the solution. Post incubation, the sample was

centrifuged at 13,000xg for 15 minutes at room temperature and the supernatant was discarded with the pellet retained. To wash the DNA pellet, 500µl of 70% (v/v) ethanol was added and the sample was centrifuged again at 13,000 x g for 2 minutes at room temperature. The supernatant was discarded, and the DNA pellet was air dried for 10 minutes at room temperature by inverting the tube and blotting against paper towel. The DNA pellet was re-suspended in 20µl of DNase and RNase free water. The resulting DNA concentration and quality was assessed using the NanoDrop as outlined in Materials and Methods 2.5.

#### *3.4.2.2 Phenol-chloroform based method*

The phenol-chloroform based DNA extraction method is outlined in Materials and Methods 2.3.

#### *3.4.2.3 ZR Fecal DNA MiniPrep™*

DNA extraction from chicken faecal samples using the ZR Fecal DNA MiniPrep™ (Zymo Research Corporation, California, USA) was carried out according to manufacturer's instructions, see **Figure 3.1**. The protocol was carried out on a single chicken faecal sample in triplicate. The method was not employed for pig faecal samples. Beta-mercaptoethanol was added to the Fecal DNA Binding Buffer (provided in the kit) to a final concentration of 0.5% (v/v) for optimal performance as advised in the manufacturer's protocol, this was done prior to starting the extraction. Briefly, 150mg of faecal sample was added to a ZR BashingBead™ Lysis Tube and 750µl of Lysis Solution (Zymo Research Corp) was added. The samples were secured in a MagNA Lyser bead-beating instrument (Roche) and processed at 6000rpm for 3 consecutive 99 second spins. The resulting samples were centrifuged, and supernatant added to the provided ZymoSpin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1 minute. The remainder of the protocol involved a spin column system designed to purify and precipitate DNA in a final elution volume of 100µl.

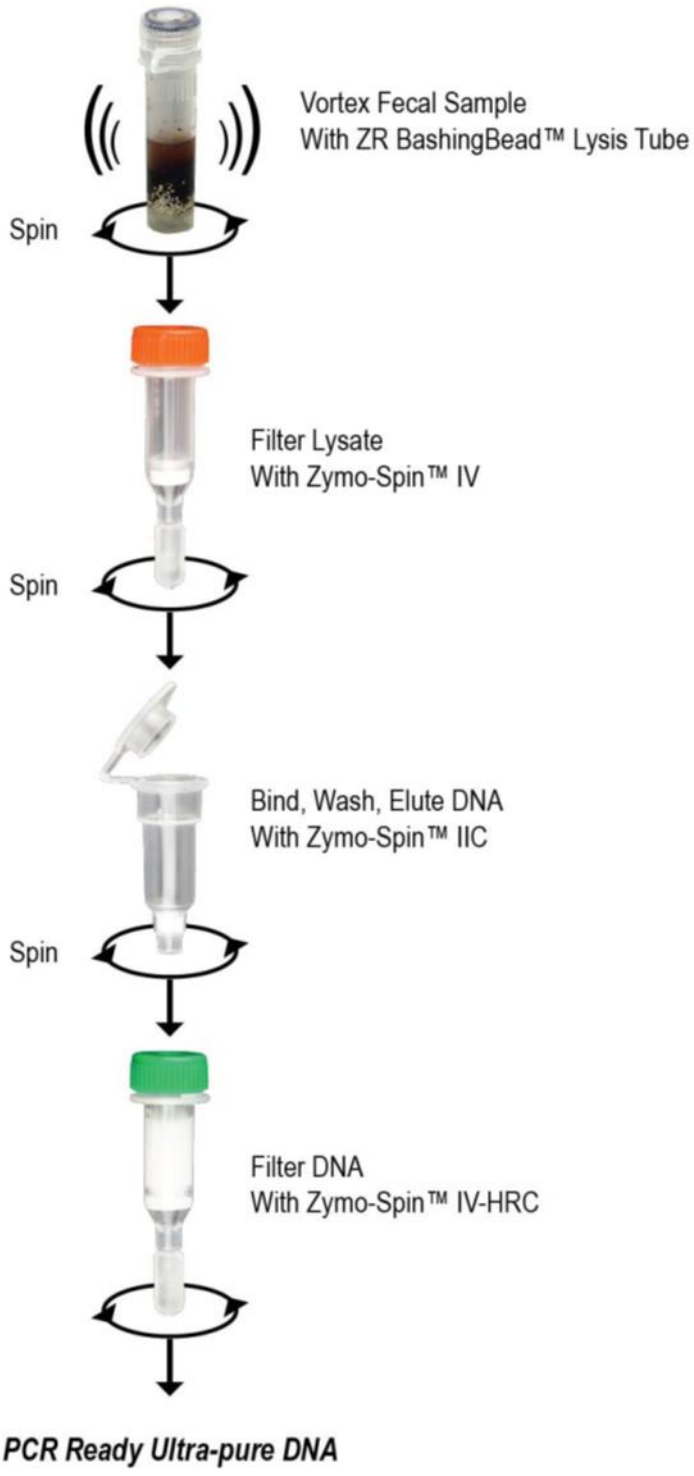


**Figure 3.1** Schematic of the ZR Fecal DNA MiniPrep™ protocol (Zymo Research Corp) from the manufacturer's instruction manual.

#### *3.4.2.4 QIAamp® DNA Stool Mini Kit*

DNA extraction from pig and chicken faecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was carried out according to the

manufacturer's instructions (see **Figure 3.2**), using the protocol for isolating DNA from stool for human DNA analysis. A hot block was set to 70˚C prior to the start of the extraction procedure, for a later enzymatic incubation step involving proteinase K (provided in the kit). All buffers were provided in the commercial kit and prepared as instructed. The appropriate volumes of 100% (v/v) ethanol were added to Buffer AW1 and Buffer AW2, and Buffer ASL and Buffer AL were incubated in a water bath at 70˚C in order to dissolve any precipitate formed. All centrifugation steps were carried out at room temperature and at 14,000xg. 200mg of frozen faeces was added to a 2ml microcentrifuge tube. Then 1.6ml of Buffer ASL was added to the tube containing the faecal sample and the tube was continuously vortexed for 1 minute. The sample was then centrifuged for 1 minute to pellet the stool particles. A total of 1.4ml of the supernatant was pipetted into a clean 2ml microcentrifuge tube and the pellet was discarded. One InhibitEX Tablet (provided in the kit) was added to the supernatant and the sample vortexed immediately and continuously for 1 minute, to completely suspend the tablet. The suspension was then incubated at room temperature for 1 minute to allow the inhibitors to adsorb to the InhibitEX matrix. Following incubation, the sample was centrifuged for 6 minutes. Immediately after the centrifuge stopped, all the supernatant was pipetted into a clean 1.5ml microcentrifuge tube and the pellet was discarded. The supernatant was centrifuged for a further 3 minutes. While the sample was being processed, 25µl of proteinase K was pipetted into a clean 2ml microcentrifuge tube. Then, 600µl of the supernatant from the last centrifuge step was added to the 2ml tube containing proteinase K. 600µl of Buffer AL was then added to the tube containing the supernatant and proteinase K and the solution was vortexed for 15 seconds, followed by an incubation at 70˚C for 10 minutes. A further 600µl of 100% (v/v) ethanol was added to the lysate and this was then mixed by vortexing for 20 seconds. The lid of a new QIAamp spin column, provided in a 2ml collection tube, was labelled and 600µl of lysate was added to the QIAamp spin column without moistening the rim. The QIAamp spin column was then centrifuged for 1 minute. The flow-through was discarded and the spin column placed into a

clean 2ml collection tube. A second aliquot of 600µl of lysate was added to the QIAamp spin column and this was centrifuged for another minute. The flow-through was discarded and the spin column placed into a clean 2ml collection tube. A final third aliquot of 600µl of lysate was added to the QIAamp spin column and this was centrifuged for 1 minute. The flow-through was discarded and the spin column placed into a clean 2ml collection tube. 500µl of Buffer AW1 was added to the spin column to wash the DNA and this was centrifuged for 1 minute. Again, the flow-through was discarded and the spin column added to a clean 2ml collection tube. Then, 500µl of Buffer AW2 was added to the QIAamp spin column to wash the DNA a final time and this was centrifuged for 3 minutes. The QIAamp spin column was then centrifuged for a further minute to prevent the chance of possible wash buffer carryover, which contains ethanol. Finally, the QIAamp spin column was added to a new, labelled, 1.5ml microcentrifuge tube and 200µl of Buffer AE was applied directly onto the QIAamp membrane. The column was incubated for 1 minute at room temperature and then centrifuged for 1 minute to elute the DNA. The spin column was then discarded, and the eluted DNA retained in the 1.5 ml microcentrifuge tube and stored at -20˚C until processed.

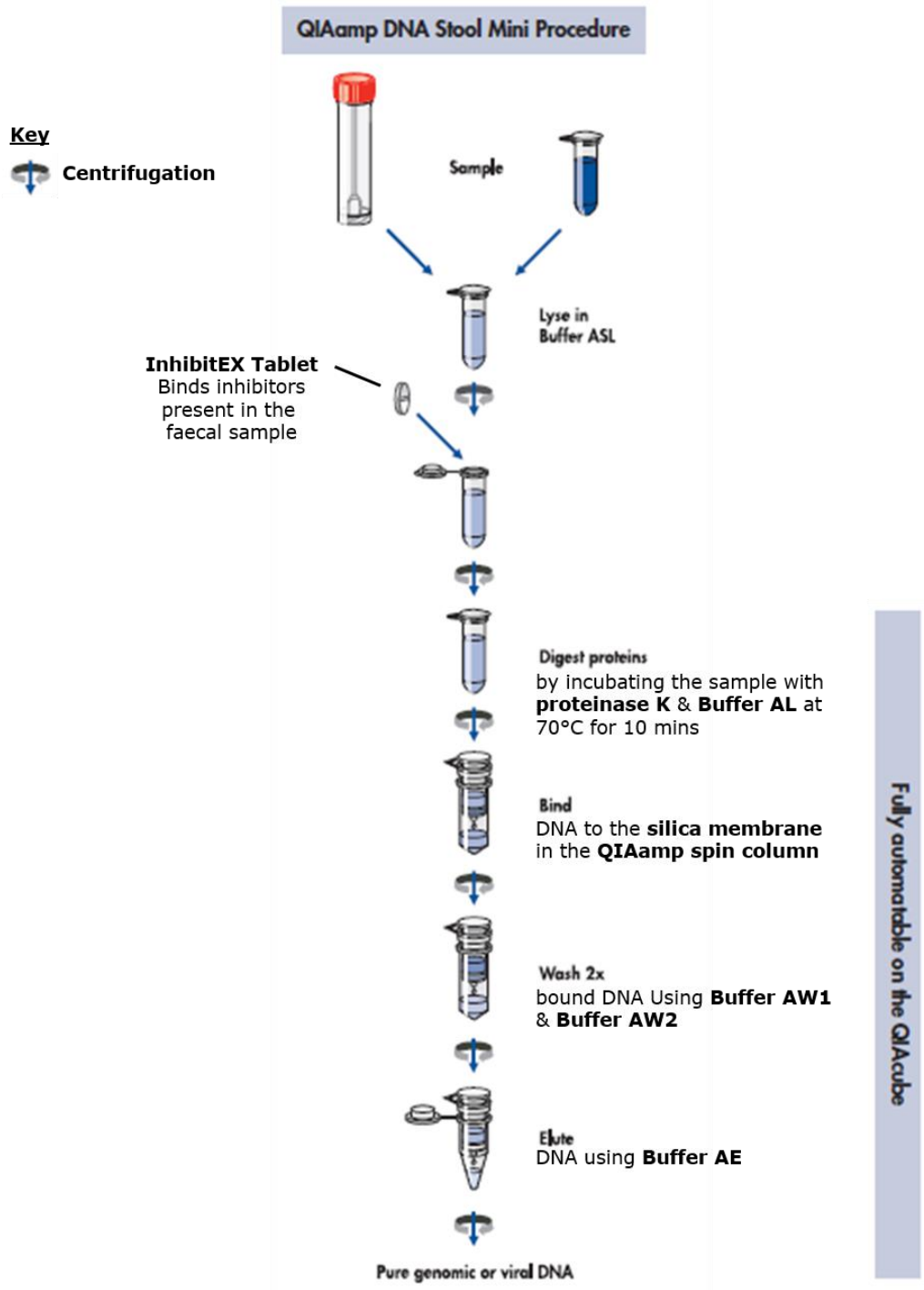


Figure 3.2 Schematic of the QIAamp® DNA Stool Mini Kit protocol (Qiagen) from the manufacturer's handbook 2012, with additional details on methodology added.

*Bead-beating modification to method*

Cell lysis is a key step in the extraction of DNA from tissues and other materials, such as faeces. Bead-beating is sometimes used in extraction protocols to aid mechanical lysis of the sample. Tubes pre-loaded with ceramic

beads from 3 different manufacturers were trialled to assess which were optimal for bead-beating prior to the QIAamp® DNA Stool Mini Kit (Qiagen) when extracting DNA from chicken faeces. The method used was the same as outlined in Material & Methods 2.4 with only the type of bead tubes used changing. The details for the bead tubes trialled are outlined in **Table 3.1**. To assess the type of bead tubes used, a single chicken faecal sample taken from a single dietary group was used and extracted in triplicate for each protocol.

Table 3.1 Details of the bead tubes used for bead-beating prior to QIAamp® DNA Stool Mini Kit (Qiagen).

| Name | Manufacturer | Material | Bead diameter (mm) | Method abbreviation |
|---|---|---|---|---|
| MagNA Lyser Green Beads Tubes | Roche Diagnostics Ltd | Ceramic | 1.4 | M |
| PowerBead Tubes | MO BIO | Ceramic | 1.4 | P |
| ZR BashingBead™ Lysis Tubes | Zymo Research Corp | Ceramic | 0.5 | ZR |

*3.4.2.5 Spiking experiments*

To further assess the differences observed in the DNA extraction procedures for pig and chicken faeces, spiking experiments were carried out to examine whether the species origin of the faeces impacts upon extractability, as well as whether there is any impact upon stability following incubation on ice. Two grams of pig or chicken faeces were spiked with 0.6mg of calf thymus DNA and homogenised using a handheld homogeniser and vortexing. Following spiking, 200mg of spiked faeces was used for DNA extraction either immediately after homogenisation or post incubation on ice for 3 hours. The incubation of samples on ice for 3 hours was examined to simulate the transportation of samples and assess how stable DNA may be from the 2 faecal sources during this process. Extractions were carried out in triplicate for each species and each extraction method. The DNA extraction method for both

species was the QIAamp® DNA Stool Mini Kit (Qiagen) with initial bead-beating using MagNA Lyser Green Beads (Roche Diagnostics) secured in a MagNA Lyser (Roche Diagnostics) instrument, see Materials and Methods 2.4.

#### *3.4.2.6 Assessment of DNA concentration and quality*

Assessments of DNA concentration and quality were carried out using the NanoDrop™ 2000 (Thermo Scientific) spectrophotometer as outlined in Materials and Methods 2.5 and 2.6.

### 3.4.3 Semi-quantitative PCR

#### *3.4.3.1 DNA polymerase enzyme optimisation*

PCR amplification was carried out using pig *actin* primers with a predicted amplicon length of 165bp, see **Table 3.4**, on genomic DNA extracted from pig's blood (see Materials and Methods 2.2). Four DNA polymerase enzymes, all originating from the *Thermus aquaticus* bacterium (*Taq* enzymes), were tested in separate PCR reactions. The enzymes were from a range of manufacturers. The enzymes tested were: GoTaq® G2 Hot Start DNA Polymerase (Promega UK), JumpStart™ DNA Polymerase (Sigma-Aldrich), AmpliTaq Gold™ DNA Polymerase (Roche Diagnostics, is currently manufactured by Applied Biosystems) and FastStart™ DNA Polymerase (Roche Diagnostics). All PCR amplification was carried out in a 25µl reaction volume and performed in a GeneAmp® PCR System 9700 (Applied Biosystems) thermocycler for 40 PCR cycles. Full details regarding the PCR constituents and concentrations as well as the PCR protocol can be found in **Table 3.2** and **Table 3.3** respectively. As Roche Diagnostics had ceased supplying AmpliTaq Gold™ DNA Polymerase and it was now being manufactured and supplied by Applied Biosystems, enzyme from the new supplier was then tested alongside the enzyme supplied from Roche Diagnostics to compare and ensure the results were indeed comparable using the same PCR protocol.

Table 3.2 PCR constituents for DNA polymerase optimisation experiments, concentrations as recommended in manufacturer's instructions.

| PCR Constituents | Final concentration of PCR constituents in a 25µl reaction volume | | | |
|---|---|---|---|---|
| | **GoTaq (Promega)** | **JumpStart (Sigma)** | **AmpliTaq Gold (Roche)** | **FastStart (Roche)** |
| PCR Buffer | 1xColourless GoTaq Flexi Buffer (Promega) | 1xPCR Buffer (Sigma) | 1xPCR Buffer II (Roche) | 1xPCR Buffer (Roche, includes 1.5mM $MgCl_2$) |
| $MgCl_2$ | 2.5mM | Included in buffer | 1.5mM | 1.5mM |
| dNTP mix | 0.2mM | 0.2mM | 0.2mM | 0.2mM |
| Forward Primer* | 0.5µM | 0.5µM | 0.36µM | 1µM |
| Reverse Primer* | 0.5µM | 0.5µM | 0.36µM | 1µM |
| DNA Polymerase | 0.05units/µl | 0.05units/µl | 0.04units/µl | 0.08units/µl |
| DNA template | 4ng/µl | 4ng/µl | 4ng/µl | 4ng/µl |

***** Primers used were the pig *actin* forward and reverse primers with an expected amplicon of 165bp, see Table 3.4. $MgCl_2$ = Magnesium chloride.

Table 3.3 PCR protocol used for each DNA polymerase tested, as recommended by the manufacturers. Each PCR reaction was carried out for 40 cycles.

| PCR Step | Temperature (˚C) and time required for each step in each DNA polymerase PCR protocol | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GoTaq (Promega) | | JumpStart (Sigma) | | AmpliTaq Gold (Roche) | | FastStart (Roche) | |
| | Temp. | Duration | Temp. | Duration | Temp. | Duration | Temp. | Duration |
| **Initial Denaturation** | 95 | 2 minutes | 94 | 1 minute | 95 | 10 minutes | 95 | 4 minutes |
| **Denaturation** | 95 | 30 seconds | 94 | 30 seconds | 95 | 15 seconds | 95 | 30 seconds |
| **Anneal** | 65 | 30 seconds | 65 | 30 seconds | 65 | 30 seconds | 65 | 30 seconds |
| **Extend** | 72 | 30 seconds | 72 | 30 seconds | 72 | 30 seconds | 72 | 1 minute |
| **Final Extension** | 72 | 5 minutes | 72 | 30 seconds | 72 | 5 minutes | 72 | 7 minutes |
| **Hold** | 4 | indefinitely | 4 | indefinitely | 4 | indefinitely | 4 | indefinitely |

Primers used were pig *actin* primers with expected amplicon of 165bp, see Table 3.4. Temp. = temperature in ˚C.

#### *3.4.3.2 Primers for host DNA detection*

*Actin*

*Actin* (*ACTB*) was chosen as a gene of interest due to its high copy number in the genome, therefore, potentially increasing the likelihood of detection despite the DNA being fragmented due to endonucleases. Sequences for a range of human *ACTB* isoforms (α, β and γ – no accession numbers recorded) were found using Ensembl release 73 (www.ensembl.org) and aligned using Clustal Omega (EMBL-EBI, Hinxton, Cambridge, UK) to find a common sequence. Human isoforms were used as there has been a more comprehensive analysis of the genes encoding *actin* in humans compared with pigs. Primers were then designed utilising the common sequence using Primer3 version 2.4.0 software (Untergasser *et al.*, 2012). Primer sequences are outlined in **Table 3.4**.

*Cytochrome b*

The gene encoding *cytochrome b* (*CYTB*) was chosen as a target as it is from the mitochondrial genome and so is likely to be better protected from the endonuclease enzymes and has been successful in other studies. Ensembl release 77 (www.ensembl.org) was utilised to find sequences for the pig and chicken *CYTB* gene and the entire porcine mitochondrial genome. The *CYTB* gene sequence for the specific breeds of pig (Large White/Landrace/Duroc) used commercially and in experimental feeding studies was also identified using Ensembl and primers designed using Primer Blast (Ye *et al.*, 2012). The primer sequences are outlined in **Table 3.4**.

Table 3.4 Primer details for PCR optimisation. F=forward primer, R=reverse primer. ACTB = actin, CYTB = cytochrome b.

| Primer name | Gene target | Accession number | Primer position in the gene (5'-3') | Primer sequence (5'-3') | Predicted amplicon length (bp) | Annealing temp. in PCR (˚C) |
|---|---|---|---|---|---|---|
| Pig_actin_F | Human ACTB | Not recorded | - | GTGGGCGACGAGGCCCAG | 165 | 65.0 |
| Pig_actin_R | Human ACTB | Not recorded | - | GGCCTCGGTCAGCAGCAC | 165 | 65.0 |
| Pig_CYTB_176_F | Sus scrofa CYTB | NC_000845.1 | 16209-16228 | GGAGTGTTGGCCCTAGTAGC | 176 | 60.0 |
| Pig_CYTB_176_R | Sus scrofa CYTB | NC_000845.1 | 16384-16365 | ATGAACGGGTGTTCTACGGG | 176 | 60.0 |
| Pig_CYTB_150_F | Sus scrofa CYTB | AY920909.1 | 382-401 | TTCATAGGCTACGTCCTGCC | 150 | 60.0 |
| Pig_CYTB_150_R | Sus scrofa CYTB | AY920909.1 | 531-512 | TCGTGTGAGGGTTGCTTTGT | 150 | 60.0 |
| Pig_CYTB_136_F | Sus scrofa CYTB | AY920909.1 | 489-508 | AATCTGAGGGGGCTTTTCCG | 136 | 60.0 |
| Pig_CYTB_136_R | Sus scrofa CYTB | AY920909.1 | 624-605 | AGGGTTGTTGGATCCGGTTT | 136 | 60.0 |
| Chicken_CYTB_F | Gallus gallus CYTB | NP_006926 | 369-388 | ACTCATAGCCACCGCCTTTG | 160 | 61.2 |
| Chicken_CYTB_R | Gallus gallus CYTB | NP_006926 | 528-509 | AAGGGTTGGGTTGTCGACTG | 160 | 61.2 |
| 16SAllBact_F | Bacterial 16S ribosomal DNA | (Mieszkin *et al.,* 2009) | Unknown – universal primers | CGGTGAATACGTTCCCGG | 142 | 56.5 |
| 16SAllBact_R | Bacterial 16S ribosomal DNA | (Mieszkin *et al.,* 2009) | Unknown – universal primers | TACGGCTACCTTGTTACGACTT | 142 | 56.5 |

#### *3.4.3.3 Bacterial DNA detection*

Bacterial primers utilised were universal *16S* primers with a predicted amplicon length of 142bp from the Mieszkin *et al.* (2009) study (see **Table 3.4**), which examined faecal contamination of water sources.

#### *3.4.3.4 Gel electrophoresis and assessment of band intensity*

DNA integrity was assessed by gel electrophoresis using 1% (w/v) agarose gels as outlined in Materials and Methods 2.6. PCR amplicons were visualised and sized by gel electrophoresis using 4% (w/v) MetaPhor® Agarose (Lonza Biologics) gels (Materials and Methods 2.8.2) and band absorbance was measured using Quantity One® software (Bio-Rad Laboratories Ltd) (Materials and Methods 2.8.3).

#### *3.4.3.5 PCR amplicon verification*

PCR amplicons were verified by methods outlined in Materials and Methods 2.9.

### 3.4.4 Quantitative PCR

Quantitative PCR was carried out using SYBR Green (Roche) methodology as outlined in Materials and Methods 2.10. Briefly, PCR was carried out on 384-well plates (Roche) and each well contained 7.5µl of SYBR Green mastermix, 1.6µl of water (DNase and RNase free), 0.45µl of both the forward and reverse primer (10µM) and 5µl of either a PCR standard, diluted DNA samples to test or DNase and RNase free water as a no template control. PCR was carried out for 45 amplification cycles and melt curves carried out, as outlined in Materials and Methods 2.10. Primers used for optimisation experiments are outlined in **Table 3.4**.

### 3.4.5 Pig Calprotectin ELISA optimisation

Faecal samples from the pig yeast-enriched protein concentrate (YPC) trial, see Pig Trials 4.2, were tested using the Pig Calprotectin (CALP) ELISA kit (CUSABIO BIOTECH Co Ltd, China). The kit does not come with a protocol for use with faecal samples and therefore needed optimising for measuring

calprotectin from the pig faeces, as advised in the manufacturer's instructions. Data from Lallès and Fagerhol (2005) were used as a guide to the dilutions to test in order to optimise the ELISA kit for faeces of newly weaned pigs. Replicate pig faecal samples from the control group and the 7.5% YPC supplemented group were used to create 2 faecal pools and these were prepared separately to ensure 2 dietary extremes were tested in the optimisation process. The sample preparation protocol followed was the manufacturer's protocol for preparation of tissue homogenates, as it was the closest to faeces when compared with the alternative protocols for serum and plasma. One hundred milligrams of faeces were homogenised in 1ml of 1x PBS by vortexing for 1 minute and then stored at -20˚C overnight. Three replicates were prepared per dietary group. After two freeze-thaw cycles, which were performed to break any remaining cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g at 4˚C. The supernatant was removed and stored overnight at -20˚C and assayed the following morning. The supernatant was diluted using Sample Diluent provided in the Cusabio ELISA kit, creating 5 different dilutions to be tested to optimise the kit. The 5 dilutions tested were: 1:5, 1:10, 1:50, 1:100 and 1:500. The remainder of the protocol was followed as outlined previously in Materials and Methods 2.11. The standard curve was loaded in duplicate and used to assess which faecal supernatant dilution was optimal.

### 3.4.6 Data analysis

The effect of species origin of the faeces on resulting DNA concentration (ng/µl) and DNA quality (260nm/280nm) when using a crude extraction method were analysed by 1-way ANOVA in Genstat 19$^{th}$ Edition. Two-way ANOVA (Genstat 19$^{th}$ Edition) was used to analyse the effect of diet (control vs. xylanase) and extraction method (phenol vs. Q + M beads) on the relative levels of pig *CYTB* DNA and bacterial *16S* DNA in the pig faeces. The relationship between extracted faecal DNA concentration (using either the phenol-based or Q + M beads method) and the relative pig *CYTB* or bacterial *16S* DNA content in the pig faeces was analysed using Pearson's Correlation in GraphPad Prism.

Differences in band intensity of chicken *CYTB* PCR amplicons run on a gel, from PCR of chicken faecal DNA extracted using several methods, were analysed by 1-way ANOVA (extraction method) in Genstat 17th Edition. The effect of DNA extraction method on DNA yield from chicken faeces was analysed by 1-way ANOVA and significant differences were further identified using the Bonferroni post hoc test, again using Genstat 17th Edition. The effects of species (pig vs. chicken) and length of incubation (no incubation vs. 3 hours) on % DNA extraction yield of spiked faeces samples were analysed by 2-way ANOVA in Genstat 19th Edition.

## 3.5 RESULTS

### 3.5.1 Effect of species on faecal DNA extraction

#### *3.5.1.1 Crude extraction*

DNA extracted from pig faeces using a crude DNA extraction protocol was of significantly (P=0.012) higher DNA concentration compared to DNA extracted from chicken faeces, **Figure 3.3A**. Interestingly, on average the concentration of DNA extracted from 50mg of pig faeces (~600ng/µl) was 6 times greater than that extracted from the same weight of chicken faeces (~100ng/µl). This suggests there is more DNA in 50mg of pig faeces, compared with the same weight of chicken faeces, or that there are differences in extractability which may be as a result of differences in faecal composition or water content as the samples were not dried prior to extraction. Conversely, there was no difference (P=0.099) in DNA quality (260nm/230nm) between samples extracted from pig or chicken faeces, **Figure 3.3B**.

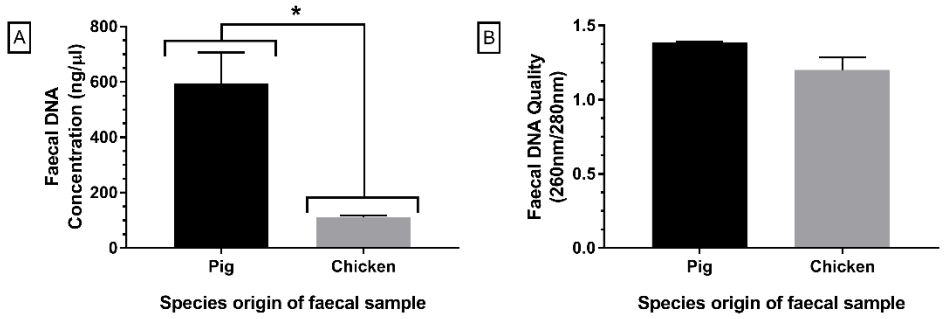


Figure 3.3 The effect of species origin of faeces on [A] DNA concentration (ng/µl) and [B] DNA quality (260nm/280nm), when extracted using a crude extraction method. *P<0.05. Mean values ± SEM, n=3 experimental replicates per group.

### *3.5.1.2 Optimal DNA extraction method from pig faeces*

The pig faeces were used to optimise the DNA extraction and PCR procedures for examining host and bacterial DNA content in the faeces, before the samples were analysed for the dietary effect. The QIAamp® DNA Stool Mini Kit (Q) (Qiagen) (Method Optimisation 3.4.2.4) was carried out in triplicate on a single faecal sample. The modified phenol-chloroform extraction method (Materials and Methods 2.3) and the Qiagen kit with prior bead-beating using MagNA Lyser Green Beads (Q + M beads) (Materials and Methods 2.4) were used to extract DNA from 4 faecal samples from each dietary treatment group (control & xylanase) singularly. The fact that the Qiagen kit alone was only carried out on a single sample is a limitation. Ideally this should have been carried out on 4 biological replicates, as were the other methods. The Q + M beads method was yielding better results than the Qiagen kit alone (and other bead-beating methods) for chicken faeces. Therefore, the decision was taken to examine this modified Qiagen kit utilising prior bead-beating with MagNA Lyser Green Beads (Roche) for pig faeces further too, as it does not require the same toxic chemicals as the phenol-chloroform based method and it performed best of the kit based methods trialled for chicken faeces (see 3.5.1.3).

The phenol-chloroform based extraction method resulted in a much higher total DNA yield (ng/mg faecal material) compared to the other two methods (Q and Q + M beads) for the same pig faecal sample (**Figure 3.4A**).

However, all three methods yielded DNA of similar high quality as measured by the 260nm/280nm ratio (**Figure 3.4B**). The ratio was approximately 1.9 for all extraction methods, which indicates high quality DNA.

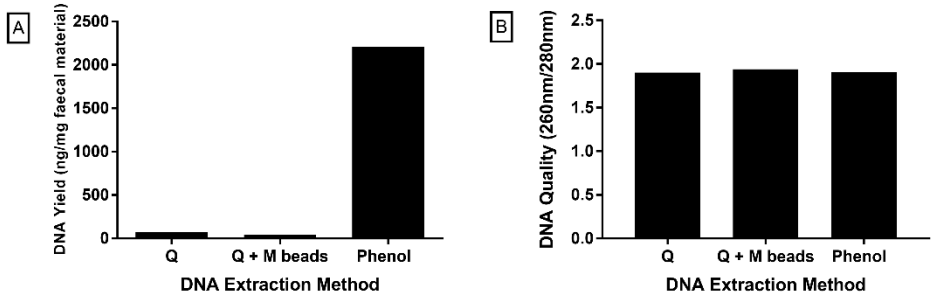


Figure 3.4 The effect of DNA extraction method on [A] DNA Yield (ng/mg faecal material) and [B] DNA Quality (260nm/280nm) of DNA isolated from pig faeces. Labelled: Q – QIAamp DNA Stool Mini Kit, Q + M beads – QIAamp DNA Stool Mini Kit with prior bead-beating using MagNA Lyser Green Beads & Phenol – phenol-chloroform based DNA extraction method. n=1 for all groups.

Although the DNA yield (ng/mg faecal material) was far greater when the phenol-chloroform based method was employed, this method does raise safety concerns. Phenol is listed as a category 3 carcinogen (IARC) and can cause phenol burns to skin, as well as other health effects such as gastrointestinal and respiratory irritation. Chloroform is a category 2B carcinogen (IARC) and inhalation can cause damage to both the respiratory and hepatic system as well as other organ systems. Therefore, more in-depth analysis was carried out using DNA extracted using the phenol-chloroform and Q + M beads method.

Quantitative PCR for pig *CYTB* and bacterial *16S* DNA detection was carried out on DNA extracted using the Qiagen kit with prior bead-beating (Q + M beads) and phenol extracted DNA, to assess whether a less toxic method could be employed. Pig *CYTB* PCR was carried out to see whether the detection in faeces was consistent between methods, no difference in *CYTB* DNA was expected. However, bacterial *16S* PCR was chosen as literature has suggested that many commercial based kits preferentially extract particular bacterial sources which would prevent total bacterial DNA, as measured using universal

primers from published literature, being used as a normalising control in this context.

When assessing the DNA quantitation of both the pig *CYTB* and bacterial *16S* genes, in the pig faecal samples from the xylanase trial, the differences between control and xylanase samples appeared similar independent of extraction method employed (**Figure 3.5A** and **Figure 3.5B**). However, the Q + M beads method resulted in a greater level of detection overall (**Figure 3.5A** and **Figure 3.5B**). When analysed by 2-way ANOVA (diet x extraction method), there was a significant effect of diet (P=0.0144) and a significant effect of extraction method (P=0.0005) on the relative pig *CYTB* DNA present in the faeces, however, there was no interaction (P=0.3062) (**Figure 3.5A**). Conversely, there was an effect of extraction (P=0.0001) on relative bacterial *16S* DNA in the pig faeces but no effect of diet (P=0.5968), nor an interaction (P=0.6315) (**Figure 3.5B**).

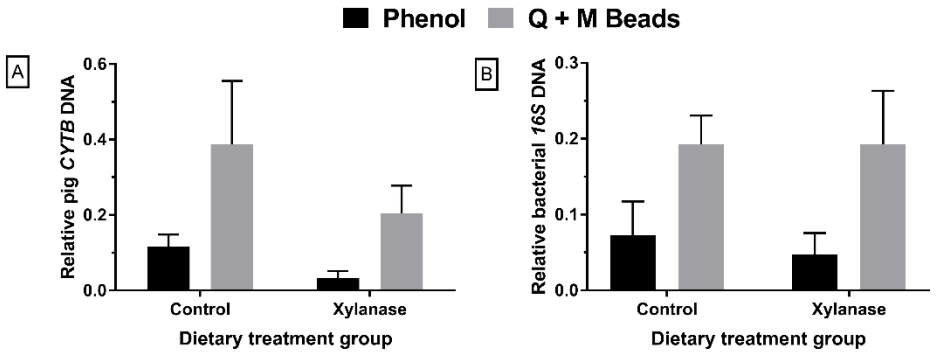


Figure 3.5 Relative [A] pig *CYTB* DNA and [B] bacterial *16S* DNA detected in pig faecal samples extracted using either a phenol-chloroform based method (Phenol) or a MagNA Lyser bead-beating method followed by QIAamp DNA Stool Mini Kit (Q + M Beads). Error bars are ± Standard Deviation, n=4 biological replicates for all groups. Two-way ANOVA P values: [A] diet = 0.0144, extraction method = 0.0005, interaction = 0.3062; [B] diet = 0.5968, extraction method = 0.0001, interaction = 0.6315.

In order for a method to be suitable for extracting DNA from faeces to then analyse gut cell loss, there should be no relationship between the amount of DNA extracted (ng/µl) and the level of gene DNA detection via quantitative PCR. When evaluating the relationship between extracted DNA concentration

(ng/µl) and the level of pig *CYTB* DNA detected there was no significant relationship observed for either the phenol-chloroform based method (P=0.4664) (**Figure 3.6A**) or the Q + M beads method (P=0.9487) (**Figure 3.6B**). These results indicate that even though there may be variability in the original DNA concentration (ng/µl) extracted, when these samples are diluted to the same concentration for PCR, the level of pig *CYTB* DNA in the sample remains representative and the dilution of the DNA is not impacted upon. However, there was a significant (P=0.0075) positive correlation between DNA concentration (ng/µl) of DNA extracted using Q + M beads and bacterial *16S* DNA detection (**Figure 3.6D**). This suggests preferential DNA extraction of bacteria with increasing DNA concentration. This is problematic as it may have a dilution effect on the host DNA content in faecal samples. However, there was no significant (P=0.2044) correlation between phenol extracted DNA concentration (ng/µl) and bacterial *16S* DNA (**Figure 3.6C**). This suggests that phenol-chloroform extraction provides a more representative profile of faecal DNA content compared to the alternative Q + M beads method. Therefore, the phenol-chloroform DNA extraction method was the most appropriate of the methods tested for extracting DNA from pig faeces and will be the chosen DNA extraction method from pig faeces in future trials, see Chapter 4.

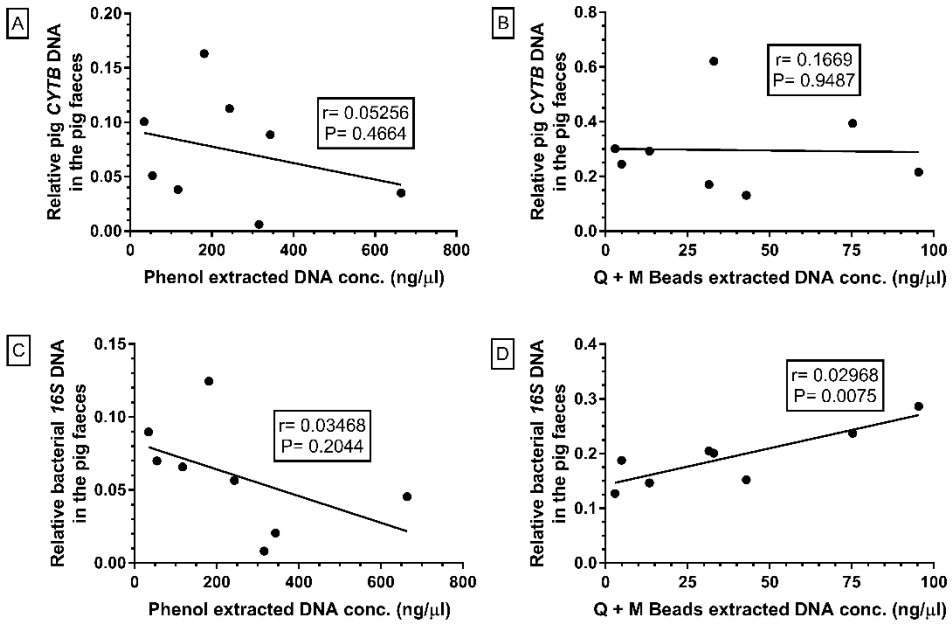


Figure 3.6 [A] The relationship between phenol extracted DNA conc. and the relative pig *CYTB* DNA in the pig faeces. [B] The relationship between Q + M beads extracted DNA conc. and the relative pig *CYTB* DNA levels in the faeces. [C] The relationship between phenol extracted DNA conc. and relative bacterial *16S* DNA in the pig faeces. [D] The relationship between Q + M beads extracted DNA conc. and the relative bacterial *16S* DNA in the pig faeces. Q + M beads = Qiagen DNA Stool Mini Kit with prior bead-beating using MagNA Lyser Green Beads. n=8 biological replicates in each graph. P and r values are displayed within each graph.

### *3.5.1.3 Optimal DNA extraction method from chicken faeces*

The phenol method was tested on chicken faecal samples and the results indicated that a phenol-based extraction protocol would not work for chicken faecal samples. The resultant DNA was of poor DNA quality (260nm/280nm) and there was consistent carry-over of substances into the eluted DNA as 2 clear peaks were visible when analysed using the NanoDrop™ 2000 spectrophotometer (**Figure 3.7A**). If DNA is of high quality, a clean single peak is expected with a 260nm/280nm ratio of approximately 1.8; pig faecal DNA extracted using the phenol-based method yielded DNA with the expected profile (**Figure 3.7B**).

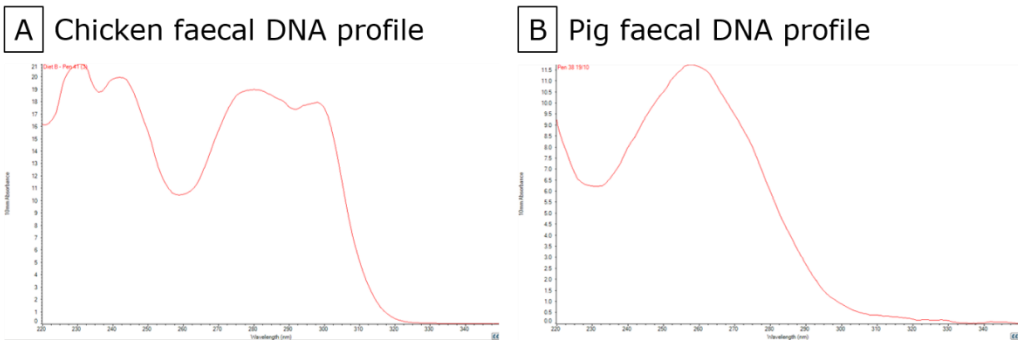


Figure 3.7 The DNA purity profiles assessed by the Nanodrop™ 2000 (Thermo Scientific) for [A] chicken faecal DNA and [B] pig faecal DNA, extracted using the modified phenol-chloroform method.

The DNA yield achieved using the phenol-chloroform based extraction was far greater than that achieved using the Qiagen and Zymo research kits (**Figure 3.8A**). However, the level of variability as observed by the error bars representing standard deviations, was much greater when using the phenol-chloroform based method compared to the alternative commercial kits. This level of variability was also evident in the DNA quality measured by the 260nm/280nm ratio on the NanoDrop™ 2000 spectrophotometer. In addition, when looking at the DNA integrity and quality when samples were run on a 1% agarose gel, the phenol-chloroform based method clearly has 2 distinct RNA bands as well as the DNA band (**Figure 3.8B**). The nature of the phenol-chloroform based extraction leads to precipitation of all nucleotides, including RNA and DNA, and therefore when looking at the 'DNA' integrity on the gel (**Figure 3.8B**) compared with the DNA yield achieved (**Figure 3.8A**) the DNA yield should be viewed with caution. The NanoDrop™ 2000 spectrophotometer does not specify between RNA and DNA and therefore the high DNA yield from the phenol-chloroform based method could be due to high levels of co-extracted RNA. The Qiagen kit resulted in lower DNA yields (ng/mg faecal material) compared with the phenol-chloroform based method but was similar to that achieved using the Zymo Research kit (**Figure 3.8A**). The DNA integrity of DNA extracted using the Qiagen kit was much better than the Zymo Research kit, even though both methods yielded similar amounts of DNA (**Figure 3.8**). The Zymo Research kit yielded much lower levels of DNA compared to the

phenol-chloroform based method but again was similar to that achieved using the Qiagen kit (**Figure 3.8A**). However, the DNA was highly degraded when extracted using the Zymo Research kit (**Figure 3.8B**).

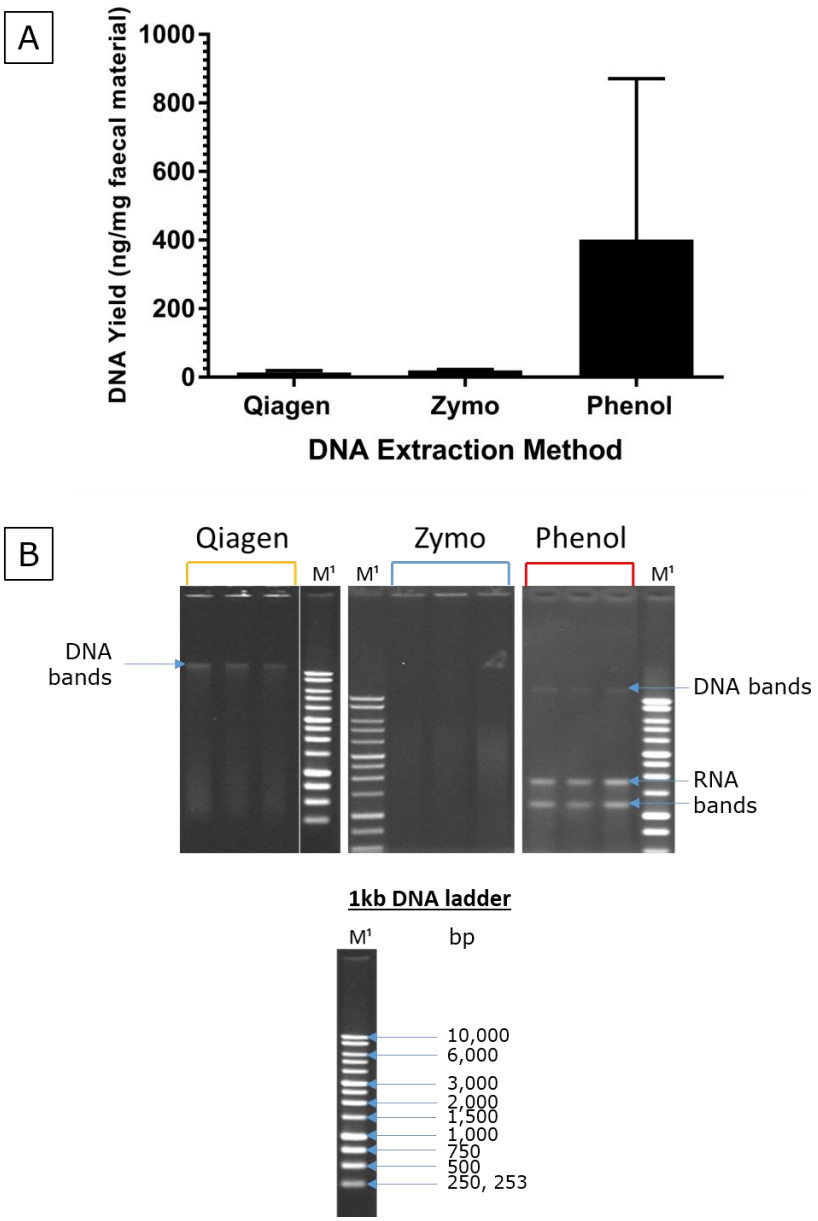


Figure 3.8 The effect of DNA extraction method employed for extracting DNA from chicken faeces on [A] DNA Yield (ng/mg faecal material) achieved and [B] the resulting DNA integrity as observed on a 1% agarose gel. M¹ - 1kb DNA ladder. n=3 experimental replicates per group, error bars are ± Standard Deviation. DNA and RNA bands on the agarose gel are indicated with arrows.

The DNA extracted from chicken faeces using the Qiagen kit, Zymo Research kit and the phenol-chloroform based method was then used for

chicken *CYTB* PCR to assess whether DNA extraction method impacted upon the ability to PCR the sample. Each DNA extraction method yielded the expected 160bp chicken *CYTB* amplicon (**Figure 3.9**). However, the Zymo Research kit yielded the 160bp amplicon with a significantly greater band intensity than the other 2 methods, suggesting that more chicken *CYTB* PCR amplicon may have been produced (**Figure 3.9** and **Table 3.5**). The Zymo Research kit resulted in a similar DNA yield to the Qiagen kit, but the DNA was more degraded with the PCR ability also improved (**Figure 3.8**, **Figure 3.9** and **Table 3.5**). The Zymo Research kit involves a bead-beating method and much of the literature around optimising DNA extraction methods from faeces involves bead-beating prior to the QIAamp® DNA Stool Mini Kit (Qiagen). Therefore, the decision was taken to assess a bead-beating step prior to the Qiagen kit to see whether the adaptation could improve resulting DNA.

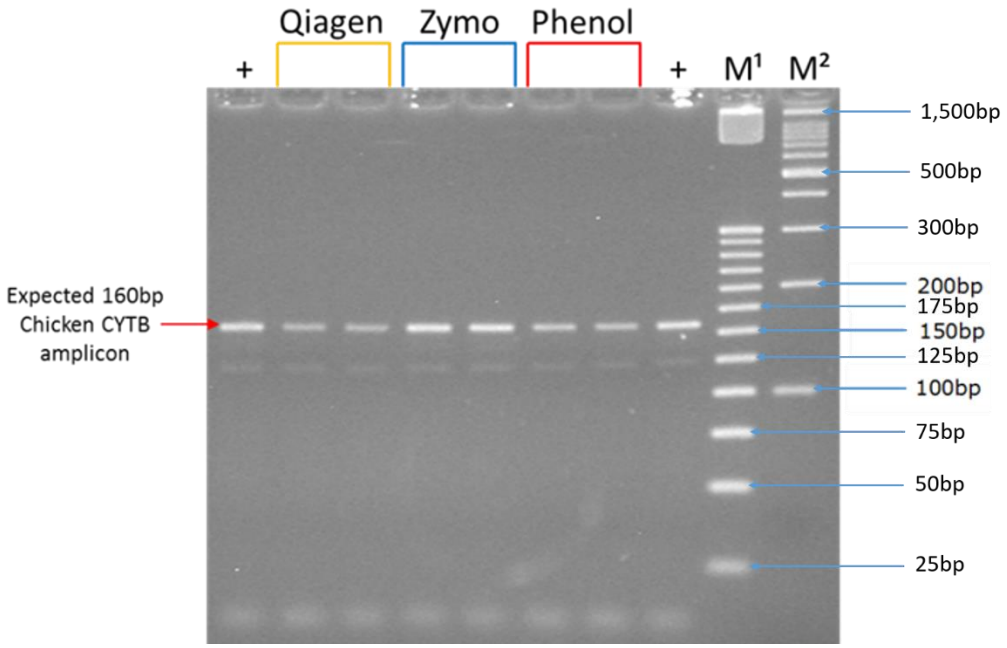


Figure 3.9 The effect of chicken faecal DNA extraction method on chicken CYTB PCR amplification. Labelled: + = positive control (chicken gDNA from chicken calf muscle), Qiagen – QIAamp® DNA Stool Mini Kit, Zymo – ZR Fecal DNA MiniPrep™, Phenol – phenol-chloroform based extraction, M¹ - 25bp DNA ladder and M² - 100bp DNA ladder. Expected chicken CYTB amplicon – 160bp, indicated by the red arrow. Replicates are experimental replicates.

Table 3.5 One-way ANOVA results analysing the effect of chicken faecal DNA extraction method on the PCR amplification of a 160bp chicken *cytochrome b* amplicon, compared to the positive control (chicken calf muscle DNA).

| DNA sample used for PCR | Average band intensity (Adj. volume) ± SD | SED | P value |
|---|---|---|---|
| Positive control | 1658.59 ± 48.84[a] | 102.5 | **0.002** |
| Qiagen extracted faecal | 871.21 ± 25.22[b] | | |
| Zymo extracted faecal | 1754.81 ± 18.02[a] | | |
| Phenol extracted faecal | 1046.28 ± 196.76[b] | | |

Means with different superscript letters in the same column differ significantly.
n=2 experimental replicates for all faecal methods and the positive control.
SD = standard deviation. SED = standard errors of the differences of the means.

*Effect of bead-beating modification on extracted chicken faecal DNA*

A single chicken faecal sample was tested in triplicate for each bead-beating method and the QIAamp® DNA Stool Mini Kit (Qiagen) alone. The commercial beads trialled were: M – MagNA Lyser Green Beads (Roche), P – PowerBead (MO BIO) and ZR – ZR BashingBead Lysis Tubes. Prior bead-beating using M and P beads resulted in similar DNA yields (ng/mg faecal material), which were significantly (P=0.003) greater than that achieved using ZR beads or the Qiagen kit alone (Q) (**Figure 3.10A**). However, there was less variability between the replicate extractions using the M beads compared to the P beads, as indicated by the error bars (**Figure 3.10A**). The Qiagen kit alone again yielded more intact DNA, whereas, all the other methods which involved bead-beating resulted in degraded DNA, similar to the results from the Zymo extracted DNA previously, where the kit includes a bead-beating step (**Figure 3.10B** and **Figure 3.8B**). However, the intensity of the DNA smears (indicative of sheared DNA), with the M and P beads were brighter than the Zymo and ZR methods, suggesting more sheared DNA present (**Figure 3.10B** and **Figure 3.8B**). DNA extracted using the Zymo Research kit was sheared but the PCR ability improved. As a result, it seemed appropriate to further investigate DNA extracted using a method involving the Qiagen kit with prior bead-beating, as the level of DNA shearing using these methods were greater and potentially the improvements in PCR could also be observed.

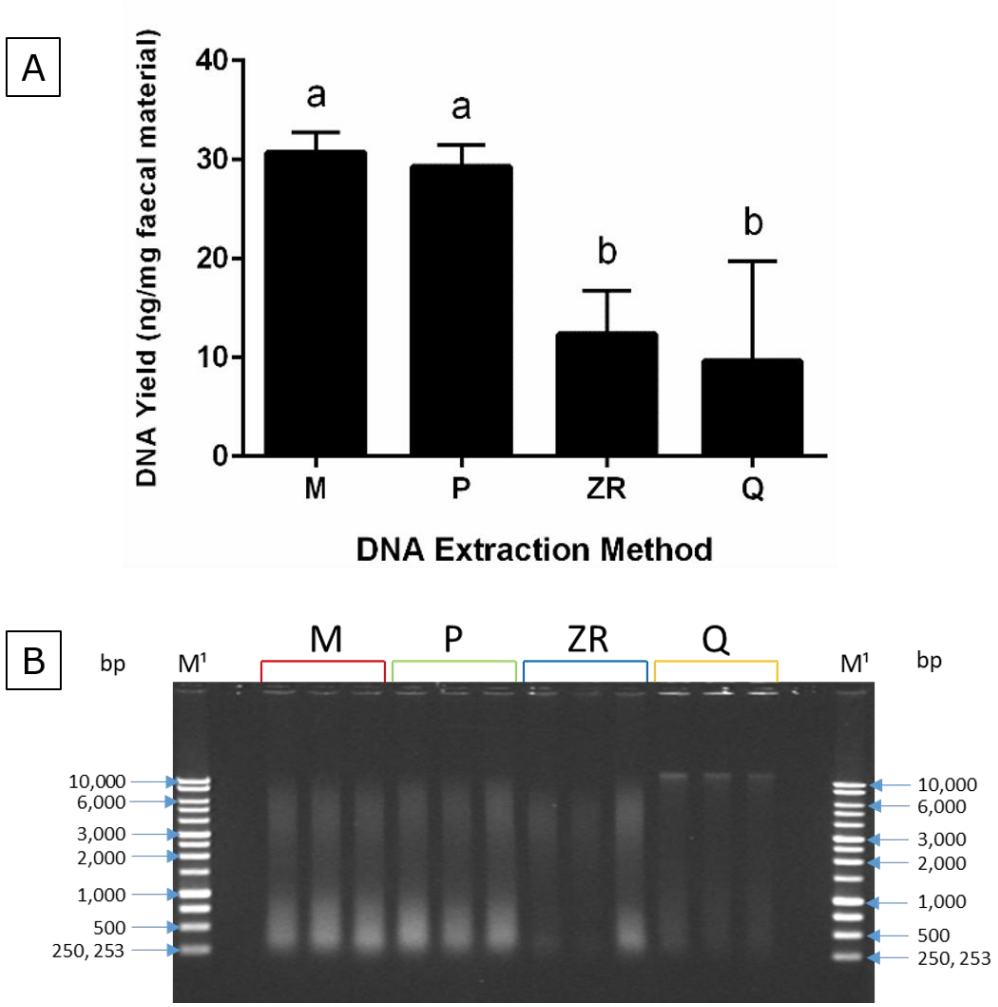


**Figure 3.10** The effect of 4 different DNA extraction methods from chicken faeces on [**A**] DNA Yield (ng/mg faecal material) and [**B**] DNA integrity achieved. Three methods involved bead-beating prior to the QIAamp DNA Stool Mini Kit (Qiagen). Beads used were: (M) – MagNA Lyser Green Beads (Roche), P – PowerBead (MO BIO), ZR – ZR Bashing Bead Lysis Tubes (Zymo Research). (Q) – QIAamp DNA Stool Mini Kit alone. DNA Yield: error bars are ± Standard Deviation, n=3 experimental replicates for each method and it is these replicates displayed on the agarose gel. M and P were significantly (P=0.003) different to ZR and Q as indicated by different letters on the graph. $M^1$ = 1kb DNA ladder.

In order to confirm which bead-beating with QIAamp® DNA Stool Mini Kit (Qiagen) DNA extraction method was optimal, chicken *CYTB* semi-quantitative PCR was carried out. This was to assess which DNA extraction protocol ensured successful and consistent amplification of the 160bp chicken *CYTB* PCR amplicon. Bead-beating with MagNA Lyser Green Beads followed by

the QIAamp® DNA Stool Mini Kit (Q + M beads) resulted in consistent amplification of the 160bp chicken *CYTB* PCR amplicon, as did the Q + P bead method (**Figure 3.11**). Amplification of DNA extracted using the Q + M beads method was more consistent compared with the Q + P beads method, as the band absorbance was more consistent between replicates (**Figure 3.11**); despite both extraction methods yielding significantly more DNA compared with other methodologies (**Figure 3.10A**). Band intensity of the 160bp amplicon was significantly greater with Q + M beads and Q + P beads compared to the alternative methods, suggesting more chicken *CYTB* DNA was amplified (**Table 3.6**). However, the standard deviation was less with Q + M beads compared to the Q + P beads, suggesting less variability with the former method (**Table 3.6**). Both the Q + ZR beads and the Q kit alone only resulted in amplification of the 160bp amplicon in 1 out of 3 replicates, indicating both of these methods are poor and should not be used for DNA extraction from chicken faeces (**Figure 3.11**). Based on these results, the best method for extracting high quality DNA from chicken faeces was bead-beating using MagNA Lyser Green Beads (Roche) followed by the QIAamp® DNA Stool Mini Kit (method outlined in Materials and Methods 2.4). With the Q + M beads method, the DNA yield (ng/mg of faecal material) was high with little variation between replicates of the same sample and the PCR ability was consistent.

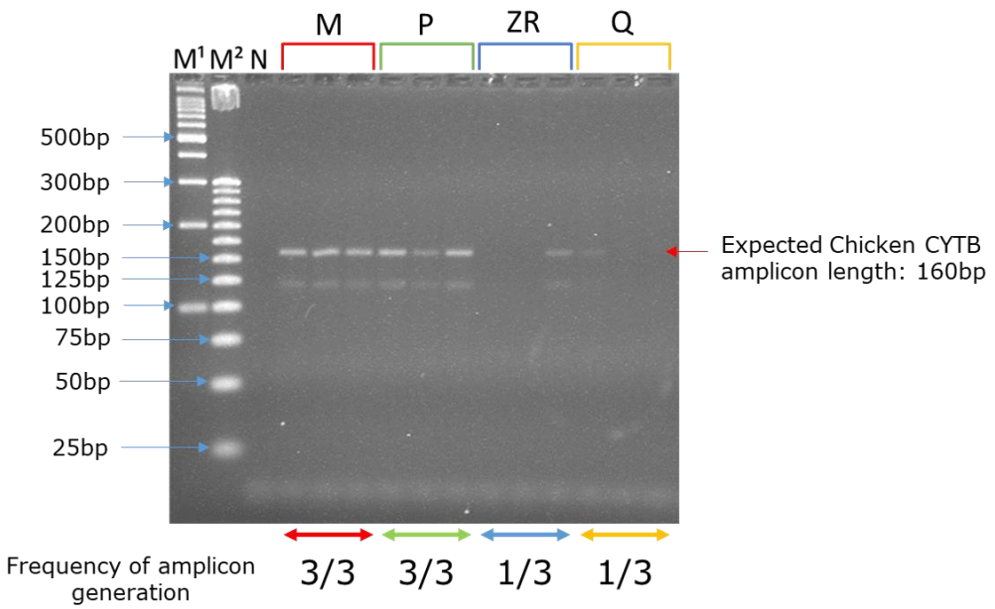


**Figure 3.11** The effects of 4 different DNA extraction methodologies on the success of chicken cytochrome (CYTB) semi-quantitative PCR. M¹ - 100bp DNA ladder, M² - 25bp DNA ladder, N – negative control, M – Bead-beating using MagNA Lyser Green Bead (Roche) + QIAamp DNA Stool Mini Kit, P – Bead-beating using PowerBead (MO BIO) tubes + QIAamp DNA Stool Mini Kit, ZR – Bead-beating using ZR BashingBead Lysis tubes + QIAamp DNA Stool Mini Kit, Q – QIAamp DNA Stool Mini Kit alone with no prior bead-beating. Predicted chicken CYTB amplicon length – 160bp, as indicated by the red arrow. Replicates are experimental replicates.

**Table 3.6** The effect of chicken faecal DNA extraction method on the average band intensity of the 160bp chicken *cytochrome b* (*CYTB*) amplicon. The greater the band intensity indicates more *CYTB* amplicon produced.

| DNA extraction method | Average band intensity of the *CYTB* amplicon (Adj. volume) ± SD | SED | P value |
|---|---|---|---|
| Q + M beads | 768.6 ± 77.7[a] | 166.1 | **0.003** |
| Q + P beads | 724.6 ± 347.7[a] | | |
| Q + ZR beads | 116.4 ± 186.1[b] | | |
| Q alone | 36.9 ± 63.8[b] | | |

Means with different superscript letters in the same column differ significantly.
n=3 experimental replicates for each method examined.
SD = standard deviation. SED = standard errors of the differences of the means.

### *3.5.1.4 Effect of species on DNA extractability and stability*

Extracting DNA from faeces of the two species posed problems, resulting in different methods being employed for the extraction from pig and chicken faeces. An additional experiment was carried out to try and elucidate a potential reason for this. As described previously pig and chicken faeces are very different in composition and consistency, with chicken faeces containing uric acid, which may impact upon extractability. In addition, as there was a difference in how long samples were stored for, but also importantly whether samples were transported prior to extraction, samples were incubated on ice for 3 hours to simulate transportation. Pig and chicken faeces were spiked with a known amount of calf thymus DNA and DNA was extracted either immediately or after 3 hours of incubation on ice in order to assess the extractability and stability of DNA from the two sources. The method used for the extractions was bead-beating with MagNA Lyser Green Beads (Roche) followed by the QIAamp® DNA Stool Mini Kit (Qiagen). There was an interaction (P=0.008) between species and incubation duration on % DNA yield from spiked faecal samples, **Figure 3.12**. The results indicate that there was less % DNA extracted from chicken faeces, but only pig faeces showed a decrease with the 3 hour incubation, see **Figure 3.12**. These results suggest that there is something in chicken faeces that inherently reduces DNA extractability, as when a known amount of DNA is added, significantly less is extracted compared with the amount extracted from pig faeces which were spiked with the same amount of DNA (**Figure 3.12**). The significant reduction in the amount of DNA extracted from pig faeces post incubation of samples on ice for 3 hours suggests DNA in pig faeces may be more susceptible to degradation during transportation, whereas chicken samples may be less so (**Figure 3.12**).

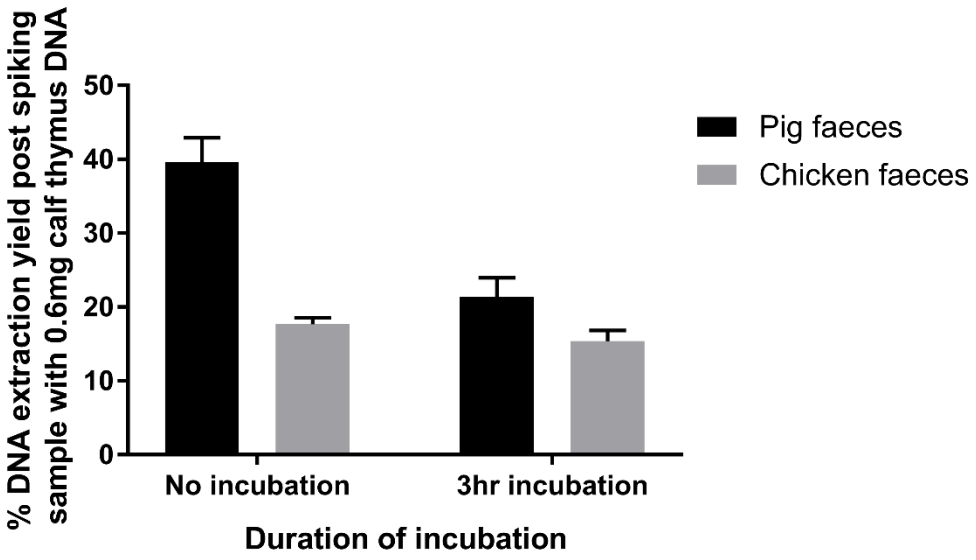


Figure 3.12 The effect of the species the faecal sample is collected from and whether the sample is extracted immediately or post 3 hours incubation on ice on the DNA yield achieved when samples are spiked with a known amount of calf thymus DNA. Mean values ± SEM, n=3 experimental replicates for all groups. P values for 2-way (species x incubation duration) ANOVA: interaction (P=0.008), species (P<0.001) and incubation duration (P=0.002).

### 3.5.2 Optimal *Taq* DNA polymerase in PCR

Following DNA extraction, conventional PCR of *actin* DNA was attempted using 4 commercial *Taq* DNA polymerases. The assessment was carried out on genomic DNA extracted from pigs' blood with the aim to assess which *Taq* enzyme was most appropriate and whether DNA extracted from pigs' blood would be an appropriate positive control for future analysis of DNA extracted from pig faeces. The 4 commercial *Taq* enzymes were GoTaq® G2 Hot Start DNA Polymerase (Promega), JumpStart™ DNA Polymerase (Sigma), AmpliTaq Gold™ DNA Polymerase (Roche) and FastStart™ DNA Polymerase (Roche). The results showed that AmpliTaq Gold™ DNA Polymerase (Roche) was the most suitable for detecting the 165bp *actin* amplicon, as the 3 remaining enzymes produced multiple bands with each enzyme producing a different banding pattern (**Figure 3.13**). Following on from this, we purchased the new AmpliTaq Gold™ DNA Polymerase, now being manufactured by

Applied Biosystems. Following a verification test to prove the new product was as good as the previous version, manufactured by Roche (data not shown), remaining PCR reactions utilised AmpliTaq Gold™ DNA Polymerase (Applied Biosystems). DNA from pigs' blood would also be used as a positive control for analysing pig DNA detection from faecal samples.

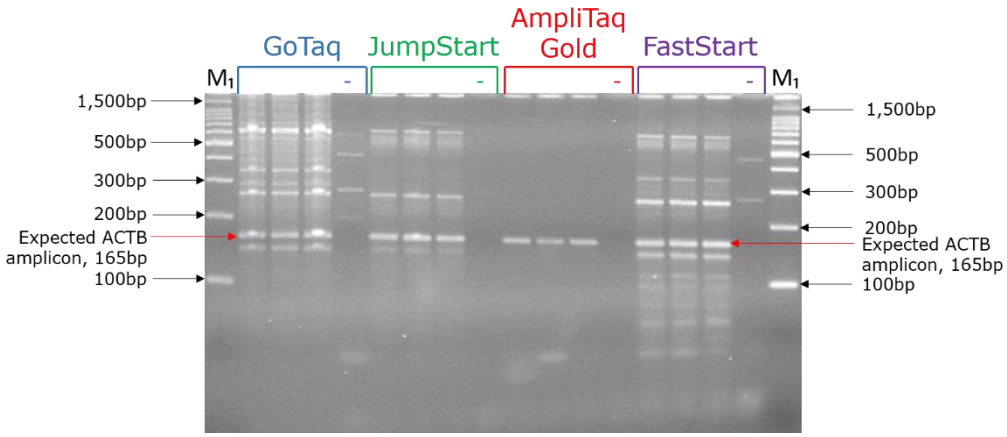


Figure 3.13 The assessment of 4 different *Taq* DNA Polymerases on the PCR amplification of pig actin (ACTB) from DNA extracted from pig's blood. The expected amplicon length was 165bp, as indicated by the red arrows. M1 = 100bp DNA ladder. Negative controls are labelled with the (-) symbol above the appropriate lane for each enzyme tested. Replicates are experimental replicates.

### 3.5.3 Assessment of the best gene target for host DNA detection

*Semi-quantitative PCR*

Semi-quantitative PCR was carried out on a 2-fold dilution series of pig faecal DNA of a known concentration with either pig *actin* or pig *cytochrome b* (*CYTB*) primers, see **Figure 3.14**. Typically, 25ng of DNA is loaded in the quantitative PCR reaction in this thesis. Therefore, the range in the amount of DNA loaded in this experiment was from 4-fold lower than 25ng, to as high as 4-fold higher, to examine the limits of detection of the 2 gene targets from faeces (**Figure 3.14**). The results illustrated that *CYTB* can be accurately detected independent of faecal DNA concentration; however, *actin* DNA detection decreased with decreasing DNA template in the PCR reaction (**Figure 3.14**). Also, the band intensity for *CYTB* did not differ greatly between the faecal

samples and positive control as well as being much greater than the *actin* bands (**Figure 3.14**). This suggests that *CYTB* is the better gene for detecting pig DNA in the pig faecal samples. Each of the 3 primer sets for pig *CYTB* DNA detection yielded an amplicon of the expected length (bp) (data not shown), but the set which yielded the 150bp amplicon was chosen for all future experiments involving pig faecal DNA.

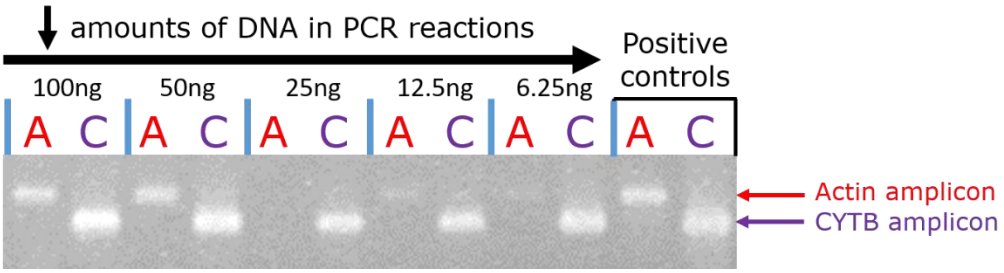


Figure 3.14 Assessment of the detection limits of PCR from pig faecal DNA using primers for actin or cytochrome b. A = *actin*, C = *cytochrome b* (*CYTB*), Positive controls = DNA from pig's blood used in the PCR reaction.

*Sanger sequencing results*

Amplicons produced following PCR amplification of faecal DNA extracted from pig and chicken faeces were verified using Sanger Sequencing and compared with predicted sequences identified in primer design. The predicted amplicon for pig *actin* was 165bp in length and the predicted amplicon for pig *cytochrome b* was 150bp in length. DNA was extracted from pig faecal samples collected from pigs on 2 different diets (a control diet and a xylanase supplemented diet) and amplified during 40 PCR cycles using pig *actin* or pig *cytochrome b* primers. DNA extracted from pig's blood was also amplified using the same primers for comparison with the faecal DNA amplicons and the predicted sequence. The bases for the *actin* amplicon from pig faecal DNA could not be called by Sanger Sequencing (data not shown), further demonstrating *actin* was not a good gene target for the analysis of host DNA in pig faeces. Alternatively, the *cytochrome b* amplicons produced from pig faecal DNA matched the sequences produced from the positive control tested originating

from pig's blood, as well as the predicted 150bp amplicon sequence, see **Figure 3.15**.

Chicken faecal DNA was only tested with *cytochrome b* primers. The predicted amplicon sequence for chicken *cytochrome b* was 160bp. Chicken faecal DNA was amplified with chicken *cytochrome b* primers and the amplicon produced was sequenced and compared with the predicted sequence. The chicken *cytochrome b* amplicon from the chicken faecal DNA matched the predicted sequence, see **Figure 3.16**, illustrating that *cytochrome b* is also a suitable target gene for the analysis of host DNA in chicken faeces as well as pig faeces.

```
CLUSTAL O(1.2.1) multiple sequence alignment

xylanase        TTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTNAGGAGCTACGGTCATCACA
cytb150         TTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACA
positive        TTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACA
control         TTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGAGGANCTACGGTCATCACA
                ************************************** **** **************

xylanase        AATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGGGGC
cytb150         AATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGGGGC
positive        AATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGGGGC
control         AATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGGGGC
                ************************************************************

xylanase        TTTTCCGTCGACAAAGCAACCCTCACACGA
cytb150         TTTTCCGTCGACAAAGCAACCCTCACACGA
positive        TTTTCCGTCGACAAAGCAACCCTCACACGA
control         TTTTCCGTCGACAAAGCAACCCTCACACGA
                ******************************
```

Figure 3.15 Alignment of pig *cytochrome b* PCR amplicon sequences identified using Sanger Sequencing. Labelled: cytb150 – predicted 150bp amplicon sequence, positive – amplicon produced from PCR using DNA from pig's blood, control – amplicon produced from pig faecal DNA from pigs on a control diet, xylanase – amplicon produced from pig faecal DNA from pigs on a xylanase supplemented diet. (*) indicates where sequences are the same.

```
CLUSTAL O(1.2.4) multiple sequence alignment

predicted       ACTCATAGCCACCGCCTTTGTGGGCTATGTTCTCCCATGGGGCCAAATATCATTCTGAGG
sequenced       ACTCATAGCCACCGCCTTTGTGGGCTATGTTCTCCCATGGGGCCAAATATCATTCTGAGG
                ************************************************************

predicted       GGCCACCGTTATCACAAACCTATTCTCAGCAATTCCCTACATTGGACACACCCTAGTAGA
sequenced       GGCCACCGTTATCACAAACCTATTCTCAGCAATTCCCTACATTGGACACACCCTAGTAGA
                ************************************************************

predicted       GTGAGCCTGAGGGGGATTTTCAGTCGACAACCCAACCCTT
sequenced       GTGAGCCTGAGGGGGATTTTCAGTCGACAACCCAACCNNN
                *************************************
```

Figure 3.16 Alignment of chicken *cytochrome b* PCR amplicon sequences identified using Sanger Sequencing. Labelled: predicted – predicted 160bp amplicon sequence, sequenced – amplicon produced from chicken faecal DNA. (*) indicates where sequences are the same.

*Comparison of detection in quantitative PCR*

A 'crossing point value' is the PCR cycle number where sufficient SYBR Green fluorescent marker is bound to enough double stranded DNA to reach the detection threshold of the PCR instrument. The earlier the cycle number

where the detection threshold is exceeded, the greater the amount of gene target DNA there was in the sample to begin with. Quantitative PCR was carried out on pig faecal DNA using primers for bacterial *16S* DNA, pig *cytochrome b* DNA and *actin* DNA. As expected, bacteria was present in high quantities in the pig faeces with the mean cycle number being 15, see **Figure 3.17**. Additionally, pig *cytochrome b* was also present in relatively high quantities with the mean cycle number being 22 compared with the *actin* amplicon being detected at greater than 35 PCR cycles (**Figure 3.17**). Beyond 35 PCR cycles it is thought that the PCR instrument is unable to accurately detect an amplicon and any signal may be due to primer dimers, where primers bind to each other. Therefore, pig *cytochrome b* was again the better target gene, compared with *actin,* for detecting host DNA in pig faeces.

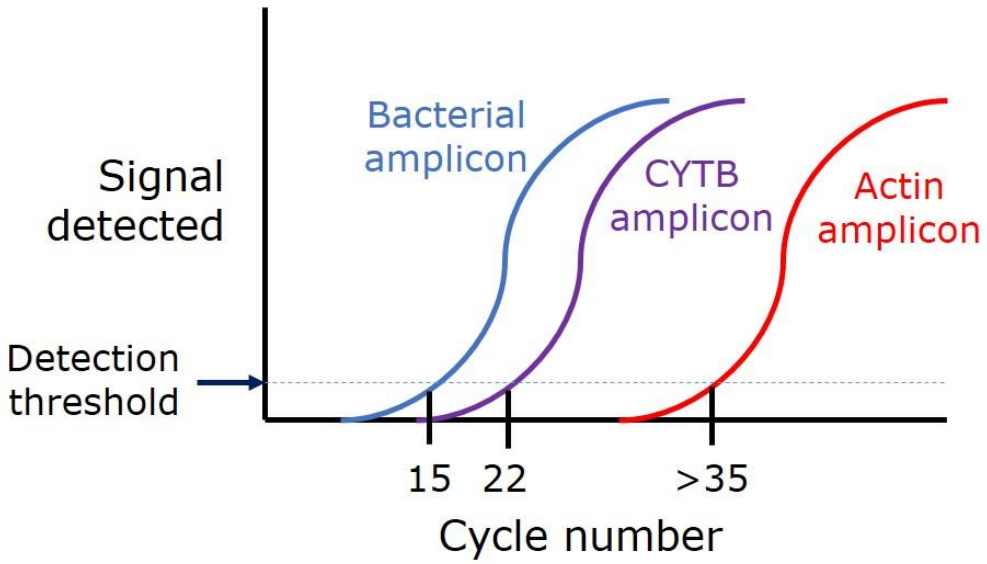


Figure 3.17 Comparison of the PCR cycle numbers where a signal was first detected from pig faecal DNA using bacterial, pig *cytochrome b* and human *actin* primers.

### 3.5.4 Bacterial DNA detection verification

The bacterial *16S* DNA primers were universal primers designed to detect a common sequence amongst a number of different bacteria (Mieszkin *et al.*, 2009). As a result, there was no predicted sequence to compare Sanger Sequencing results to. Equally, it was difficult for the instrument to call the bases of the amplicon, likely due to the fact there were numerous PCR products

produced which were 142bp in length. Therefore, smaller portions of the amplicon sequence, that had been reported, were put through nucleotide BLAST analysis. All predicted sequences were bacterial ribosomal DNA sequences (data not shown), and therefore the universal primers were deemed suitable for the amplification of bacterial DNA from faeces in this study.

### 3.5.5 Pig Calprotectin ELISA optimisation

The standard curve for the Pig Calprotectin (CALP) ELISA Kit (Cusabio) was produced using a 2-fold dilution series of the Standard provided as indicated in the manufacturer's instructions (**Figure 3.18**). The standard curve was linear between optical densities of approximately 0.4 and 1.2, see **Figure 3.18**. To use this standard curve to analyse the concentrations of CALP (ng/ml) in samples, the optical density results for unknowns should fall within the linear range, the mid-point being 0.8, see **Figure 3.18**. Use of the kit for pig faecal samples needed optimisation, therefore, supernatant from faecal samples of pigs on 2 dietary extremes were diluted over a range and tested to see which dilution should be used for future experiments. Using the results from the standard curve (**Figure 3.18**) and the faecal supernatant dilution optimisation (**Table 3.7**), the 1:5 dilution was chosen as the dilution to use for future experiments assessing the levels of CALP in pig faeces.

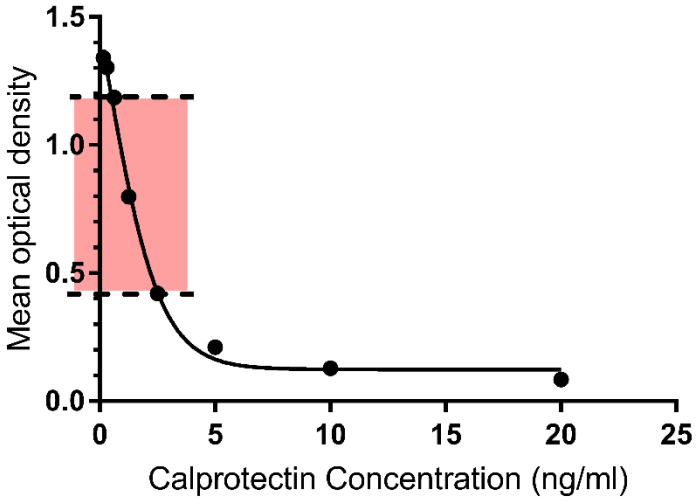


Figure 3.18 Standard curve from the Pig Calprotectin (Cusabio) ELISA Kit. Shaded area indicates the range where the curve is linear.

Table 3.7 Optimisation of which faecal supernatant dilution to use in the Pig Calprotectin ELISA Kit (Cusabio).

| | Mean optical density ± standard deviation | |
|---|---|---|
| **Dilution** | **Faeces from control pigs** | **Faeces from 7.5% YPC pigs** |
| 1:5 | 0.684 ± 0.199 | 0.760 ± 0.138 |
| 1:10 | 0.853 ± 0.109 | 1.153 ± 0.116 |
| 1:50 | 1.097 ± 0.140 | 1.168 ± 0.357 |
| 1:100 | 1.212 ± 0.301 | 1.152 ± 0.142 |
| 1:500 | 1.112 ± 0.199 | 1.271 ± 0.233 |

YPC = yeast-enriched protein concentrate supplemented.

## 3.6 DISCUSSION

### 3.6.1 DNA extraction procedures

*Extractability and stability*

As predicted, different DNA extraction procedures needed to be optimised for both pig and chicken faeces. It has previously been observed by other researchers that the species of interest can impact upon the chosen DNA extraction protocol to be used (Hart *et al.*, 2015). The crude extraction results in this study indicated that there was more DNA present in the single pig faecal sample compared with a chicken faecal sample, but this may also be due to differences in extractability. Additionally, samples are extracted on a frozen weight basis without prior drying, therefore drying samples prior to extraction may resolve this difference. However, although DNA may not be as prone to degradation as RNA, care must be taken if any drying technique was to be employed as the spiking experiments in this study illustrated that even incubating samples on ice for 3 hours can lead to a significant decrease in the recovery of DNA from pig faeces (but not chicken faeces). Maksimov *et al.* (2017) highlighted the difficulties of giving a definitive limit of detection in spiking experiments due to freeze thawing of samples and the potential release of additional DNA from the samples. Equally in our studies there would have already been an unknown amount of DNA present in the faecal sample and so the crude extraction and spiking results in this chapter should be taken with caution. Nevertheless, the results are interesting and suggest that there may be something unique about chicken faeces that reduces the DNA extractability compared with pig faeces, this may be uric acid content. In addition, it appeared that DNA in pig faeces may be more susceptible to degradation, even where samples may be on ice for several hours such during transportation. Taken together the results do illustrate that further research around determining the appropriate storage technique, and transportation method, for faecal samples are required and whether samples should be dried prior to processing. A study examined using commercial products for the preservation of human faeces including RNAlater™ (Ambion), Paxgene™ (PreAnalytiX), Whatman FTA cards

(Whatman) and drying over silica gel, as well as traditional preservation techniques such as refrigeration or immediate freezing in liquid nitrogen, and found RNAlater™ was the best preservative when used with the QIAamp® DNA Stool Mini Kit, as PCR amplification was also examined alongside DNA yield (Nechvatal *et al.*, 2008). Conversely, another study found that RNAlater significantly reduced DNA yield and decided that freezing within 15 minutes of defecation was a suitable storage method, as long as samples were processed within 3 days and homogenised prior to extraction (Gorzelak *et al.*, 2015). Future experiments should investigate the appropriate storage technique for pig and chicken faeces, combined with the DNA extraction procedure, to enable the potential for samples to be collected on farm and sent to the laboratory for analysis.

*DNA extraction from pig faeces*

A number of studies have assessed a selection of commercial kits and identified the QIAamp® DNA Stool Mini Kit (Qiagen) as the best commercial protocol for DNA extraction from faeces (Li *et al.*, 2003; Maksimov *et al.*, 2017; McOrist *et al.*, 2002; Nechvatal *et al.*, 2008). However, it is important to recognise most comparative studies are assessing the faecal microbiome rather than host faecal DNA content, therefore, it was important to optimise the extraction procedures for the purpose of analysing faecal DNA as a marker of gut cell loss in pig and chicken faeces. Our results showed that the modified phenol-chloroform extraction method resulted in much higher DNA yields (ng/mg of faecal material) for extracting DNA from pig faeces than the Qiagen Kit or a modified method, but there appeared to be no difference in DNA quality (260nm/280nm values). Similarly, a study comparing extraction methods for the effective examination of *16S* ribosomal DNA in pig faeces devised a method which didn't involve expensive reagents like Proteinase K and equally avoided the use of phenol and found that this method resulted in higher DNA yields that the QIAamp® DNA Stool Mini Kit protocol (Tang *et al.*, 2008). Alternatively, another study experienced problems with both phenol-chloroform extraction and the QIAamp® DNA Stool Mini Kit and determined that the FAST DNA Spin

Kit for Soil by MP Biomedicals, which included beat-beating, was the optimal method out of 6 methods attempted to analyse *Cryptosporidium* oocysts in water concentrates (Jiang *et al.*, 2005). Taken together, this information confirms that DNA extraction procedures need to be optimised for the required samples to be analysed.

Phenol is used in DNA extraction because DNA does not dissolve in phenol and therefore stays in the aqueous phase, whereas, the proteins and other compounds in faeces will dissolve in the presence of phenol and settle in the organic phase enabling effective separation. Commercial kits on the other hand employ chaotropic salts in solutions known as 'lysis buffers', these solutions disrupt cell membranes and the salts cause the DNA to become hydrophobic and bind to a solid matrix, such as silica columns (Altayari, 2016). Other non-DNA compounds in the sample are then removed via washing steps, followed by elution of the DNA (Altayari, 2016). Therefore, the modified phenol-chloroform method used in this study may have resulted in increased DNA yield from the pig faeces as a commercial lysis buffer (Promega) was employed prior to the standard phenol-chloroform extraction, so maybe any un-degraded cells were broken down leading to a greater release of DNA for purification. In addition, the modified phenol-chloroform based method did not result in preferential extraction of bacterial *16S* DNA, unlike the devised method involving bead-beating of the faeces in the presence of lysis buffers followed by the QIAamp® DNA Stool Mini Kit. In this study the modified phenol-chloroform method resulted in the most reproducible results so was chosen as the best method to extract DNA from pig faeces to analyse faecal host DNA as a marker of gut cell loss in pigs. Although there are safety concerns of using phenol, if it is handled safely with the correct personal protective equipment, there are no issues with its use.

*DNA extraction from chicken faeces*

Although a number of studies suggest the QIAamp® DNA Stool Mini Kit (Qiagen) to be the most effective for DNA extraction from a range of sources, other papers found other kits to be better, including the FastDNA® SPIN Kit for

Soil (MP Biomedicals) (Ariefdjohan *et al.*, 2010; Burbach *et al.*, 2016; Ferrand *et al.*, 2014; Jiang *et al.*, 2005) and the ZR Fecal DNA MiniPrep™ Kit (MO BIO) (Claassen *et al.*, 2013; Maksimov *et al.*, 2017), which both involve a bead-beating step. But many papers have also utilised a bead-beating step prior to the QIAamp® DNA Stool Mini Kit and this improved DNA yields and PCR quantification (Grześkowiak *et al.*, 2016; Li *et al.*, 2007; Smith *et al.*, 2011). Bead-beating is thought to lead to a greater DNA yield due to the mechanical lysis of cells rather than just using chemical or enzymatic methods alone.

In our study we examined 6 methods for extracting DNA from chicken faeces and found that bead-beating using MagNA Lyser Green Bead Tubes (Roche) in the presence of lysis buffer followed by the QIAamp Kit was the best method for extracting DNA from chicken faecal samples, suitable for PCR. Other methods were comparable such as the Zymo Research kit, but the modified phenol-chloroform method used for pig faecal samples did not work with the chicken faecal samples. Although we were still able to amplify the gene target, the measurements for DNA concentration were inaccurate, meaning normalising the DNA for PCR would not have been accurate and could lead to biases. Phenol extraction can lead to co-extraction of DNA with RNA and assessments of DNA integrity in our study indicated RNA contamination, which is in agreement with that of Yu and Morrison (2004) who also observed RNA bands when examining DNA integrity of phenol-extracted DNA. The chicken faecal samples also contained sawdust bedding, and in some cases feathers. The poor DNA results achieved when employing phenol-chloroform based extraction for chicken faecal DNA may be due to the sawdust present in the samples (Jadhav and Vanjara, 2004). A study examining bacterial DNA using PCR methodology from chicken faeces, contaminated with litter, which had been extracted using phenol were unable to amplify the gene target when the faeces were contaminated with wood-shaving bedding (Cisar *et al.*, 2010). Phenol binds to sawdust and this process potentially interferes with the DNA extraction process, leading to contaminants and carry-over of substances into the eluted DNA as observed using the NanoDrop™ software (Jadhav and Vanjara, 2004).

Phenol extraction is also pH dependent. As chicken excreta is a mixture of waste from the digestive system and uric acid, the pH is likely to cause problems. Future experiments should measure pH of collected pig and chicken faeces at the time of collection and prior to processing, as well as potentially aim to alter the pH in a titration type experiment to analyse the full extent of pH on phenol-chloroform extraction.

As mentioned previously, in agreement with other studies, a method involving bead-beating for chicken faeces in the presence of lysis buffer prior to the QIAamp® DNA Stool Mini Kit was the optimal method in this study. Smith *et al.* (2011) examined 2 different bead-beating methods prior to the QIAamp kit, and the kit alone, and also found pre-treatment to improve DNA yields. Out of the 3 bead tubes tested in the present study, the MagNA Lyser Green Bead Tubes resulted in consistent amplification of the chicken *CYTB* gene target with the least variation in band absorbance across replicates. The fact that different methods are being adopted for different species corresponds with the results from Hart *et al.* (2015), which examined 5 extraction methods for faeces from 5 species and showed that faecal DNA extraction methods need to be matched to the host species as different species have different DNA extraction and PCR inhibitors present in their faeces.

### 3.6.2 Polymerase chain reaction

*DNA polymerase*

One study created inhibitor pools using common inhibitors found in a range of substances such as humic acid, hematin and collagen, to attempt to identify the mechanisms by which inhibitors affect the polymerase chain reaction (Opel *et al.*, 2010). Mechanisms hypothesised included inhibitor binding to the DNA to reduce the amount of template available in the reaction, inhibitor binding to the polymerase itself or interfering with the DNA during the extension phase of PCR (Opel *et al.*, 2010). Other studies have assessed the ability of different DNA polymerase enzymes to amplify DNA via PCR from varying starting samples, with or without PCR enhancers/facilitators (Abu Al-

Soud and Rådström, 1998; Abu Al-Soud and Rådström, 2000). In the present study 4 DNA polymerases originating from the *Thermus aquaticus* bacterium (*Taq* enzymes) were examined. The AmpliTaq Gold™ DNA polymerase was clearly identified as the best polymerase tested. Previous studies which have used increasing levels of faeces in PCR as a way of manipulating inhibitors levels, suggested *Taq* DNA polymerases to be less successful at PCR amplification compared with enzymes originating from a different bacterium (Abu Al-Soud and Rådström, 2000). This may explain why the other 3 *Taq* polymerases tested in this study resulted in poor results, but, in this study all the enzymes led to successful amplification of the 165bp *actin* amplicon, the problem was a lack of specificity so potentially any inhibitors were affecting the extension phase of the amplification reaction. In the study by Abu Al-Soud and Rådström (2000), with the addition of bovine serum albumin (BSA) both *Taq* and *rTth* polymerases enabled amplification in the presence of 4% faeces as opposed to 0.4% in the PCR reaction. Although AmpliTaq Gold™ (Applied Biosystems), a *Taq* polymerase, worked very well in our study without the need for PCR facilitators, future optimisation experiments could potentially assess whether facilitators such as BSA might further maximise amplification by protecting the *Taq* from inhibitors present in faeces. Inhibitors present in faeces may bind to the BSA substrate rather than interfere with the polymerase (Kreader, 1996).

Another study assessed the suitability of different DNA polymerases in the presence of buffer saturated with phenol, the results found that *Tth* DNA polymerase was unaffected by buffer containing phenol, whereas, *Taq* DNA polymerase was inactive (Katcher and Schwartz, 1994). Conversely, our results show that DNA extracted utilising phenol can be amplified using *Taq* enzymes, as AmpliTaq Gold™ also originates from *Thermus aquaticus* and was the most successful polymerase trialled in these experiments. The difference in the results observed could be due to the level of phenol carry over from the purification process, as it is assumed in our study that there was not a significant carry-over of phenol from the extraction process into the PCR.

*Detection targets*

*Actin* and *cytochrome b* were chosen as target genes for the detection of pig DNA in pig faecal samples due to their high copy number in the genome and number of copies per cell respectively (Nygard *et al.*, 2007; Andréasson *et al.*, 2002). *Actin* did not prove an appropriate gene for detection of pig DNA in faeces. Potentially the animal DNA is too fragmented by endonucleases present in the gastrointestinal tract for *actin* detection by short primers in PCR, this degradation poses a problem in other experiments such as drug delivery (Borges *et al.*, 2005; Loretz *et al.*, 2006). *Cytochrome b* is in the mitochondrial genome and was found to be the optimal gene for PCR amplification from faeces in our study, as it was successfully detected even when very low levels of template DNA were included in the PCR. These findings correspond to other studies where mitochondrial genes have been appropriate targets in PCR, including meat authentication (Matsunaga *et al.*, 1999) and studies of water contamination (Caldwell and Levine, 2009), and is likely due to mitochondrial DNA being robust and potentially better protected from endonucleases (Rodríguez *et al.*, 2004). Another factor increasing the usefulness of mitochondrial genes for detection in PCR is that sections of mitochondrial DNA are conserved across species with only slight variations, enabling species-specific primers to be designed (Gerber *et al.*, 2001).

### 3.6.3 Calprotectin ELISA for faeces

In this chapter we optimised the Cusabio Pig Calprotectin ELISA Kit for use with faeces as there was not already a protocol in place and the manufacturer advises to optimise protocols for use with samples not already specified in their instructions. As tissues, serum or plasma were the only starting samples with a protocol supplied by the manufacturer we utilised previous work by Lallès and Fagerhol (2005) as a starting point for faecal dilutions to test for samples originating from weaner pigs. We established 1:5 as the optimal dilution of faeces to prepare pig faecal samples prior to loading

the 96-well plates for the assay. For future analysis of calprotectin in pig faeces the devised protocol for faecal sample preparation will be followed.

### 3.6.4 Summary

- Crude extraction results illustrated significantly more DNA was extracted from pig faeces, compared with chicken faeces.
- A modified phenol-chloroform DNA extraction method was optimal for extracting DNA from pig faeces.
- Bead-beating chicken faeces with Buffer ASL (Qiagen) in MagNA Lyser Green Bead Tubes (Roche), followed by the QIAamp® DNA Stool Mini Kit (Qiagen) was the best method for extracting DNA from chicken faeces.
- When spiking both pig and chicken faecal samples with a known amount of DNA, there was significantly greater recovery of DNA from the pig faeces compared with the chicken faecal samples. Conversely, there was a significant decrease in the recovery of spiked DNA from pig faeces following a 3-hour incubation, but no effect of incubation on the recovery of DNA from spiked chicken faeces.
- The optimal DNA polymerase enzyme tested in semi-quantitative PCR was AmpliTaq Gold™ DNA Polymerase (Applied Biosystems).
- *Cytochrome b* was identified as the better target gene for the analysis of host DNA in faeces, compared with *actin*.
- The optimal dilution of pig faeces with diluent for analysis using the Pig Calprotectin ELISA Kit (Cusabio) was 1:5.

### 3.6.5 Conclusion

In this chapter, methodologies for the extraction of DNA from pig and chicken faeces, as well as the PCR protocol for examining host and bacterial DNA content in the faeces have been optimised. Also, a protocol for using the Pig Calprotectin ELISA Kit by Cusabio for assessing the levels of pig calprotectin in pig faeces was also optimised. The methodologies developed in this chapter will be used to analyse faeces from pig and chicken feeding studies where

differences in gut cell sloughing are expected between experimental diets. Any differences should be measurable by detecting host *CYTB* DNA levels in the faeces and potentially normalising this to total bacterial DNA content.

# CHAPTER 4. PIG TRIALS

## 4.1 XYLANASE TRIAL

### 4.1.1 Introduction

As the methods for DNA extraction and PCR amplification of both pig DNA and bacterial DNA from pig faeces had been optimised (see Chapter 3) the overall aim of this chapter was to apply the developed methodology to pig trials where pigs would receive diets thought to impact upon gut cell loss, which could potentially be measured.

Non-starch polysaccharides (NSPs) present in feed ingredients increase digesta viscosity and therefore limit nutrient availability (Zhang *et al.*, 2018) Xylanase is a feed enzyme added to pig and poultry diets designed to hydrolyse NSPs, enabling the animal to access and utilise more of the nutrients (Zhang *et al.*, 2018). Feed to gain ratio is often reduced in animals fed a diet supplemented with xylanase, and although there is greater nutrient availability, the exact mechanism is yet to be fully understood, particularly with regards to gut health (Zijlstra *et al.*, 2010). Growing pigs supplemented with a mixture of non-starch polysaccharide degrading enzymes including xylanase had significantly improved growth performance, along with significant upregulation of proteins associated with nutrient metabolism and immunity, and a down-regulation of proteins associated with transcription and translation in the intestinal mucosa (Zhang *et al.*, 2017). A decrease in protein synthesis associated with the gut mucosa leads to greater energy availability to the animal for growth, as previously discussed.

Equally as described in Chapter 1, crypt cell proliferation in the intestine is associated with increased epithelial cell migration, likely leading to increased gut cell sloughing. Broilers supplemented with either 10g/kg or 30g/kg of xylanase on rye-based diets had significantly reduced crypt cell proliferation compared to the control group, with no differences observed between the two inclusion levels (Silva and Smithard, 2002). However, the effect on crypt cell proliferation was not significant when xylanase was included at the commercial

level of 1g/kg (Silva and Smithard, 2002). Similarly, broilers fed a wheat based diet supplemented with xylanase at 500, 1000 and 5000 units per kg had significantly ($P<0.05$) reduced feed:gain ratio compared to birds which received the control diet, as well as, having significantly improved villus height and the ratio of villus height to crypt depth (Luo *et al.*, 2009). Changes in gut morphology such as increased crypt cell proliferation and reduced villus height could lead to a greater replacement of epithelial cells. These degraded cells would be excreted into the faeces and could potentially be assessed using the faecal DNA assay. As a result, faecal samples were collected from a xylanase inclusion trial of newly weaned piglets which formed part of Katherine May's PhD thesis and faecal DNA was extracted and composition analysed.

### 4.1.2 Hypotheses

1. There will be no effects of dietary treatment on the total faecal DNA yield and quality.
2. Pigs consuming diets containing a xylanase inclusion diet will have a reduced level of host DNA in their faeces.
3. The levels of total bacterial DNA content will be unaffected by the dietary treatment.

### 4.1.3 Objectives

1. Assess the effects of a xylanase inclusion diet and duration of supplementation post weaning on total faecal DNA yield and quality.
2. Assess the effects of a xylanase inclusion diet and duration of supplementation post weaning on faecal DNA composition (host DNA and bacterial DNA).

### 4.1.4 Materials and Methods

#### *4.1.4.1 Animal trial and sample collection*

A pig xylanase growth trial was carried out by Katherine May as part of her PhD research project (May, 2017). Sixteen female Camb12 type (Landrace x Duroc x Large White genotype) newly weaned piglets (8.8±1.38 kg) were

randomly assigned to either a control (n=8) or treatment group (n=8). The control group was subjected to a standard pig feeding programme, with pigs receiving feed containing 24.3% protein and 16.2MJ/kg digestible energy (DE), during days 0-13 of the trial, followed by 22.7% protein and 15.2MJ/kg DE during days 14-27 and then 22.5% protein and 14.4MJ/kg DE for days 28-42. The treatment group received the same feed over the same time period, but with a commercial preparation of xylanase (Econase XT 25, AB Vista, Marlborough, UK) included at 26000, 18600 and 17800 birch xylan units (BXU)/kg, at days 0-13, 14-27 and 28-42 respectively. All pigs were given *ad libitum* access to feed and water over the whole trial. Excreted faecal samples from individual animals were collected at the end of week 2, 4 and 6 of the trial from the pen floor. Animals were weighed at these time points, equally these time points were when the diets the pigs were receiving changed in line with the standard commercial feeding programme (starter, grower, finisher), and so it seemed appropriate to collect faecal samples at these time points. Firstly, from a logistical point of view, but also to try and correspond any differences observed in faecal DNA measurements to the animal performance of the animals. Collected faecal samples were frozen and stored at -20˚C until processed.

#### *4.1.4.2 DNA extraction*

DNA was extracted from 30mg of frozen pig faeces utilising a phenol-chloroform based method as outlined in Materials and Methods 2.3. DNA concentration and quality were then assessed as outlined in Materials and Methods 2.5 and 2.6, and then samples were stored at -20°C until processed. DNA yield was calculated and expressed as µg of DNA per mg of faeces for DNA extracted from the xylanase trial.

#### *4.1.4.3 Quantitative PCR*

To ensure all the faecal DNA samples from the xylanase trial could be amplified by qPCR, each faecal sample was diluted to 20ng/µl and tested singularly using bacterial *16S* primers (Mieszkin *et al.*, 2009). A 5µl volume of each diluted sample was tested using the bacterial *16S* primers with the

protocol outlined in Materials and Methods 2.10. As each faecal DNA sample was successfully amplified a 5µl aliquot of each faecal sample (diluted to 20ng/µl) was then used to create a faecal DNA pool, which was then used to create a 1 in 2 standard curve for qPCR analysis of the faecal DNA. Faecal DNA samples (diluted to 20ng/µl) were then further diluted 1 in 8 onto a 96-well plate. Ten microliters of mastermix (see Materials and Methods 2.10) was loaded onto the plate using a multi-dispense pipette, and then 5µl of each faecal sample transferred from the 96-well plate into the appropriate well on the 384-well plate. Each faecal sample was tested in triplicate against the standard curve. The negative control used was 5µl of water (RNase and DNase free) (Sigma) tested in triplicate at multiple points on the plate. The faecal samples were tested using pig *CYTB* DNA and bacterial *16S* DNA primers, see Materials and Methods 2.7 and 2.10. Quantitative analysis of the cycle where the fluorescent signal exceeds the threshold (crossing point, Cp values) and melt curve analyses were then carried out in LightCycler® 480 (version 1.5.1) software.

#### *4.1.4.4 Data analysis*

All data were analysed using Genstat statistical software (16$^{th}$ Edition), with significance at $P<0.05$. One-way ANOVA was used to assess the effect of diet on animal body weight. Two-way ANOVA (diet x time) was used to assess the effects of diet (control vs. xylanase) and time (2, 4 or 6 weeks of the trial) on the DNA yield (µg/mg faeces), DNA quality (260nm/280nm) and the faecal DNA composition (*CYTB*, *16S* and *CYTB/16S*).

### 4.1.5 Results

#### *4.1.5.1 Animal performance*

Body weights were not significantly ($P=0.949$) different between animals on the control or xylanase inclusion diet throughout the trial (**Figure 4.1**). Measures of feed intake were attempted in this study but due to the feeders used at the time the measurements for this were deemed inaccurate as feed from troughs was spilled onto the floor or contaminated with urine or

faeces (see Katherine May PhD thesis (May, 2017)). Therefore, no measurements of FCR were analysed in the present thesis.

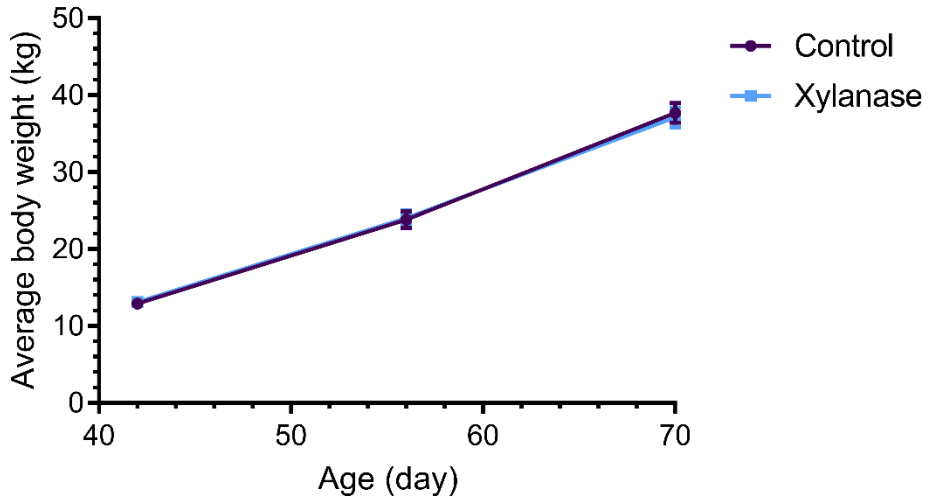


Figure 4.1 Line graph indicating the pig body weights (kg) on either the control or xylanase diet throughout the study. Means ± SEM, n=8 biological replicates per group.

#### *4.1.5.2 Effect of a xylanase inclusion diet, and duration of supplementation, on pig faecal DNA*

*Total faecal DNA content and quality*

There was no interaction (P=0.947) between treatment and time on DNA yield (µg/mg faecal material) (**Figure 4.2A**). However, there was a trend (P=0.062) for increased DNA yield (µg/mg faecal material) from the faecal samples in the xylanase group, with no significant (P=0.278) effect of time (**Figure 4.2A**). There was no significant effect of treatment (P=0.615) or time (P=0.159) on the 260nm/280nm DNA quality assessment, and no interaction between treatment and time (**Figure 4.2B**).

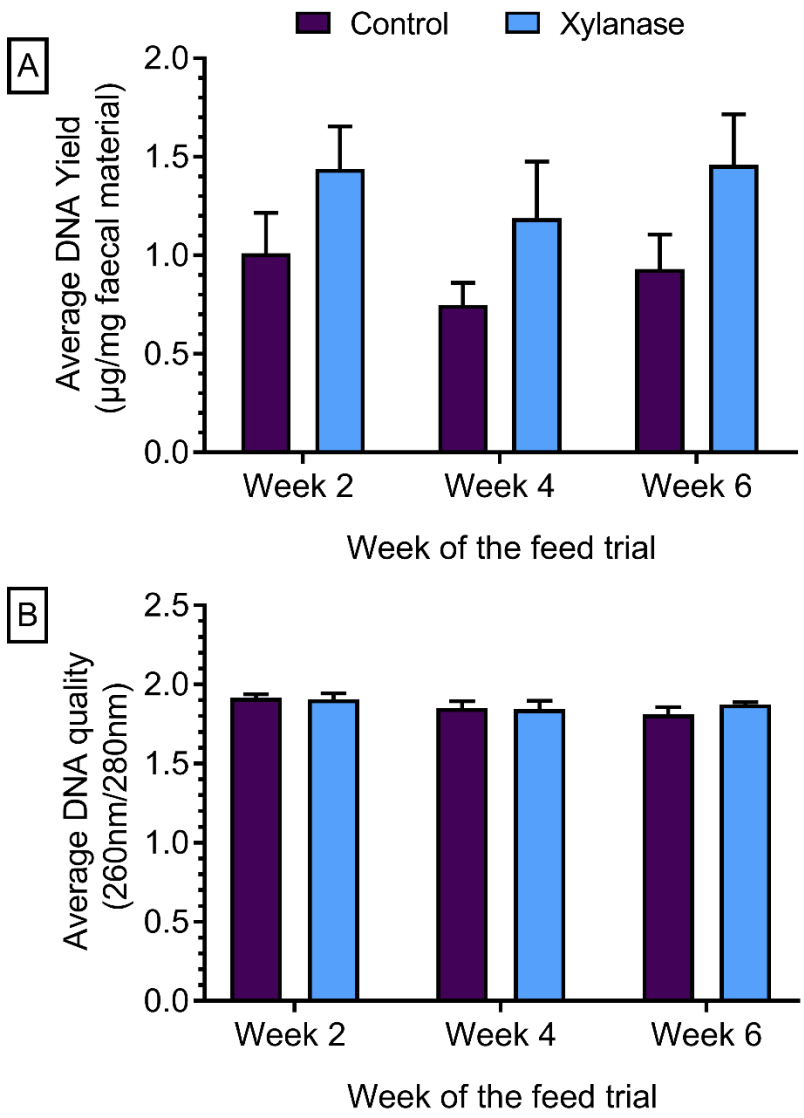


**Figure 4.2** The effect of a xylanase inclusion diet and time on [**A**] Average DNA yield (µg DNA/mg faeces) and [**B**] Average DNA quality (260nm/280nm) of the DNA extracted from pig faeces. There was a trend (P=0.062) for increased DNA yield (µg/mg faecal material) from the faecal samples in the xylanase group. Means ± SEM, n=8 biological replicates per group.

*Faecal DNA composition*

There was a significant (P=0.039) effect of diet on pig *CYTB* DNA content in the faecal samples and a weak trend (P=0.087) for an effect of time, however, there was no interaction (diet x time) (P=0.225) (**Figure 4.3A**). A xylanase inclusion diet significantly reduced faecal pig *CYTB* DNA levels. The effect of the

xylanase supplementation only appeared significant at the first 2 time points, but there appeared to be no difference by week 6 (**Figure 4.3A**). Conversely, there was no interaction (P=0.698) between time and diet, as well as no singular effects of time (P=0.145) or diet (P=0.974) on bacterial *16S* DNA content in the faeces (**Figure 4.3B**). As the bacterial DNA content did not differ between different dietary treatment groups or over time it was used as a normalising control for the pig *CYTB* DNA content. There was a significant (P=0.002) effect of diet on the faecal pig *CYTB* DNA normalised for bacterial *16S* DNA, although, there was no effect of time (P=0.213) or an interaction (diet x time) (P=0.648) (**Figure 4.3C**). Xylanase supplementation significantly reduced the normalised *CYTB* DNA levels in the faeces compared with the control group (**Figure 4.3C**).

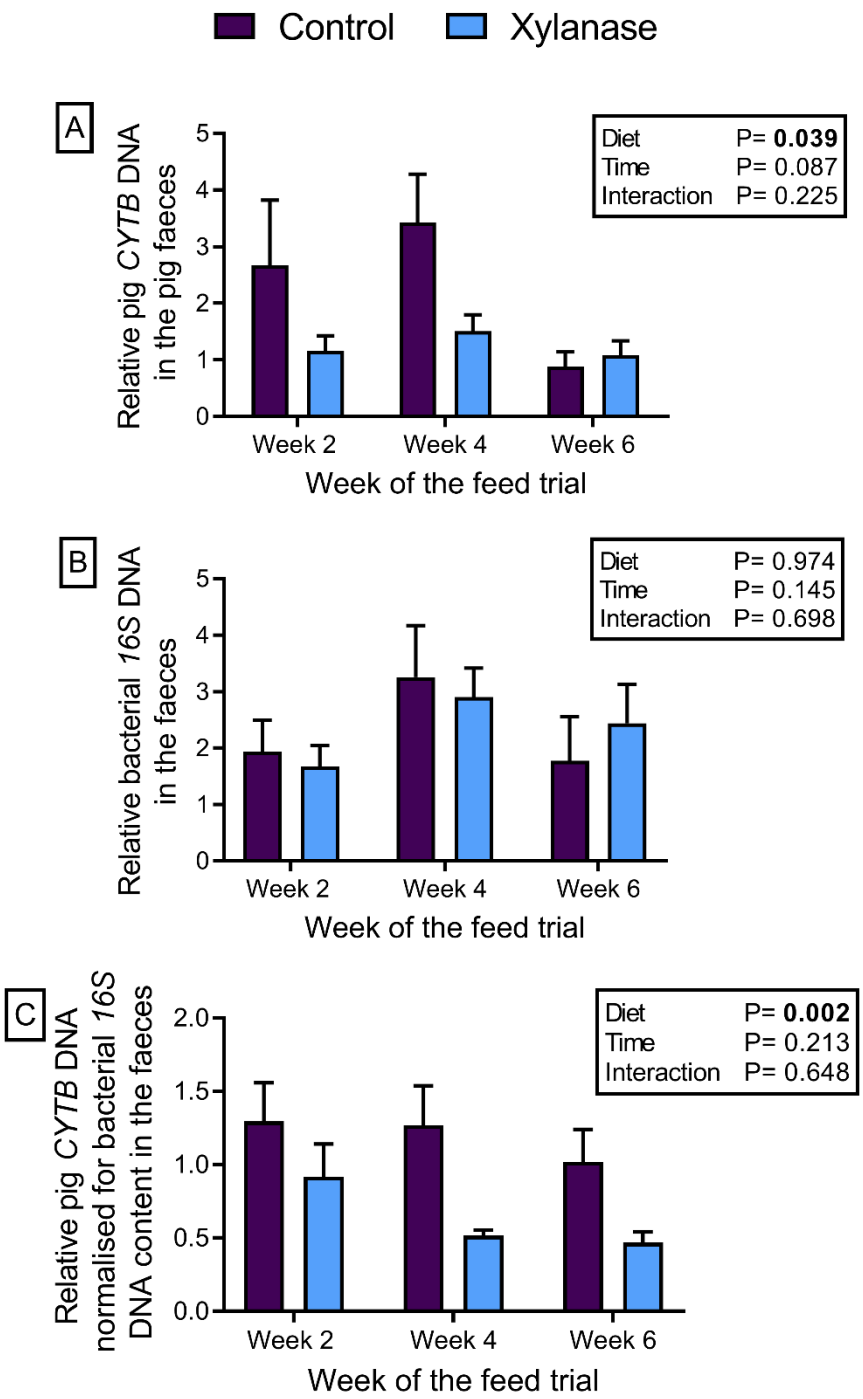


**Figure 4.3** The effect of a xylanase inclusion diet and time on [**A**] Relative pig *CYTB* DNA in the faeces, [**B**] Relative bacterial *16S* DNA in the faeces and [**C**] Relative pig *CYTB* DNA normalised for bacterial *16S* DNA. Means ± SEM. n=8 biological replicates for all groups other than control week 6 where n=7. P values from the two-way ANOVA (Diet x Time) are displayed in each graph.

### 4.1.6 Discussion

As expected, following the method optimisation results outlined in Chapter 3, the DNA yields achieved from the pig faecal samples were high, with an average of 0.9µg/mg faeces for control samples and 1.4µg/mg faeces for xylanase samples. There was a trend for an effect of diet on DNA yield, but no effect of time. The slightly higher DNA yield from faeces of xylanase treated animals could be due to an increased level of feed DNA present in the faeces, as xylanase hydrolyses xylans present in feed ingredients as discussed previously (Zhang *et al.*, 2018). Alternatively it could also be as a result of xylanase aiding the degradation of NSPs which may decrease the extractability of the DNA (Kumar *et al.*, 2007). Future work could assess the extractability of spiked DNA from pig faeces with varying fibre content to attempt to quantify the effect of complex carbohydrate upon DNA extraction from pig faeces. DNA quality (260nm/280nm) was not affected by diet or time, with an average value of 1.87 which is very close to the value of pure DNA (1.8). Therefore, the phenol-based DNA extraction is a suitable method for the extraction of DNA from faeces of pigs on different experimental diets.

As predicted, there was a significant decreasing effect of xylanase on pig *CYTB* DNA in the faeces compared to the control group and a trend for an effect of time, with greater faecal host DNA closer to weaning. In terms of the trend for an effect of time, Bedford & Cowieson (2012) suggest that as the digestive capacity of the small intestine increases as animal's age, there may be a reduced capacity for xylanases to decrease the starch presence and therefore the benefit of these enzymes is reduced. Weaning is a stressful event and can have a negative impact upon gut barrier function (Smith *et al.*, 2010). Potentially xylanase attenuated the detrimental effects of the post-weaning period on the intestinal barrier function. This may be attributed to xylanase reducing the levels of NSPs, which have been shown to increase intestinal epithelial cell proliferation rate (Kerr and Shurson, 2013). As previously discussed, an increase in cell proliferation rate is associated with an increased level of gut cell loss and increased energy utilisation, and therefore the results

described here may provide further evidence as to a potential mechanism for the improved animal performance observed in xylanase supplemented pigs. Unfortunately, due to inaccuracies with the measurement of feed intake in this study, comparisons cannot be made against FCR. In future experiments it would be interesting to interrogate any relationship between faecal host DNA and FCR in swine.

Additionally, the reducing effect of xylanase on *CYTB* DNA was greater when host DNA was normalised for bacterial *16S* DNA, which did not change between dietary treatment groups. However, the trend for an effect of time on faecal pig *CYTB* DNA was lost with normalisation. It is interesting however that no differences were observed in the total bacterial *16S* DNA in the faeces as measured by PCR, as xylanase hydrolysis products are thought to stimulate beneficial bacteria by a 'prebiotic-like' mechanism and therefore shift the microbial profile (Bedford and Cowieson, 2012). Conversely, although there may have been changes in the microbial profile, there may not have been differences in the total bacterial levels or not all the microbial groups may have been detected using the universal bacterial *16S* primers (Kiarie *et al.*, 2013). As a result, further work is needed to assess whether it is acceptable to normalise faecal host DNA with bacterial *16S* DNA content, as there may be important effects, such as the effect of time, which may be missed.

## 4.2 YEAST-ENRICHED PROTEIN CONCENTRATE TRIAL

### 4.2.1 Introduction

Inclusion of yeast extract in livestock diets has previously been shown to affect animal performance and gut morphology (Muthusamy *et al.*, 2011). Muthusamy *et al.* (2011) demonstrated in broilers that supplementing the diet with 1g/kg yeast cell wall and hydrolysed whole yeast resulted in improved FCR (P=0.003), and also significantly increased average villus height (P=0.02) in the jejunum and average width of the villi (P=0.034) in the ileum. Gut maturation leads to gut morphological changes such as increased villus height, which also leads to decreased gut cell losses. Yeast contains substantial amounts of mannan and glucan in the cell wall. These components have been suggested to exert a 'pre-biotic like' effect, with varied performance-enhancing effects, such as inhibition of pathogenic invasion, modulation of the host immune response and, as previously discussed, enhancing the host gut morphology, thereby limiting the number of pathogens in the gut lumen (Roto *et al.*, 2015). The main mechanism by which this occurs is thought to involve the mannan and glucan from the yeast cell wall binding to pathogenic bacteria, preventing their attachment to the intestinal epithelia and therefore enabling the host to reserve energy for growth, rather than repair and replenishment of epithelial cells (Roto *et al.*, 2015). Therefore, inclusion of yeast-enriched protein concentrate (YPC) was used as a dietary treatment in this study to test the hypothesis, since a significant reduction in gut cell sloughing was expected in YPC treated animals, which should then be detected using the *CYTB* DNA assay.

Faecal calprotectin (CALP) levels have previously been used as a marker of gut inflammation in pigs (Lallès and Fagerhol, 2005). Studies in humans have assessed human DNA content in faeces as a marker of bowel inflammation (Vincent *et al.*, 2015). Since gut turnover might be influenced by gut inflammation, we also assessed the calprotectin levels in pig faeces. As there were issues with accurately measuring feed intake in the xylanase trial, no comparisons between pig faecal *CYTB* DNA content and FCR were possible. As a result, an additional aim of this study was to investigate whether there were

any relationships between faecal calprotectin, *CYTB* DNA and animal performance, particularly FCR.

### 4.2.2 Hypotheses

1. Dietary yeast-enriched protein concentrate will improve animal performance.
2. There will be no effects of the dietary treatment or time on the DNA yield and quality.
3. Dietary yeast-enriched protein concentrate will reduce pig DNA in the faeces compared to the control group, but there will be no effects on bacterial *16S* DNA content.
4. Pig faecal calprotectin contents will be reduced in animals receiving a diet containing yeast-enriched protein concentrate and there may potentially be an effect of time due to animals being newly weaned.
5. Measures of pig DNA in the faeces will correlate to animal performance measures, particularly FCR.

### 4.2.3 Objectives

1. Assess the effects of a yeast-enriched protein concentrate inclusion diet on animal performance.
2. Assess the effects of a yeast-enriched protein concentrate inclusion diet and duration of supplementation on pig faecal DNA content, composition and faecal calprotectin levels.
3. Determine whether there was any relationship between faecal biomarker measurements and animal performance.

### 4.2.4 Materials and Methods

#### *4.2.4.1 Animal trial and sample collection*

The feeding study was carried out at Harper Adams University and was approved by the internal ethical review committee (Approval number: 00583940-STAFF-1-201607). The study did not use any 'regulated procedures' and was therefore not carried out under a Home Office Licence. Newly weaned

Large White x Landrace x Pietrain piglets (8.37kg ±1.10, n=180) were assigned to one of four dietary treatment groups (n=9 pens per treatment, 5 animals per pen). The pigs were in single sex pens with both males and females used in the study but balanced across the treatment groups. There were 5 pens of males and 4 pens of females on each treatment. The pens were 1.5m² with plastic slatted floors, in line with the commercial Assured Food Standard requirement of 0.3m² per animal up to 30kg in body weight. The diets differed in yeast-enriched protein concentrate (YPC, AB Agri, Peterborough, UK) content, as follows: 0% (control), 2.5%, 5% and 7.5% (w/w) (**Appendix 2**). The YPC product is a maize based protein concentrate with a high content (30%) of inactive yeast from the bioethanol production industry. Its nutritional value is shown in **Appendix 3**. Diets were formulated by AB Agri Limited. All diets were formulated to be equivalent in net energy (NE) and standard ileal digestible (SID) lysine, methionine, threonine, tryptophan and valine and were balanced using potato protein and wheat. Diet 1 was offered from weaning (~26.5 days) to day 14 of the trial and diet 2 was offered from day 14 to day 28 (**Appendix 2**). No therapeutic zinc oxide or dietary antibiotics were used. Animals were weighed at the beginning of the trial (at weaning), on day 14 and day 28. Feed intake was determined by offering a specified weight of feed, measuring the amount of feed weighed back at the end of each week and using these values to calculate feed eaten. Feed conversion ratio (FCR) was calculated as feed intake divided by animal body weight gain and provided as an average for each experimental pen. A single fresh faecal sample was taken immediately after defecation from a minimum of 3 pigs from each pen at weighing and these samples were then pooled for the pen. Weighing of the pigs was typically carried out at midday. The pooled sample was approximately 40g in weight and this was stored at -20˚C post collection. Samples were then transported, frozen and packed with ice packs, to Kimberley Slinger at the University of Nottingham. Once delivered samples were again stored at -20°C until processed.

#### *4.2.4.2 DNA extraction*

DNA was extracted from 30mg of frozen pig faeces utilising the phenol-chloroform based method as outlined in Materials and Methods 2.3. As indicated in Materials and Methods 2.3.1, faecal samples from the pig yeast trial had to be treated with RNase A (Promega) to remove RNA contaminants. DNA concentration and quality were then assessed as outlined in Materials and Methods 2.5 and 2.6, and stored at -20˚C until processed. DNA yield was calculated and expressed as ng of DNA per mg of faeces for DNA extracted from the YPC trial.

#### *4.2.4.3 Quantitative PCR*

All the faecal DNA samples from the YPC trial were analysed by qPCR using primers for pig *CYTB* DNA and bacterial *16S* DNA using SYBR Green (Roche) qPCR methodology in a LightCycler® 480 (Roche) instrument, as outlined in Materials and Methods 2.7 and 2.10. A pool of extracted faecal DNA (diluted to 10ng/µl) was used in a 4-fold dilution series to create standard curves for both pig *CYTB* and bacterial *16S* DNA. Pig faecal samples (already diluted to 10ng/µl) were then diluted 1 in 8 and tested in triplicate against the standard curve. This was to ensure a constant amount of DNA was used in the qPCR so that DNA composition between samples could be compared. Negative template controls (DNase and RNase free water) were also tested in triplicate at 2 points on the plate.

#### *4.2.4.4 Pig Calprotectin ELISA*

Pig faecal samples from the YPC trial were analysed for their calprotectin content using a Pig Calprotectin (CALP) ELISA Kit (CUSABIO BIOTECH Co Ltd, China), as outlined in Materials and Methods 2.11.

#### *4.2.4.5 Data analysis*

All analysis of variance (ANOVA) analyses were analysed using Genstat statistical software (17th Edition), with significance at $P<0.05$. One-way ANOVA was used to assess the effects of diet on animal performance (BW, daily feed intake, daily live weight gain and FCR) at each time point separately. Two-way ANOVA (factors: diet x day of trial, blocking for pen) was used to assess whether

there were any effects on the resulting DNA concentration (ng/µl), DNA quality (260nm/280nm), DNA yield (ng/mg faecal material) and DNA contents (*CYTB*, *16S* and *CYTB:16S* ratio). Three-way ANOVA (factors: diet x day of trial x plate, blocking for pen) was used to analyse the faecal pig calprotectin levels. Where significant differences were observed, post hoc Bonferroni tests were then performed. The relationships between pig faecal *CYTB* DNA content and FCR and pig faecal calprotectin and FCR were assessed by Pearson's correlation for both time points separately, using GraphPad Prism (version 7.03).

### 4.2.5 Results

#### *4.2.5.1 Animal performance*

There were no observable health issues to report and there were no significant treatment differences in daily intake or growth rate (**Table 4.1**). Pigs fed diets containing 2.5, 5 or 7.5% YPC had significantly reduced FCR recorded from wean to day 14 ($P<0.001$) and over the entire 28 day trial (wean-day 28, $P=0.019$) for 5% and 7.5% YPC inclusion compared to animals on the control diet (**Table 4.1**). However, there was no effect ($P=0.220$) of YPC on FCR from day 14 to day 28 (**Table 4.1**).

Table 4.1 The effect of dietary yeast-enriched protein concentrate on animal performance.

| | YPC inclusion level (%) | | | | | P |
|---|---|---|---|---|---|---|
| **Animal performance** | **0** | **2.5** | **5.0** | **7.0** | **SED** | **value** |
| Average BW at weaning (kg) | 8.38 | 8.33 | 8.38 | 8.37 | 0.025 | 0.175 |
| Average BW at day 14 (kg) | 12.30 | 12.91 | 13.03 | 12.79 | 0.291 | 0.081 |
| Average BW at day 28 (kg) | 21.95 | 21.66 | 22.68 | 21.84 | 0.666 | 0.457 |
| DFI wean-day 14 (g/pig/day) (diet 1) | 340 | 339 | 338 | 317 | 19.0 | 0.561 |
| DFI day 14-day 28 (g/pig/day) (diet 2) | 830 | 776 | 823 | 791 | 41.0 | 0.542 |
| DFI wean-day 28 (kg/pig/day) (treatment diets) | 580 | 557 | 580 | 554 | 27.0 | 0.585 |
| DLWG wean-day 14 (g/pig/day) (diet 1) | 280 | 327 | 332 | 316 | 21.1 | 0.076 |
| DLWG day 14-day 28 (g/pig/day) (diet 2) | 687 | 625 | 689 | 646 | 36.4 | 0.240 |
| DLWG wean-day 28 (kg/pig/day) (treatment diets) | 483 | 476 | 511 | 481 | 24.6 | 0.510 |
| FCR wean-day 14 (diet 1) | $1.24^{a}$ | $1.04^{b}$ | $1.02^{b}$ | $1.01^{b}$ | 0.048 | **<0.001** |
| FCR day 14-day 28 (diet 2) | 1.21 | 1.25 | 1.20 | 1.23 | 0.025 | 0.220 |
| FCR wean-day 28 (treatment diets) | $1.21^{a}$ | $1.17^{ab}$ | $1.14^{b}$ | $1.15^{b}$ | 0.023 | **0.019** |

Means with different superscript letters in the same row differ significantly.

#### *4.2.5.2 Effect of a yeast-enriched protein concentrate inclusion diet, and duration of supplementation, on pig faecal DNA and calprotectin*

*Total faecal DNA content and quality*

DNA extracted from pig faecal samples collected on day 14 of the trial was of a significantly (P=0.014) higher concentration (ng/µl) compared to samples collected on day 28. However, there was no effect (P=0.581) of diet on the extracted DNA concentration and all interactions were not statistically significant (P=0.674). DNA yield (ng/mg faecal material) was significantly (P=0.016) higher at day 14 compared with day 28, in accordance with the increased DNA concentration observed in samples collected on day 14 (**Figure 4.4A**). However, there was no effect of dietary treatment (P=0.551) on faecal DNA extraction yield (ng DNA/mg faecal material) nor any interaction (0.739)

between day and dietary treatment (**Figure 4.4A**). When analysing DNA quality (260nm/280nm) there were no significant (P>0.05) interactions or singular effects of either diet or day of the trial (**Figure 4.4B**).

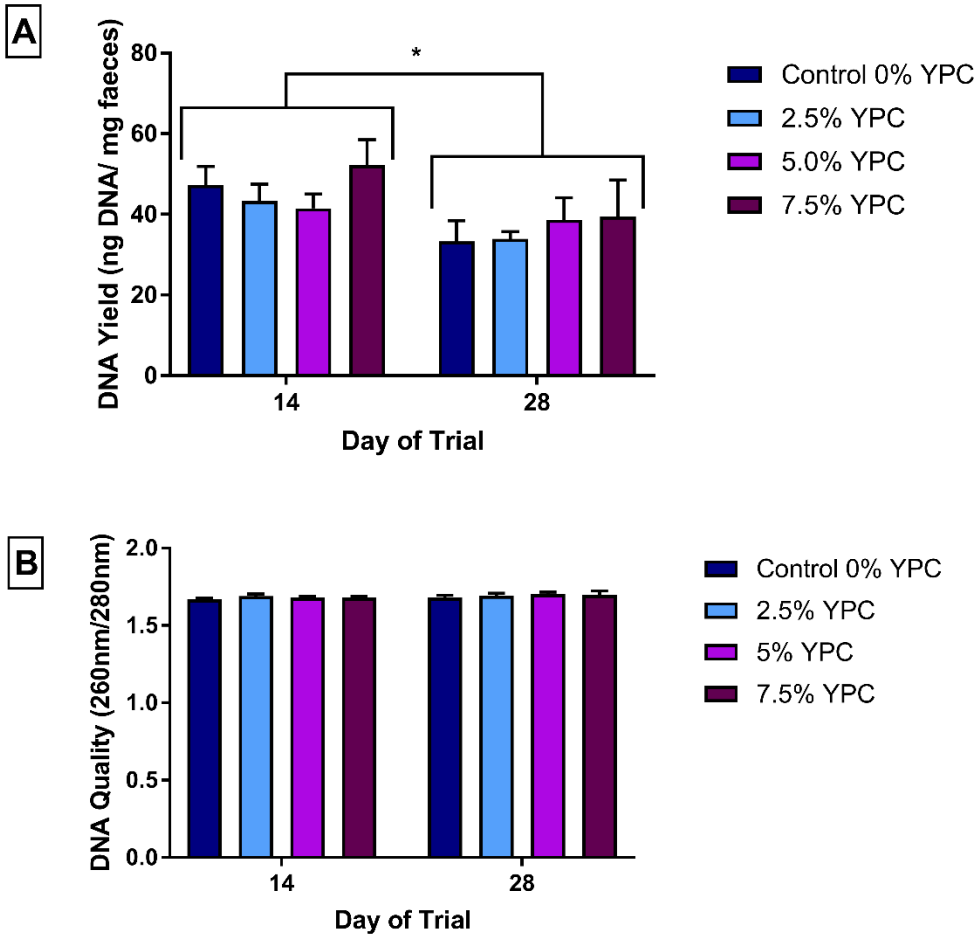


Figure 4.4 The effect of yeast-enriched protein concentrate (YPC) and day of the trial on [A] DNA yield (ng DNA/mg faeces) and [B] DNA quality (260nm/280nm). * P<0.05. Means ± SEM, n=9 biological replicates for all groups.

*Faecal DNA composition and calprotectin content*

There was no day x YPC interaction (P=0.222) nor any effect of day (P=0.997) on pig *CYTB* DNA in the faeces, but there was a significant (P=0.008) effect of YPC diet (**Figure 4.5A**, **Table 4.2**). Bonferroni post hoc analysis showed that faeces from animals on the control diet (0% YPC) contained significantly (P<0.01) more pig *CYTB* DNA than those on the 2.5% YPC diet (**Figure 4.5A**, **Table 4.2**). Interestingly, even though there was no day x YPC interaction, all 3

YPC diets appeared to reduce faecal pig *CYTB* DNA contents at day 14, but only 2.5% YPC did so at day 28 (**Figure 4.5A**).

In contrast, there were no significant effects of diet (P=0.348), day (P=0.205) nor an interaction (P=0.602) on bacterial *16S* DNA content in the faeces (**Figure 4.5B**). As there were no differences in bacterial *16S* DNA, bacterial DNA was used to normalise the pig *CYTB* DNA content (**Figure 4.5C**). There was still a significant (P=0.045) effect of YPC diet observed when *CYTB* DNA was normalised for bacterial *16S* DNA content, with animals on the control diet (0% YPC) having significantly (P<0.05) more normalised *CYTB* DNA in their faeces compared to those on the 2.5% YPC diet (**Figure 4.5C**, **Table 4.2**), with no effect of day (P=0.368) nor a day x YPC interaction (P=0.206).

Pig faecal calprotectin is a marker of inflammation and the levels were significantly higher at day 14 compared to day 28 (P=0.009), indicating more intestinal inflammation closer to weaning (**Figure 4.5D**). However, there was no significant (P=0.709) effect of YPC diet nor a day x YPC interaction (P=0.391).

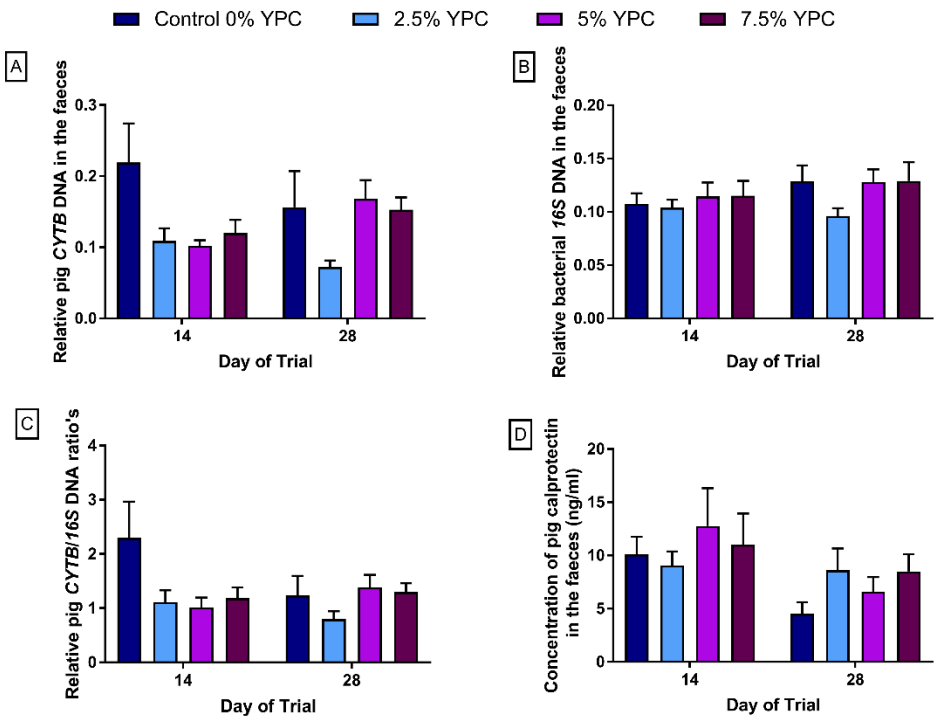


**Figure 4.5** The effect of yeast-enriched protein concentrate (YPC) and day of trial on [A] faecal pig *CYTB* DNA content, [B] faecal bacterial *16S* DNA content, [C] faecal pig *CYTB* DNA content normalised for bacterial *16S* DNA, [D] faecal calprotectin content. Means ± SEM, n=9 biological replicates for all groups.

**Table 4.2** Post hoc analysis of the effect of diet on pig faecal DNA content.

| | Mean faecal DNA content in dietary groups | | | | | P value for effect of diet (ANOVA) |
|---|---|---|---|---|---|---|
| **Faecal DNA component** | **Control 0% YPC** | **2.5% YPC** | **5% YPC** | **7.5% YPC** | **SED** | |
| **Pig *CYTB* DNA** | $0.188^{a}$ | $0.091^{b}$ | $0.135^{ab}$ | $0.136^{ab}$ | 0.0259 | 0.008 |
| **Pig *CYTB* DNA normalised for 16S DNA** | $1.77^{A}$ | $0.96^{B}$ | $1.20^{AB}$ | $1.24^{AB}$ | 0.278 | 0.045 |

$^{a-b}$ Samples differing in lowercase letters indicate significant statistical differences at P<0.01.

$^{A-B}$ Samples differing in uppercase letters indicate significant statistical differences at P<0.05.

#### *4.2.5.3 Relationship between host DNA, calprotectin and FCR*

Pearson correlations were used to investigate relationships between the faecal DNA or calprotectin contents and feed efficiency. There was a trend (P=0.093) for a correlation (r=0.2839) between the faecal pig *CYTB* DNA content (at day 14) and the recorded FCR (wean-day 14) over the early stages, where a significant effect of diet on FCR was observed (**Figure 4.6A**, **Table 4.1**). It is important to note there is a outlier (a result >2 standard deviations from the mean) for *CYTB* DNA at day 14 included in the complete analysis displayed in **Figure 4.6A**, analysis has been carried out whereby this result was removed and this altered the P value to P=0.0191, indicating a statistically significant relationship between *CYTB* DNA at day 14 and FCR. However, there was no correlation (P=0.393) at the later phase (day 14-28; **Figure 4.6B**), where no effect of diet on FCR was observed (**Table 4.1**). There is also an outlier for *CYTB* DNA at day 28 included in the complete analysis displayed in **Figure 4.6B**, analysis has been carried out whereby this result was removed and this did not change the results. Likewise, there were no significant correlations between faecal calprotectin concentrations and recorded FCR at either day 14 or day 28 (**Figure 4.6C** & **Figure 4.6D**).

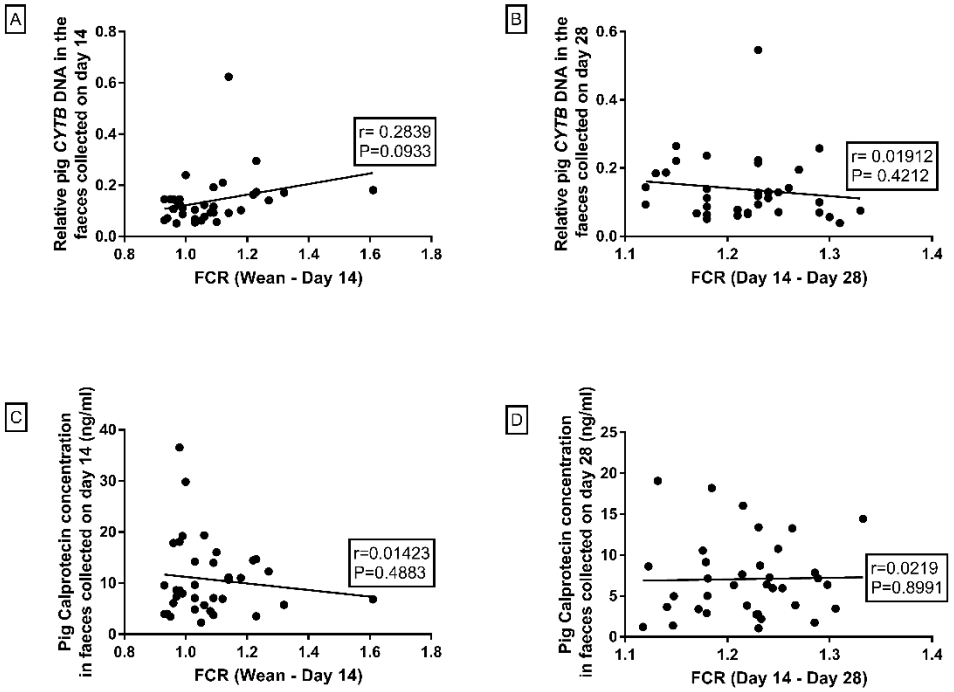


Figure 4.6 [A] The relationship between the pig *CYTB* DNA content in faeces at day 14 and recorded FCR (wean-day 14). [B] The relationship between the pig *CYTB* DNA content in faeces at day 28 with FCR (day 14-day 28). [C] The relationship between pig faecal calprotectin concentration (ng/ml) on day 14 and FCR (wean-day 14). [D] The relationship between pig faecal calprotectin (ng/ml) on day 28 and FCR (day 14-day 28). P values and r values are provided within each graph.

### 4.2.6 Discussion

As expected, animals on the YPC diets had a significantly improved FCR compared to the control group, however, this was not in a dose dependant manner and only between wean to 14 days of the trial but not between 14 days and 28 days. DNA yield was significantly greater in samples extracted from day 14 compared to samples extracted on day 28. The reason for this is unclear but it may be due to there being a greater level of feed digestion at day 28 due to there being a more mature gut. Faecal dry matter was not measured and while it is unlikely to impact upon PCR, as measurements are performed on a constant DNA basis, this may account for the difference in DNA yield, although this is purely speculation. It is important to note that the units for DNA yield from the current YPC trial are ng DNA per mg of faeces, compared with the units for DNA

yield from the xylanase trial samples which were µg DNA per mg of faeces. This suggests that more DNA was extracted from pig faeces in the xylanase study, compared with the amount of DNA extracted from samples in the YPC study. However, the NanoDrop instrument used to measure DNA concentration cannot distinguish between RNA and DNA. Samples in the current study were treated with RNase A which removes RNA from the sample, and so differences in DNA yield between the 2 studies should be viewed with this in mind as it could be a factor of removing RNA which may have otherwise interfered with the DNA concentration reading, used to calculate DNA yield. Alternatively, the fact that samples in the current YPC study were transported on ice, while samples from the xylanase study were not, may have impacted on the amount of DNA available in the samples for extraction. This in correspondence with the results obtained in the DNA spiking experiment in Chapter 3 where pig faecal samples stored on ice for 3 hours, much like the conditions during transportation of the samples, had significantly less DNA extracted from them compared with samples which were extracted immediately after spiking. Although the method for DNA extraction in the spiking experiment was not the phenol-chloroform based method used in the current chapter, which is important to recognise. In the current YPC study, the DNA quality was unaffected by either diet or day of the trial the samples were collected.

Pig *CYTB* DNA was significantly ($P<0.01$) higher in faeces collected from the pigs on the control diet compared with pigs on the YPC inclusion diets, but bacterial *16S* DNA content was unaffected by either the diet or the age of the pigs. The fact that the bacterial *16S* DNA content remains unchanged suggests it could potentially be used as a normalising control for the analysis of host DNA content in livestock faeces. Unlike in the xylanase study, normalising host DNA for bacterial *16S* did not create or lose any effects that were not apparent when assessing host DNA singularly, however the P value for the effect of diet was increased ($P=0.045$ as opposed to $P=0.008$). The level of host DNA in faeces was hypothesised to be directly proportional to the amount of gut cell sloughing. The results of this study indicate that there is significantly more gut cell

sloughing in the control fed pigs than the animals on a yeast inclusion diet, as with the xylanase inclusion study discussed previously. It is therefore possible that the yeast component exerted a protective effect on the gut epithelium as previously described. As these animals are newly weaned pigs, the results suggest that inclusion of yeast-enriched protein concentrate in the diet helps the animal cope better with the problems observed when animals are transitioning to solid feed (Fairbrother *et al.*, 2005).

There was a trend for a relationship between FCR measured over the first 14 days of the trial and the pig *CYTB* DNA content in the faeces at day 14, although the relationship was statistically significant when an outlier was removed from the analysis. The first 14 days of the study were also where significant differences in FCR between dietary treatments were observed. These results suggest that the levels of host DNA content in faeces may be a useful method for assessing gut cell losses and their impact on feed efficiency.

Faecal calprotectin levels are a recognised measure of intestinal inflammation and have been widely studied in humans (Fagerberg *et al.*, 2005). However, fewer studies have been carried out in animals. Lallès & Fagerhol (2005) determined reference values for faecal calprotectin levels in pigs of different ages and lactation stage but the present study has assessed dietary intervention of newly weaned piglets. Pig faecal calprotectin was significantly higher at day 14 of the trial compared with day 28, with no influence of the YPC diet. Previous studies have shown that dietary inclusion of chitosan can reduce faecal calprotectin and therefore intestinal inflammation in pigs (Xiao *et al.*, 2014), but the YPC had no effect in the present study. The only differences in pig faecal calprotectin concentration (ng/ml) were caused by the age of the animal (day 14 > day 28), whereas differences in pig *CYTB* DNA were caused by diet. This shows that the pig *CYTB* DNA content is unrelated to the levels of calprotectin in the faeces, which is a marker of inflammation. Conversely, others found a significant, but weak, correlation between human faecal DNA content and calprotectin content and hypothesised the weak relationship was due to the different origins of the markers (Varela *et al.*, 2009).

In conclusion, dietary YPC positively influenced FCR in newly weaned piglets and reduced faecal *CYTB* DNA content. These results suggest that dietary YPC may reduce gut cell sloughing, which is an energy demanding process. However, further validation experiments are required to verify the origin of faecal *CYTB* DNA and ensure sloughed epithelial cells are the only source.

# CHAPTER 5. CHICKEN TRIALS

## 5.1 PHYTASE TRIAL

### 5.1.1 Introduction

Assessments of host DNA in pig faeces from dietary intervention studies were successful and significant differences, which showed some relationship with FCR, were measured. Therefore, the aim of this chapter was to apply the developed methodology for the analysis of host DNA in chicken faeces to poultry dietary intervention studies where effects on gut cell loss were also expected.

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is a complex molecule whereby an inositol is surrounded by 6 phosphorus atoms (Ravindran *et al.*, 1994). When the phytic acid is bound to a mineral, such as magnesium, zinc or calcium, it is known as phytate (Ravindran *et al.*, 1994; Reddy *et al.*, 1982). Phytate is the primary storage form of phosphorus and inositol in seeds and this is hydrolysed by the enzyme phytase, present in the seeds, to enable the seedling to utilise the products for growth (Ravindran *et al.*, 1994). However, bioavailability of phosphorus in phytate form has very poor substrate solubility in the gastrointestinal tract and therefore cannot be utilised effectively by monogastric animals and humans (Cowieson *et al.*, 2011). Equally, the complexes that are formed when phytic acid binds to minerals are largely insoluble and so this also decreases bioavailability of minerals to the animal (Reddy *et al.*, 1982). In order to combat the issue of phytate in monogastric nutrition, exogenous phytase is added to diets to release bound minerals and increase phosphorus availability to the animal (Bedford and Schulze, 1998).

As previously described in Chapter 1, dietary energy is required to replenish the epithelial cells lining the gastrointestinal tract and therefore a high level of gut cell loss can negatively impact upon feed efficiency. In addition, gut maturation results in changes to the gastrointestinal tract morphology, such as increased villus height which is observed alongside decreased gut cell

losses (Xu *et al.*, 2003). Wu *et al.* (2004) was the first study to assess the effect of microbial phytase on gut morphology, until then much of the research was surrounding the effect of exogenous xylanase. It was observed that phytase not only significantly improved broiler chicken performance compared with controls, despite the diets containing adequate phosphorus, but equally there were significant effects on the gross structure of the gastrointestinal tract and morphology (Wu *et al.*, 2004). In birds supplemented with phytase there was a significant decrease in the weight of the duodenum and jejunum and collectively the small intestine, as well as a significant decrease in the length of each section of the small intestine, which corresponded with an increased villus height in the duodenum (Wu *et al.*, 2004). Increased villus height leads to greater nutrient absorption due to the greater surface area (Caspary, 1992), this explains the decreases in the lengths of the small intestine. The overall aim of this study was to assess whether a phytase inclusion diet would affect feed efficiency, as well as the levels of chicken *CYTB* DNA present in the faecal samples, as the host faecal DNA content presumably reflects gut cell losses influenced by changes in gut morphology. An additional aim was to examine whether there was a relationship between faecal host DNA and FCR.

### 5.1.2 Hypotheses

1. A phytase inclusion diet will be beneficial to bird performance.
2. There will be no effects of dietary treatment on the total faecal DNA yield and quality.
3. Chickens consuming the phytase inclusion diet will have a reduced level of host DNA in their faeces.
4. The levels of total bacterial DNA content will be unaffected by the dietary treatment.
5. There will be a relationship between FCR and host DNA content.

### 5.1.3 Objectives

1. Assess the effects of a phytase inclusion diet on bird performance.

2. Assess the effects of a phytase inclusion diet on total faecal DNA yield and quality from faeces collected at 42 days of age.
3. Assess the effects of a phytase inclusion diet on faecal DNA composition (host DNA and bacterial DNA).
4. Identify whether there is a relationship between bird performance, particularly FCR, and chicken *CYTB* DNA content in faeces.

### 5.1.4 Materials and Methods

#### *5.1.4.1 Animal trial and sample collection*

The phytase trial was carried out at Drayton Animal Health Ltd (owned by ADAS UK at the time of the experiment), an external contract research facility, for AB Vista. Faecal samples were collected from a subset of pens from a larger scale trial, the details of the subset of pens will be explained. One hundred and forty-four Ross 308 broiler chicks (40.6 ± 0.7g) were assigned to either a control or a phytase inclusion diet on day of hatch, with n=72 birds per treatment with 18 birds in 4 replicate pens. The trial lasted from 0 to 42 days of age. The birds on the phytase inclusion diet received the same diet as the control fed animals throughout the trial but with an additional commercial preparation of 2500FTU (500 grams/tonne) of Quantum Blue (AB Vista, Marlborough, UK). A crumble starter diet was fed between 0 and 21 days of age and a pelleted grower diet fed between 21 and 42 days of age. The diet compositions for the starter and grower diets were formulated by Target Feeds and are indicated in **Appendix 4**. Feed and water were offered *ad libitum*. Pens were 1.5m x 1.3m with an initial stocking density of 9 birds/$m^2$, in accordance with Home Office legislation detailing the housing requirements for birds under experimentation. Bird weight and pen feed intake was measured at 1, 21 and 42 days of age and feed conversion ratio then calculated on a pen basis as total bird body weight gain (all birds in the pen) divided by the total feed intake of the pen. Three pooled faecal samples were collected from the pen floor of each pen on day 42 of the trial, resulting in 12 pooled faecal samples per dietary treatment group. Faecal samples were pooled to ensure faecal DNA analysis was representative of the pen, which is the replicate in this study. Pooled faecal

samples were stored at -20˚C post collection. Then, samples were transported, frozen and packed with ice packs, to Kimberley Slinger at the University of Nottingham. Once delivered samples were again stored at -20°C until processed.

#### *5.1.4.2 DNA Extraction*

DNA was extracted from the chicken faecal samples using the method outlined in Materials and Methods 2.4, which briefly involved bead-beating ~200mg of faeces in a MagNA Lyser Green Beads Tube (Roche Diagnostics) with Buffer ASL (Qiagen) in a MagNA Lyser instrument (Roche Diagnostics), followed by an adapted version of the QIAamp® DNA Stool Mini Kit (Qiagen). DNA was also extracted from chicken calf muscle as outlined in Materials and Methods 2.2, this was used as a positive control for the PCR experiments. DNA concentration (ng/µl) and quality (260nm/280nm) were then measured using the NanoDrop™ 2000 (Thermo Scientific) spectrophotometer and a value for DNA yield (ng DNA/mg faeces) was then calculated. DNA extraction was carried out on each pooled faecal sample (n=24) a total of 3 times on 3 different days to assess the inter-assay coefficient of variation (CV) for the DNA extraction procedure from chicken faeces, this was 26% for DNA concentration. Results for DNA quality were too variable, including some very large or negative values, therefore values for CV are not reported as they were deemed too inaccurate to be meaningful. Fifty microliter aliquots from the 3 replicate extractions of the same sample were pooled together and the concentration of the DNA pool assessed using the NanoDrop. DNA integrity was also assessed by gel electrophoresis on a 1% agarose gel, see Materials and Methods 2.6.

#### *5.1.4.3 Quantitative PCR*

A single extracted chicken faecal DNA sample and the chicken calf muscle DNA (positive control) were each diluted to a concentration of 20ng/µl and then each diluted again to create a two 4-fold dilution series, to be used as a standard curves to assess the qPCR efficiency when tested with chicken *CYTB* DNA and bacterial *16S* DNA primers (see Materials and Methods 2.7 and 2.10). The resulting qPCR efficiencies for the chicken *CYTB* DNA standard curves were

1.93 for faecal DNA and 2.08 for chicken muscle DNA. For the bacterial *16S* DNA standard curves, the qPCR efficiency for faecal DNA was 1.98 and 2.74 for chicken muscle DNA. As a result of the qPCR efficiencies a 4-fold dilution series of a pool of chicken faecal DNA was used to create the standard curves for final qPCR analysis. Each extracted combined faecal DNA sample was diluted to 5ng/µl, except for 3 samples which already had a concentration value below 5ng/µl. An aliquot of each of the faecal DNA samples, diluted to 5ng/µl, were pooled and used to create the 4-fold dilution series for the standard curve as previously described, except the 3 samples with a concentration less than 5ng/µl. All the faecal DNA samples to test against the standard curve were then diluted a further 1 in 8 so that the results would be in the linear range of the standard curve. The 3 faecal samples with a concentration <5ng/µl were tested neat. The qPCR was carried out for each target gene, chicken *CYTB* DNA and bacterial *16S* DNA, on 384-well plates (Roche Diagnostics) using SYBR Green qPCR methodology (Roche Diagnostics) as outlined in Materials and Methods 2.10. The standard curve and the faecal DNA samples were tested in triplicate. A no template control (DNase and RNase free water) was also tested in triplicate, at 2 different points, on the qPCR plate.

#### *5.1.4.4 Data analysis*

All data were analysed in Genstat 19$^{th}$ Edition, apart from Pearson's correlation analysis which was performed in GraphPad Prism (version 7.03). Significance was defined as P≤0.05 and a trend as a P value >0.05 and <0.1. The effects of dietary treatment on bird performance (body weight, feed intake, body weight gain, or feed conversion ratio) were assessed by 1-way ANOVA. The effect of diet on faecal DNA assessments (DNA yield and DNA quality) and composition (*CYTB*, *16S* and *CYTB*/*16S*) were assessed by 1-way (diet) ANOVA, blocking for pen, as there were 3 faecal samples collected and analysed per pen. The relationship between faecal host DNA at day 42 and corrected FCR (21-42 days) was calculated using Pearson's correlation.

### 5.1.5 Results

#### *5.1.5.1 Bird performance*

There were no significant effects of the phytase (2500FTU) inclusion diet on bird performance (body weight, feed intake, body weight gain, or feed conversion ratio) throughout the entire 42 day trial when compared to birds receiving the control diet (**Table 5.1**).

Table 5.1 The effect of a phytase (2500FTU) inclusion diet on bird performance.

| | Dietary treatment | | | P value from one-way ANOVA (diet) |
|---|---|---|---|---|
| **Bird performance** | **Control** | **Phytase (2500FTU)** | **SED** | |
| Mean BW day 0 (g) | 40.8 | 40.5 | 0.54 | 0.643 |
| Mean BW day 21 (g) | 855.9 | 886.9 | 24.65 | 0.254 |
| Mean BW day 42 (g) | 3385.3 | 3469.6 | 45.66 | 0.114 |
| FI 0-21 days (g/bird) | 1097 | 1099 | 21.71 | 0.940 |
| FI 21-42 days (g/bird) | 3892 | 3889 | 161.92 | 0.984 |
| FI 0-42 days (g/bird) | 4902 | 4996 | 80.74 | 0.287 |
| BWG 0-21 days (g/bird) | 792 | 820 | 30.89 | 0.394 |
| BWG 21-42 days (g/bird) | 2462 | 2497 | 47.97 | 0.485 |
| BWG 0-42 days (g/bird) | 3340 | 3424 | 44.99 | 0.112 |
| Corrected pen FCR 0-21 days (FI/BWG) | 1.36 | 1.31 | 0.047 | 0.361 |
| Corrected pen FCR 21-42 days (FI/BWG) | 1.55 | 1.57 | 0.022 | 0.359 |
| Corrected pen FCR 0-42 days (FI/BWG) | 1.51 | 1.52 | 0.034 | 0.948 |

BW – body weight, FI – feed intake, BWG – body weight gain, FCR – feed conversion ratio.

#### *5.1.5.2 Effect of a phytase inclusion diet on chicken faecal DNA*

*Total faecal DNA content and quality*

Two values had to be removed from the data set prior to analysis due to there being negative values for 260nm/280nm (-0.34 and -16.49), these results were deemed inaccurate. There was no significant (P=0.737) effect of a phytase inclusion diet on the DNA yield (ng DNA/mg faeces) extracted from chicken faeces (**Figure 5.1A**). Equally, there was no significant (P=0.436) effect

of a phytase inclusion diet on DNA quality assessed by 260nm/280nm (**Figure 5.1B**).

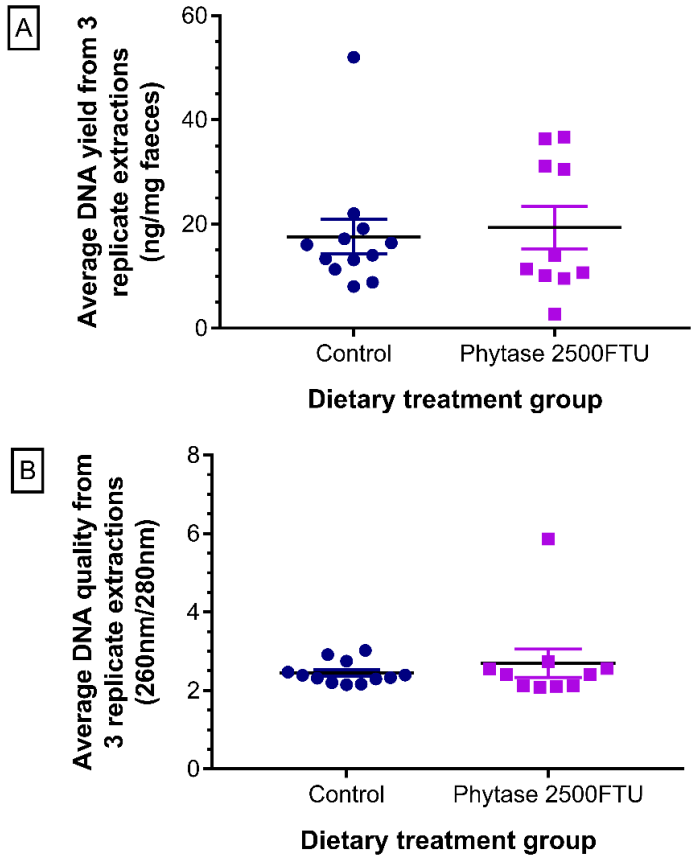


**Figure 5.1** The effect of a phytase (2500FTU) inclusion diet on [**A**] DNA yield (ng DNA/mg faeces) and [**B**] DNA quality (260nm/280nm) of the DNA extracted from chicken faeces from 3 replicate extraction procedures. Error bars are ± SEM. n=12 biological replicates for the control group. n=10 biological replicates for the phytase group, as 2 values were deemed inaccurate and removed due to negative values for 260nm/280nm.

*Faecal DNA composition*

There was no significant effect of a phytase (2500FTU) inclusion diet on the relative chicken *CYTB* DNA (P=0.493) content in the chicken faeces, however, there was a trend for an effect of diet on the relative bacterial *16S* DNA (P=0.077) content in the chicken faeces (**Figure 5.2A** and **Figure 5.2B** respectively). As the effect of the diet on bacterial *16S* DNA was only a trend and not statistically significant, faecal chicken *CYTB* DNA was normalised for

bacterial *16S* DNA content in the faeces. There was still no significant effect of diet on the normalised chicken *CYTB* DNA, although the P value decreased compared to the P value prior to normalisation (P=0.166 vs. P=0.493) (**Figure 5.2C**).

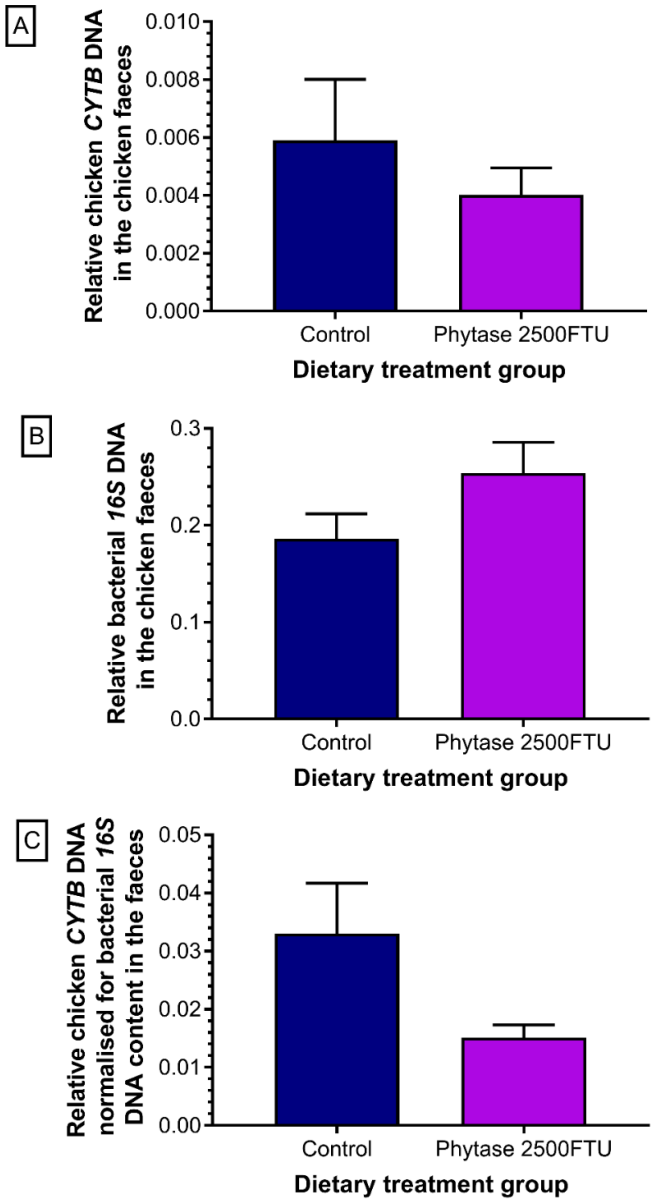


Figure 5.2 The effect of a phytase (2500FTU) inclusion diet on [A] Relative chicken *CYTB* DNA in the chicken faeces, [B] Relative bacterial *16S* DNA in the chicken faeces and [C] Relative chicken *CYTB* DNA normalised for bacterial *16S* DNA content in the faeces. There was a trend for an effect of diet on the relative bacterial *16S* DNA (P=0.077) content. Means ± SEM. n=12 biological replicates for all groups.

*5.1.5.3 Relationship between host faecal DNA and animal performance*

There was no significant correlation (P=0.9751, r=0.01327) between chicken *CYTB* DNA in the faeces from birds collected at 42 days of age and the FCR corrected for mortality (21-42 days) (**Figure 5.3**).

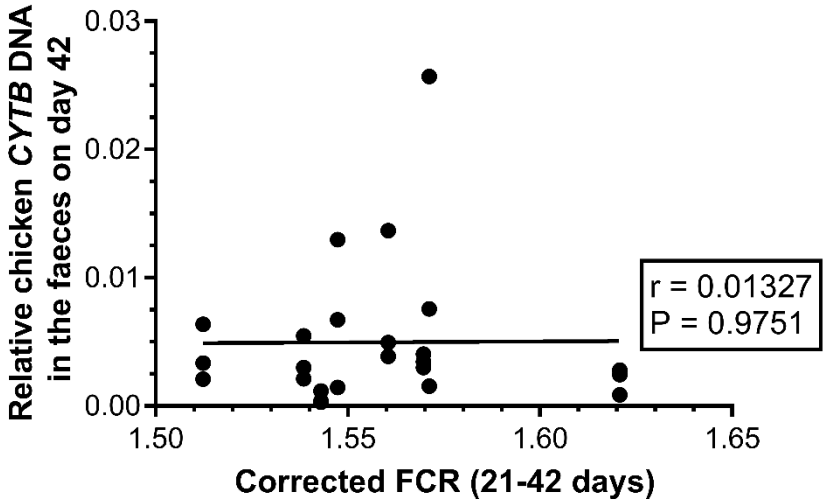


Figure 5.3 The relationship between chicken *CYTB* DNA at in the faeces at day 42 and the corrected FCR between days 21 and 42. P and r value displayed on the graph.

### 5.1.6 Discussion

The inclusion of phytase at 2500FTU had no impact upon animal performance in this study. Levels of phytase greater than 1,500FTU are known as 'super-dosing' concentrations as this is an inclusion level much higher than that typically added in commercial diets (e.g. 500FTU and 1000FTU), although some authors believe super-dosing is an inclusion level of 2500FTU or higher (Adeola and Cowieson, 2011; Walk *et al.*, 2014; Beeson *et al.*, 2017). Therefore, the levels of phytase supplemented in this study would be considered a super-dose regardless. As a result, the lack of a response in animal performance is unexpected as many studies have found benefits of phytase super-dosing, especially an improved FCR, even at levels below that used in this study (Walk *et al.*, 2014; Beeson *et al.*, 2017). A potential lack of effect in this study could be due to the number of replicates being limited to 4 per dietary treatment, as this is a subsection of a larger experiment.

As expected, there was no significant effect of a phytase inclusion diet on the DNA yield or quality of DNA extracted from faecal samples collected at 42 days of age. In order for the developed methodology to be utilised to assess the effect of an experimental diet on gut cell loss, the DNA extraction procedure should not be influenced by the factor being examined, in this case dietary manipulation. In addition, there was no impact of the phytase inclusion diet on chicken *CYTB* DNA present in the faecal samples. There was however a trend for an effect of phytase on bacterial *16S* DNA present in the faeces, whereby the relative levels were increased in the phytase treated group. There have been fewer studies assessing the effect of phytase on intestinal and faecal microflora compared with other exogenous enzymes such as xylanase. Ptak *et al.* (2015) observed a significant increase in the total bacterial content of ileal digesta in broilers fed a phytase inclusion diet compared with birds on a diet adequate in phosphorus, indicating the phytase itself may stimulate changes in the microflora. Examination of the bacterial composition found that there were specific bacterial species that were affected, either increased or decreased, in birds fed a phytase inclusion diet (Ptak *et al.*, 2015). In this study, normalisation of chicken *CYTB* DNA for the total bacterial *16S* DNA was only carried out as there was not a significant effect on bacterial DNA, and while this did not elude any significant differences the P value decreased for the effect of a phytase inclusion diet.

As previously discussed, phytase super-dosing has been shown to positively influence FCR, as well as, the gross structure and morphology of the small intestine (Wu *et al.*, 2004). Conversely, another study showed no effects on gut morphology of phytase supplementation at 5000FTU in broilers compared with birds on a diet adequate in phosphorus, despite a decrease in feed intake from 15-28 days of age leading to an improved FCR during this period (Khodambashi Emami *et al.*, 2013). In the present study, there were no effects of 2500FTU phytase on FCR and equally no effects on faecal chicken *CYTB* DNA nor a relationship between the two measurements. If our hypothesis is correct with regards to host DNA present in faeces being directly proportional

to the levels of gut cell sloughing, which negatively impacts upon feed efficiency, then the results of this study are as expected due to neither component having a differential which could be measured by the *CYTB* DNA assay. Alternatively, this data shows that further work is required to identify the sensitivity of the chicken *CYTB* DNA.

## 5.2 WHEAT BRAN TRIAL

### 5.2.1 Introduction

Following the results of the broiler phytase trial whereby no significant differences were observed, indicating a lack of an effect of phytase on gut cell sloughing or limitations to the sensitivity of the *CYTB* DNA assay, a further poultry trial was conducted. The additional study was conducted to determine the effects of a wheat bran inclusion diet on bird performance, faecal and digesta DNA content, as well as the relationship between bird performance and the chicken *CYTB* DNA content in the faeces.

Wheat bran is the outer layer of a wheat grain, which encases the energy dense endosperm containing starch for germination, and is separated from the endosperm during the milling of wheat for food and feed (Stevenson *et al.*, 2012). Wheat bran has a very high fibre content, particularly insoluble fibre which makes up 35-48% of wheat bran compared with only 1.5-4% of soluble fibre (Vitaglione *et al.*, 2008). Insoluble dietary fibre decreases gut transit time and decreases nutrient digestibility alongside elevating endogenous losses (Montagne *et al.*, 2003). The decreased transit time can lead to an increased feed intake which will impact upon FCR. In contrast, the soluble fibre content of wheat bran increases digesta viscosity which reduces nutrient utilisation (Bedford, 1996) and may therefore impede body weight gain. Wheat bran also contains large amounts of phytic acid (Stevenson *et al.*, 2012) which is thought to be an anti-nutrient due to the complexes it forms with minerals, leading to poor solubility in the gastrointestinal tract (Cowieson *et al.*, 2011) and this can impair nutrient absorption. These effects on the digestive process have been shown to increase the lengths and weights of the sections of the gastrointestinal tract, increase cellular proliferation and negatively impact upon gut morphology in pigs and poultry, which may lead to an increased maintenance energy requirement due to the metabolic demand for visceral organ development and maintenance (Jha *et al.*, 2019; Kerr and Shurson, 2013). Jin *et al.* (1994) fed pigs either a control diet or 10% wheat straw and observed significantly ($P<0.03$) more crypt cell nuclei labelling with bromodeoxyuridine

(BrdU) in the jejunum and colon tissues indicating a greater level of DNA synthesis and cell proliferation. In addition, levels of RNA were higher in the colon of the wheat straw treated group, also indicating a greater level of protein synthesis, although no differences were observed in animal performance (Jin *et al.*, 1994).

The aim of this study was to assess whether a diet containing 20% wheat bran would influence bird performance and the DNA composition of both digesta and faecal samples. Particularly whether there would be an impact of a wheat bran inclusion diet upon the *CYTB* DNA content of both digesta and faeces, potentially indicating effects on gut cell sloughing. Analysing digesta DNA composition throughout the gastrointestinal tract may elucidate the relative levels of chicken *CYTB* DNA at various sections within the tract and provide a clearer picture as to where the faecal chicken *CYTB* DNA may originate from.

### 5.2.2 Hypotheses

1. Birds receiving the wheat bran inclusion diet will have a reduced performance, particularly FCR, due to either an increase feed intake or reduced body weight gain.
2. There will be no effect of diet or time on faecal DNA yield and quality, and no differences between samples collected from individual birds or the pen floor.
3. There will be no effect of diet or gut section on the digesta DNA yield and quality.
4. Birds receiving a wheat bran inclusion diet will have a greater level of host DNA in their faeces compared to the controls.
5. Pen floor faecal samples will have a faecal DNA composition which corresponds to those samples collected from individual birds.
6. There will be a relationship between faecal host DNA and FCR.
7. Birds receiving a wheat bran inclusion diet will have a greater level of host DNA in their digesta from each section of the digestive tract, and

the relative levels of host DNA and bacterial DNA will increase from the duodenum through to the colon.

### 5.2.3 Objectives

1. Assess the effect of a wheat bran inclusion diet on bird performance.
2. Assess whether there are any effects of a wheat bran inclusion diet on chicken faecal and digesta DNA yield and quality, and whether date of collection or how the faecal samples were collected impacts upon these measures.
3. Assess whether there are any effects of a wheat bran inclusion diet and length of time on the diet on chicken faecal DNA composition, host DNA and bacterial DNA content.
4. Determine whether collecting samples from the pen floor provides faecal DNA results which correspond to those collected from known individual birds.
5. Identify whether there is any relationship between faecal host DNA and bird performance, particularly FCR.
6. Assess whether there is an effect of a wheat bran inclusion diet and gut section of origin on digesta DNA composition and relative DNA levels.

### 5.2.4 Materials and Methods

#### *5.2.4.1 Animal trial and sample collection*

The trial was carried out at the University of Nottingham and approved by the internal ethical review committee (ethical approval number: 000171). No 'regulated procedures' were carried out and therefore the study was carried out under DEFRA regulations and did not require a Home Office licence. The feeding trial design is outlined in **Figure 5.4**. Birds were sourced from PD Hook's Cote hatchery in Bampton Oxfordshire (Postcode: OX18 2EG). Birds arrived at the unit on day of hatch and were given a wing tag on arrival day so that individual birds could be identified throughout the study. Diets were formulated by Target Feeds Ltd, UK (**Appendix 5**). Birds were housed in floor level pens with dimensions of 1m x 2m and the bedding used was Eco Chip Pure

Grade 5 (Datesand Ltd, Manchester, UK). The lighting conditions were a 12-hour light: 12-hour dark cycle and the temperature started at approximately 31˚C on day 0, and controlled to decrease by approximately 2˚C each week, humidity was also controlled according to the appropriate guidelines. Thirty six Ross 308 birds (46.3±0.5 g) were brought in on day of hatch and provided with the control diet (**Appendix 5**) until 4 days of age. At 4 days of age, 18 birds (80.4±2.2 g) were assigned to a control group and continued to receive the control diet and the other 18 birds (81.4±1.7 g) were assigned to a diet containing 20% wheat bran, diets were balanced for energy and protein (**Appendix 5**). Birds were housed in pairs, meaning there were 9 replicate pens per treatment. Birds were weighed at 0, 4, 11, 18, 25 and 32 days of age and then on the day of cull, which was either at 33 days of age (5 pens per treatment) or 34 days of age (4 pens per treatment). Feed and water were offered *ad libitum*. For the first 2 weeks of the trial period, birds were offered feed in open troughs which were large enough for the birds to jump into and this led to some feed spillage into the bedding and therefore some measurements of feed intake in the first half of the study may be slightly inaccurate. At the beginning of the third week of the trial period, feed was offered in feed hoppers which prevented the feed spillage problems. In order to measure feed intake a set amount of feed was weighed out for each pen of birds at the beginning of each week, feed troughs/hoppers were filled up as required throughout the week and the feed remaining in both the feed bags and troughs/hoppers at the end of each week were weighed back and subtracted from the total available feed for the week, the difference being the value for feed eaten. Feed conversion ratio was calculated on a weekly basis as the pen feed intake divided by the pen body weight gain, as although individual bird weights were taken a measure for individual feed intake was not possible.

Faecal samples were collected from individual birds at weighing by collecting faeces from the weighing bucket which was cleaned between different birds using a sterile wipe. At the later stages of the trial (days 18 and 25 of age), two faecal samples were collected from each pen floor as well.

Faecal samples were only collected from the pen floor at the later stages of the trial, rather than from the beginning, as this was when the faeces were large enough to be differentiated and separated from the bedding. Faeces were collected from the pen floor as well as the weighing bucket so that comparisons on the DNA assay results could be made between clean faeces and that contaminated with bedding. The days of faecal sample collection and the number collected per dietary treatment is illustrated in **Figure 5.4**. Faeces were collected into sealable plastic bags and stored at -20°C until processed. At the end of the study (days 33 and 34), birds were euthanised by the injection of an overdose of barbiturate by wing vein. Dissections were carried out by the same researcher (Kimberley Slinger) to ensure consistency in the measurements, and therefore birds were culled over two days to ensure there was not too much to do on a single day. Approximately 1.5ml of digesta was collected for each bird into 2ml tubes from the following sections of the gastrointestinal tract: the duodenum, jejunum, ileum and colon (see **Figure 5.4**). The duodenum is defined as the section of the small intestine surrounding the pancreas, and digesta was taken from the latter half of the duodenal loop in order to obtain enough digesta for subsequent analysis. The jejunum is defined as the section of the small intestine from the end of the duodenal loop to the Meckel's diverticulum, and digesta was obtained from the midpoint of the jejunum. The ileum is defined as the small intestine section from Meckel's diverticulum to the ileocecal junction, and digesta was collected from the ileum approximately 3cm distal of Meckel's diverticulum. The colon is defined as the section of the gastrointestinal tract from the ileocecal junction to the cloaca, and digesta was collected from the end of the colon just prior to the cloaca. Digesta samples were immediately put on dry ice and then stored at -70°C until processed.

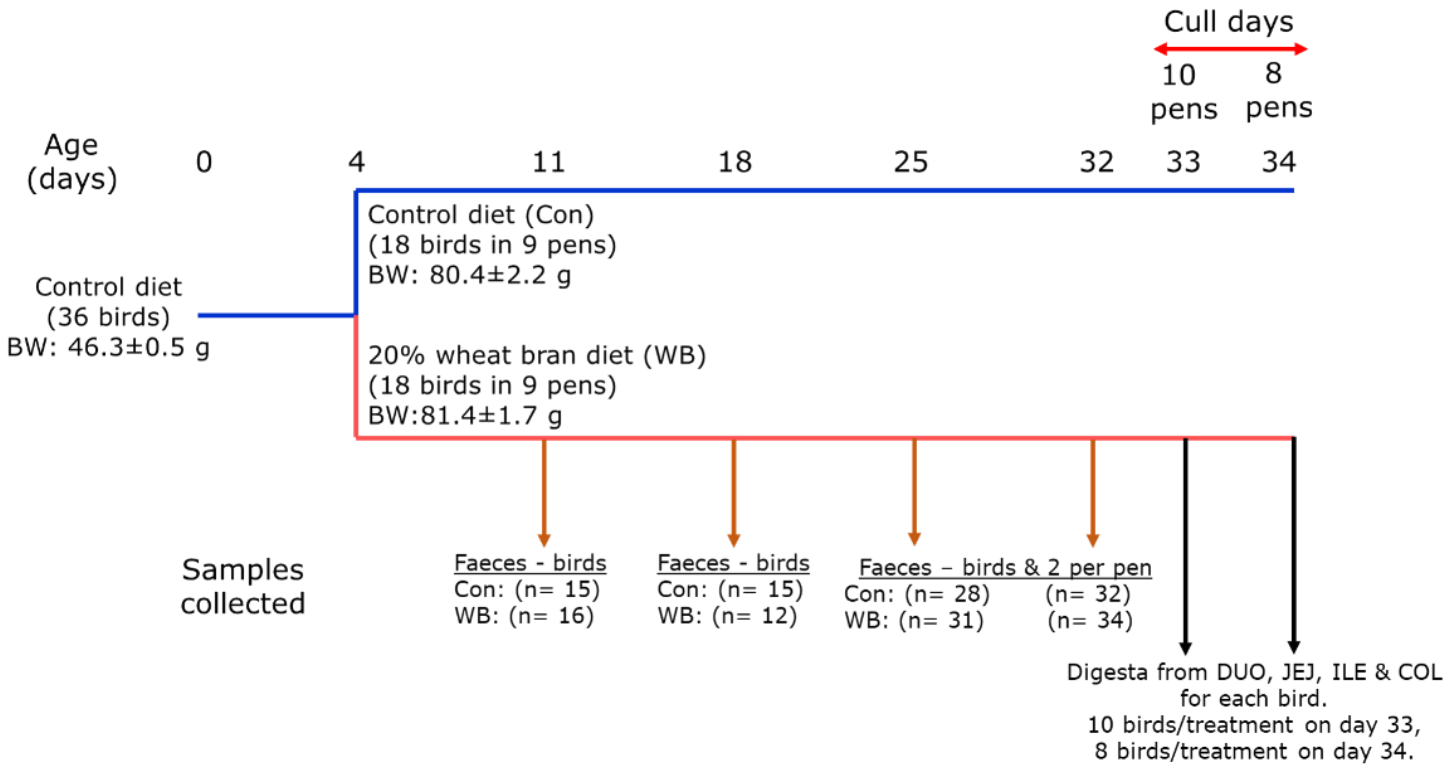


Figure 5.4 The chicken wheat bran study design and sample collection. Faecal samples were collected from known individual birds at each time point, by collection from a bucket. Two faecal samples were collected from each pen floor on days 18 and 25, when faecal samples were large enough for clear differentiation and separation from the bedding. and Labels: BW – body weight, DUO – duodenum, JEJ – jejunum, ILE – ileum and COL – colon. 'n' numbers displayed are biological replicates.

#### *5.2.4.2 DNA Extraction*

DNA was extracted from each faecal sample collected from known individual birds (n=111) or the pen floor (n=72) and from digesta samples from 1 bird per pen (n=72). DNA was extracted using the method outlined in Materials and Methods 2.4, which briefly involved bead-beating ~200mg of faeces or digesta in MagNA Lyser Green Bead Tubes (Roche Diagnostics) with lysis Buffer ASL (Qiagen) secured in a MagNA Lyser instrument (Roche Diagnostics), followed by an adapted protocol for the QIAamp® DNA Stool Mini Kit (Qiagen) as previously described (see 2.4.1). DNA yield and quality was assessed by spectrophotometry and gel electrophoresis as previously described (Materials and Methods 2.5 and 2.6).

#### *5.2.4.3 Quantitative PCR*

*Initial test run of samples*

Initially each DNA sample from faeces or digesta was diluted to 5ng/µl onto a 96-well plate. Samples with a starting concentration of <5ng/µl were loaded onto the 96-well plate neat. Each sample was then tested singularly on a 384-well qPCR plate (Roche Diagnostics) using SYBR Green qPCR methodology with either chicken *CYTB* primers or bacterial *16S* primers to assess the range of crossing point (Cp) values, as qPCR from digesta hadn't been attempted previously (Materials and Methods 2.7 and 2.10).

*Assessment of standard curves*

Following the initial test run of each sample with each primer set, it was clear that there was an effect of the different DNA sample origin (the 4 gut sections and faeces) on the range of Cp values for each gene target. Therefore, the next step was to analyse the linearity of standard curves originating from the 5 different sample types for each gene of interest. Five separate DNA pools were created (faecal, duodenum, jejunum, ileum and colon) by taking a 5µl aliquot of each diluted DNA sample from the appropriate origin and mixing the total volume by vortexing. The 5 DNA pools were then diluted to create a 1 in 4 dilution series to be used as standard curves for qPCR using both the chicken

*CYTB* DNA and bacterial *16S* DNA primer sets. Ten standard curves were produced and analysed for the range in which the standard curve remained linear and the PCR efficiency within the linear phase (**Figure 5.5**, see Materials and Methods 2.10 for an explanation of PCR efficiency). As observed in **Figure 5.5** there was a large range in starting Cp values between the different standard curves. For example, the average starting Cp value for bacterial *16S* DNA from faeces was 14, but it was 26 from duodenum samples, indicating a 4,096 fold difference in the levels of bacterial *16S* DNA between samples originating from these 2 sources (**Figure 5.5B**). Equally, the linear range of the *16S* DNA standard curves were greatly reduced from samples originating from digesta of the duodenum and this improved the further down the avian digestive tract digesta samples were collected from (**Figure 5.5B**). Conversely, the linearity and PCR efficiencies of *CYTB* DNA standard curves were comparable between different sample origins (**Figure 5.5A**).

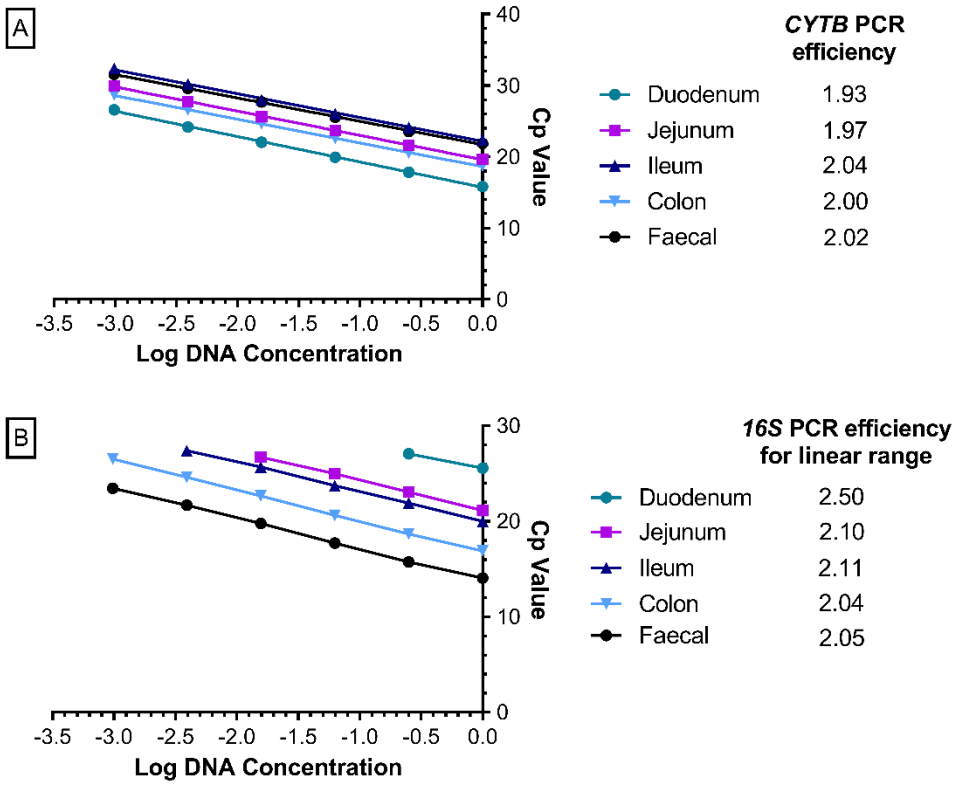


**Figure 5.5** The qPCR standard curves produced from a 1 in 4 dilution series of 5 different DNA pools when amplified with either [**A**] chicken *CYTB* DNA primers or [**B**] bacterial *16S* DNA primers. Only the linear range of the standard curves are displayed in the figures and the PCR efficiencies for this range are also displayed.

In order to be able to compare DNA composition between different DNA sources, one standard curve would need to be used to compare results from individual samples to. In addition to analysing the PCR efficiencies of the different standard curves, the standard curves were also compared to each other using the delta Cp method. Similar to comparing PCR efficiencies, the delta Cp method assesses whether the PCR used to create the standard curve values behaves the same way between different samples. Although there may be a difference in the levels of specific gene target (e.g. chicken *CYTB*) in the sample, resulting in the different starting Cp values, the standard curves should have the same PCR efficiency in order for them to be comparable to each other. Delta Cp was calculated as the difference between a Cp value from one standard curve to another and this was done for each log DNA concentration

value present on the individual standard curves. The delta Cp values can then be plotted on a graph with a trend line through the values for comparison (**Figure 5.6**). Typically standard curves with the lowest starting Cp value and the largest linear range were used to compare the other standard curves to, i.e. colon for chicken *CYTB* and faecal for bacterial *16S* (**Figure 5.6**). The slope of the trend line indicates whether the standard curves are comparable, a slope value between -0.15 and 0.15 was accepted as the two standard curves behaving the same way, as a value of 0 would indicate no difference between the 2 standard curves (**Figure 5.6**). As observed in **Figure 5.6** the duodenum standard curves did not behave the same way as any other standard curve assessed, this was likely due to the extremes in the levels of each gene target present in samples from this region. However, all other DNA pools behaved similar to each other in the PCR reaction with slope values within the permitted range (see **Figure 5.6**). Based on all the analysis carried out, it was decided that the 1 in 4 dilution series for colon DNA was used as the standard curve for chicken *CYTB* DNA analysis, as the PCR efficiency was 2 and the standard curve began at a Cp value of approximately 18, the lowest after the duodenum samples (**Figure 5.5A**). For bacterial *16S* DNA analysis, a 1 in 4 dilution series for faecal DNA was used as the standard curve, as the PCR efficiency was 2.05 and the standard curve began with the lowest Cp value of approximately 14 (**Figure 5.5B**).

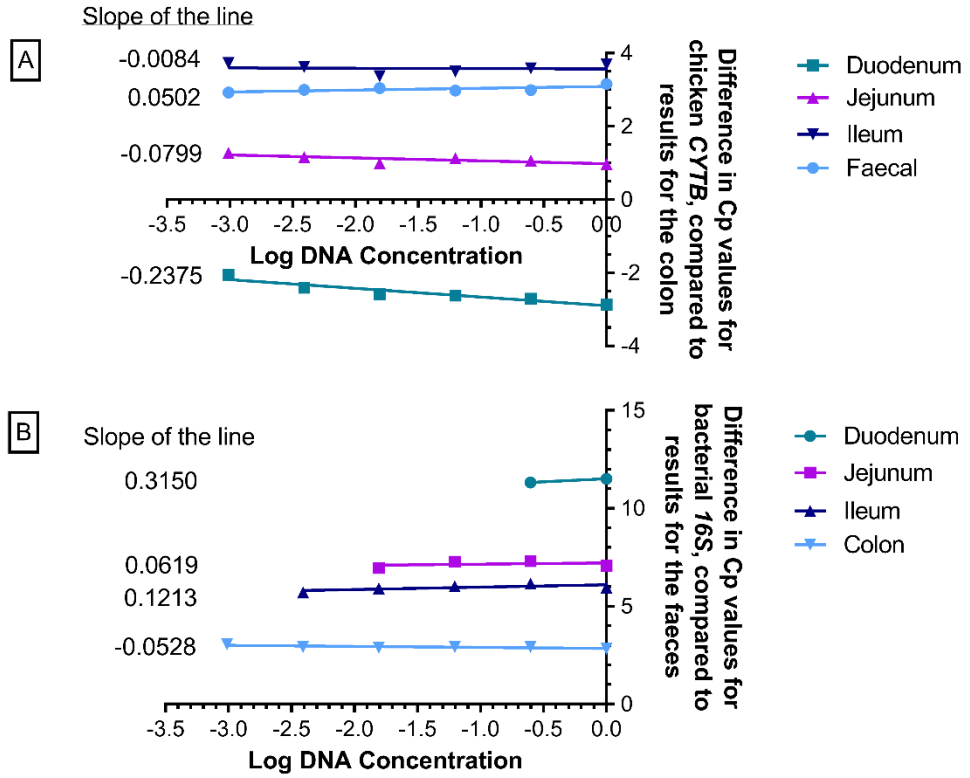


**Figure 5.6** Comparison of qPCR standard curves created from different DNA pools using either [**A**] chicken *CYTB* DNA primers or [**B**] bacterial *16S* primers. Slopes from the equation of the line are displayed in the graphs. A slope value of between -0.15 and 0.15 was accepted as the standard curves behaving the same way in the qPCR reaction independent of the DNA pool used. The duodenum standard curve was not comparable to any other standard curve assessed.

*Final analysis of samples*

The results for the Cp value range from the initial test run of samples and the results from the standard curves were used to decide how to dilute samples in order for them to be detected within the linear range of the chosen standard curves for each gene target. The chosen dilutions are presented in **Table 5.2**. For final qPCR analysis for either chicken *CYTB* DNA or bacterial *16S* detection samples, standard curves were produced fresh on the day of analysis. Mastermix was loaded into appropriate wells on two 384-well plates (Roche Diagnostics) per gene of interest (see Materials and Methods 2.7 and 2.10 for primer details and mastermix composition). The appropriate standard curve for

the gene of interest was then loaded in triplicate on each qPCR plate. Samples to be tested were loaded evenly and in duplicate over the two qPCR plates so that effectively half the trial was tested on each plate. No template controls (NTC) were used on each plate, analysed at 2 different points on the plate and in triplicate; these contained mastermix and 5µl of RNase and DNase free water. The qPCR cycle conditions are outlined in Materials and Methods 2.10. A melt curve cycle was also included at the end of each reaction. The standard curves were used to calculate the relative levels of the genes of interest present in the different samples, where a sample had been diluted the results were multiplied by the dilution factor.

Table 5.2 Dilution factors for DNA samples from different origins used for final quantitative PCR analysis with either chicken *CYTB* DNA or bacterial *16S* DNA primers.

| | **Dilution used for each PCR reaction** | |
|---|---|---|
| **Origin of DNA samples** | **Chicken *CYTB* DNA using standard curve created from pooled colon DNA samples** | **Bacterial *16S* DNA using standard curve created from pooled faecal DNA samples** |
| **Faeces** | 1 in 16 | 1 in 32 |
| **Duodenum** | 1 in 64 | Not diluted – tested neat |
| **Jejunum** | 1 in 16 | Not diluted – tested neat |
| **Ileum** | 1 in 4 | Not diluted – tested neat |
| **Colon** | 1 in 16 | 1 in 16 |

#### *5.2.4.4 Data analysis*

Statistical analysis by ANOVA and associated post hoc tests were analysed using Genstat 19th Edition. Pearson's correlations were assessed using GraphPad Prism (version 7.03). The effect of diet on animal performance measures (body weight, pen feed intake, pen body weight gain and pen FCR) was assessed by one-way ANOVA. The effects of diet and time on faecal DNA yield (ng DNA/mg faeces) and quality (260nm/280nm) were analysed by two-way ANOVA for an unbalanced design, for samples collected from individual birds and samples collected from the pen floor separately. The effects of diet

and gut section on digesta DNA yield (ng DNA/mg digesta) and quality (260nm/280nm) were analysed by two-way ANOVA. Quantitative PCR analyses of faecal DNA (chicken *CYTB* DNA, bacterial *16S* DNA and *CYTB* DNA normalised for *16S* DNA) were analysed by two-way (date x diet) ANOVA for an unbalanced design, blocking for plate. Quantitative PCR analyses of digesta DNA (chicken *CYTB* DNA, bacterial *16S* DNA and *CYTB* DNA normalised for *16S* DNA) were analysed by two-way (diet x gut section) ANOVA, blocking for plate. Bonferroni post hoc analysis was used to analyse significant effects. Pearson's correlation was used to assess the relationship between faecal chicken *CYTB* DNA content and FCR. Significance was taken as $P \leq 0.05$ and trends reported as between $P > 0.05$ and $P < 0.10$.

### 5.2.5 Results

#### *5.2.5.1 Bird performance*

There was a 5.5% mortality over the trial, with 1 bird from the control group dying unexpectedly and 1 bird from the wheat bran group being culled due to poor health. This was probably due to the fact birds were in pairs, rather than larger groups. Both birds were replaced with another (spare) bird of a similar weight which had been on the appropriate diet for the same length of time. There was no effect of a 20% wheat bran inclusion diet on bird performance throughout the trial (see **Table 5.3**) when assessing body weight, pen feed intake, pen body weight gain or pen FCR, except for the feed intake between 11 and 18 days of age, where there was a significant ($P < 0.001$) reduction in the wheat bran group.

Table 5.3 Bird performance data for the wheat bran broiler trial.

| Bird performance | Dietary treatment | | SED | P value from one-way ANOVA (diet) |
|---|---|---|---|---|
| | Control | 20% Wheat Bran | | |
| Mean BW at day 4 (g) | 80.4 | 81.4 | 2.82 | 0.711 |
| Mean BW at day 11 (g) | 211.7 | 203.6 | 11.70 | 0.490 |
| Mean BW at day 18 (g) | 489 | 490 | 31.7 | 0.982 |
| Mean BW at day 25 (g) | 952 | 949 | 51.2 | 0.948 |
| Mean BW at day 32 (g) | 1580 | 1581 | 78.6 | 0.990 |
| Pen FI D4-D11 (g) | 492 | 461 | 33.1 | 0.374 |
| Pen FI D11-D18 (g) | 1352 | 1186 | 26.8 | **<0.001** |
| Pen FI D18-D25 (g) | 1315 | 1321 | 82.9 | 0.943 |
| Pen FI D25-D32 (g) | 1943 | 1921 | 102.9 | 0.833 |
| Pen BWG D4-D11 (g) | 267 | 245 | 20.1 | 0.304 |
| Pen BWG D11-D18 (g) | 558 | 573 | 52.0 | 0.777 |
| Pen BWG D18-D25 (g) | 926 | 899 | 58.7 | 0.654 |
| Pen BWG D25-D32 (g) | 1256 | 1264 | 65.7 | 0.898 |
| Pen FCR D4-D11 (Pen FI/ Pen BWG) | 1.91 | 1.92 | 0.224 | 0.993 |
| Pen FCR D11-D18 (Pen FI/ Pen BWG) | 2.48 | 2.21 | 0.27 | 0.321 |
| Pen FCR D18-D25 (Pen FI/ Pen BWG) | 1.42 | 1.47 | 0.038 | 0.202 |
| Pen FCR D25-D32 (Pen FI/ Pen BWG) | 1.55 | 1.53 | 0.059 | 0.783 |
| Final BW Day 33 (g) | 1627 | 1662 | 120.4 | 0.777 |
| Final BW Day 34 (g) | 1838 | 1796 | 115.9 | 0.722 |

BW- body weight, FI – feed intake, D – bird days of age, BWG – body weight gain, FCR – feed conversion ratio.

### *5.2.5.2 DNA extraction results*

*Total faecal DNA content and quality*

There were consistent issues with the faecal DNA extraction from the chicken faeces of the wheat bran trial. The limit of detection for DNA concentration for the NanoDrop (Thermo Scientific) is published at 2ng/µl.

Regularly the DNA concentration following extraction from faeces in this study were below 5ng/µl with some below 2ng/µl and sometimes reported as negative values, due to being well below the limits of detection of the instrument. As a result, some values for DNA yield (ng DNA/mg faeces) were negative and therefore excluded from the statistical analysis. Equally, some values for DNA quality (260nm/280nm) were also negative or very large (e.g. 75.40) and were also deemed inaccurate. Hence any DNA quality values which were reported as negative or above a value of 3 were removed from the statistical analysis.

Faecal DNA results were analysed by two-way (diet x time) ANOVA for an unbalanced design. There was no interaction (P=0.734) between diet and time and no singular effects of diet (P=0.803) or time (P=0.310) on DNA yield (ng DNA/mg faeces) of samples collected from individual birds (**Figure 5.7A**). Conversely, there was a significant (P<0.001) effect of time on the DNA quality (260nm/280nm) of faecal samples collected from individual birds, but no interaction between diet and time (P=0.105) or a singular effect of diet (P=0.895) (**Figure 5.7B**). The optimal 260nm/280nm value for 'pure' DNA is 1.8. Bonferroni post hoc analysis for the effect of time on faecal DNA quality of samples collected from individual birds showed that samples collected at week 3 were of significantly poorer quality than those collected at the other 3 time points (**Table 5.4**).

When analysing the results for faecal samples collected from the pen floor there was a significant effect of time (P=0.05) on DNA yield, but no effect of diet (P=0.731) or an interaction between time and diet (P=0.628) (**Figure 5.7C**). The DNA yield was significantly greater in samples collected from the pen floor at week 4 compared with week 3 (**Figure 5.7C**). In contrast, there was no effect of time in samples collected from individual birds, and potentially a greater DNA yield at week 3 compared with week 4, however, there appeared to be a greater level of error in results collected from individual birds (see **Figure 5.7A**). There was no interaction (P=0.682) between diet and time on DNA quality of samples collected from the pen floor and no singular effects of diet

(P=0.992) or time (P=0.943) (**Figure 5.7D**). In addition, the DNA quality was marginally better in samples collected from the pen floor (mean value of 2.12) compared with samples collected from individual birds (mean value of 2.19), but this is unlikely to be significant.

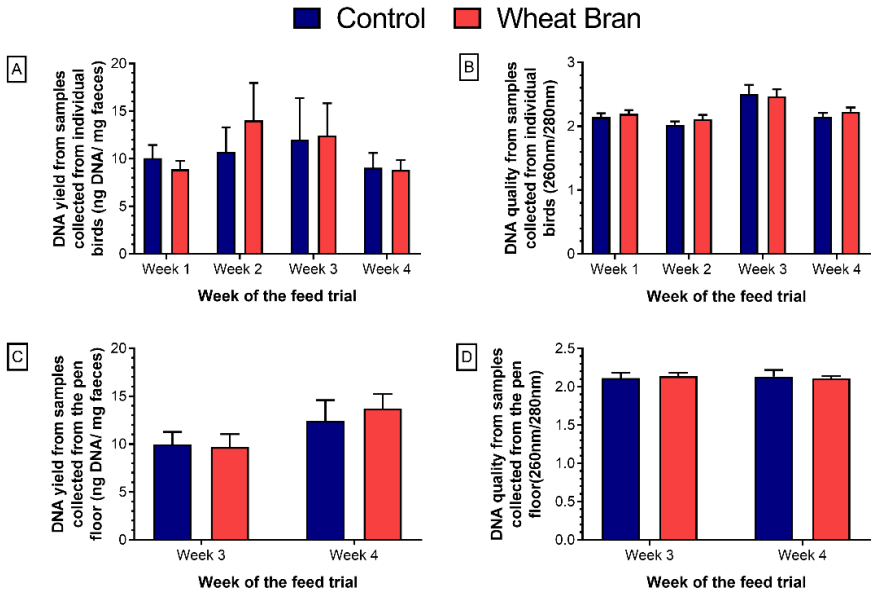


Figure 5.7 The effect of a wheat bran inclusion diet on [A] the DNA yield and [B] the DNA quality from faecal samples collected from individual birds and [C] the DNA yield and [D] the DNA quality from faecal samples collected from the pen floor. DNA yield is expressed as ng DNA/mg faeces and DNA quality is displayed as the 260nm/280nm value. Means ± SEM. Inaccurate values removed from the analysis. [A&B] n= 14, 14, 13, 9, 5, 7, 14 and 13 respectively. [C&D] n= 15, 16, 16 and 18, respectively. n numbers = biological replicates.

Table 5.4 Post hoc analysis for the effect of time on faecal DNA quality (260nm/280nm) of samples collected from individual birds.

| | **Week of the feed trial** | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **SED** | **P value** |
| Mean DNA quality (260nm/280nm) | 2.17[a] | 2.06[a] | 2.48[b] | 2.19[a] | 0.07328 | **<0.001** |

Means with different superscripts differ significantly.

*Total digesta DNA content and quality*

There was a significant (P<0.001) effect of gut section the digesta was taken from on the DNA yield (ng DNA/mg digesta) achieved, however, there was no effect of diet (P=0.348) and no interaction (P=0.792) between diet and gut section (**Figure 5.8A**, **Table 5.5**). Post hoc analysis for the effect of gut section showed that the DNA yield achieved from the digesta samples originating from the duodenum was significantly higher than those originating from other sections of the gastrointestinal tract (**Table 5.5**). Similarly, there was a significant (P<0.001) effect of gut section on the DNA quality assessed by 260nm/280nm (**Figure 5.8B**, **Table 5.5**). Post hoc analyses for the effect of gut section on DNA quality (260nm/280nm) are indicated in **Table 5.5**. However, there was no effect of diet (P=0.217) on DNA quality (260nm/280nm) or an interaction between diet and gut section (P=0.446) (**Figure 5.8B**).

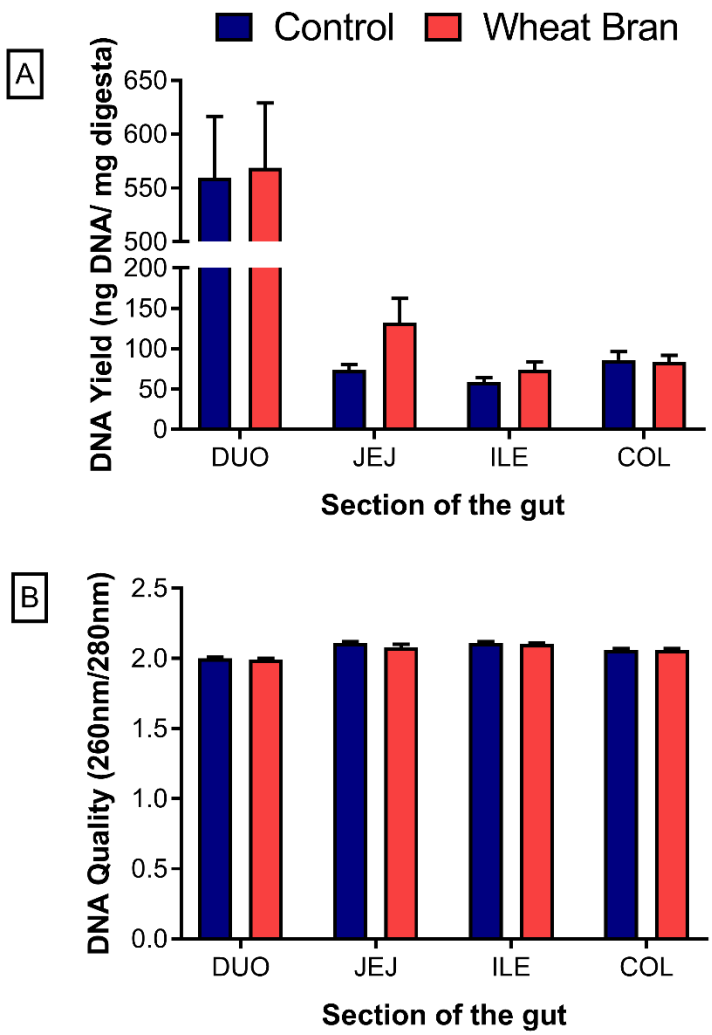


**Figure 5.8** The effect of a wheat bran inclusion diet on digesta [**A**] DNA yield (ng DNA/mg digesta) and [**B**] DNA Quality (260nm/280nm). Means ± SEM, n=9 biological replicates for all samples. There was a significant ($P<0.001$) effect of gut section on both DNA yield and DNA quality, see **Table 5.5** for post hoc results, however there was no effect ($P>0.05$) of diet or an interaction.

Table 5.5 Post hoc analysis of the effect of gut section on chicken digesta DNA assessments of DNA yield and quality.

| Digesta DNA measurement | Mean digesta DNA measurement in different sections of the gastrointestinal tract | | | | SED | P value for the effect of gut section (ANOVA) |
|---|---|---|---|---|---|---|
| | Duodenum | Jejunum | Ileum | Colon | | |
| **DNA yield (ng DNA/mg digesta)** | 564.2[b] | 102.9[a] | 66.5[a] | 84.7[a] | 32.44 | **<0.001** |
| **DNA quality (260nm/280nm)** | 1.99[a] | 2.10[c] | 2.11[c] | 2.06[b] | 0.011 | **<0.001** |

Means with different superscripts in the same row differ significantly.

#### 5.2.5.3 *Effect of a wheat bran inclusion diet, and duration on the diet, on faecal DNA composition*

There was a significant effect of time (P=0.047) on relative chicken *CYTB* DNA content in the faeces collected from individual birds, however, there was no effect of diet (P=0.338) or an interaction between time and diet (P=0.798) (**Figure 5.9A**). Bonferroni post hoc analysis showed that faecal samples collected at the end of week 2 contained significantly higher levels of chicken *CYTB* DNA compared with samples collected at the end of week 4 (**Table 5.6**). In contrast, there were no effects of time (P=0.310) or diet (P=0.103) nor any interaction (P=0.286) for relative bacterial *16S* DNA content in the faeces collected from individual birds (**Figure 5.9C**).

There was no diet x time interaction (P=0.937) or effects of time (P=0.369) or diet (0.322) on chicken *CYTB* DNA content in faecal samples collected from the pen floor, which corresponds with the results obtained from the individual birds samples (**Figure 5.9B** and **Figure 5.9A** respectively, **Table 5.6**). Equally the effect of a wheat bran inclusion diet on chicken *CYTB* DNA appears to be the same between faecal samples of individual birds and from the pen floor for the 2 latter time points, i.e. slightly increased level of *CYTB* DNA in the samples from birds who consumed a wheat bran inclusion diet compared with the control diet at weeks 3 and 4 (see **Figure 5.9A** and **Figure 5.9B**), although this wasn't statistically significant. However, there was a significant effect of time (P=0.031) on bacterial *16S* DNA content of the faeces collected from the pen floor, which differs from the results obtained from the individual bird samples taken on the same days (weeks 3 and 4) (**Figure 5.9D** and **Figure 5.9C** respectively, **Table 5.6**). There was significantly more bacterial *16S* DNA in the samples collected at week 3 compared with week 4, when collected from the pen floor (**Figure 5.9D**, **Table 5.6**). In addition, there was no interaction (diet x time) (P=0.823) or an effect of diet (P=0.342) on the bacterial *16S* DNA content in the faeces collected from the pen floor (**Figure 5.9D**).

As there was no effect of diet on bacterial *16S* DNA content, chicken *CYTB* DNA was normalised for bacterial *16S* DNA content as done previously in

the phytase trial analysis. There was no interaction (P=0.253) between diet and time on normalised *CYTB* DNA content in the faeces from individual birds, nor any singular effects of diet (P=0.136) or time (P=0.170) (**Figure 5.9E**). Similarly, there was no interaction (P=0.352) between diet and time on normalised *CYTB* DNA content in the faeces collected from the pen floor, nor any singular effects of diet (P=0.204) or time (P=0.190) (**Figure 5.9F**).

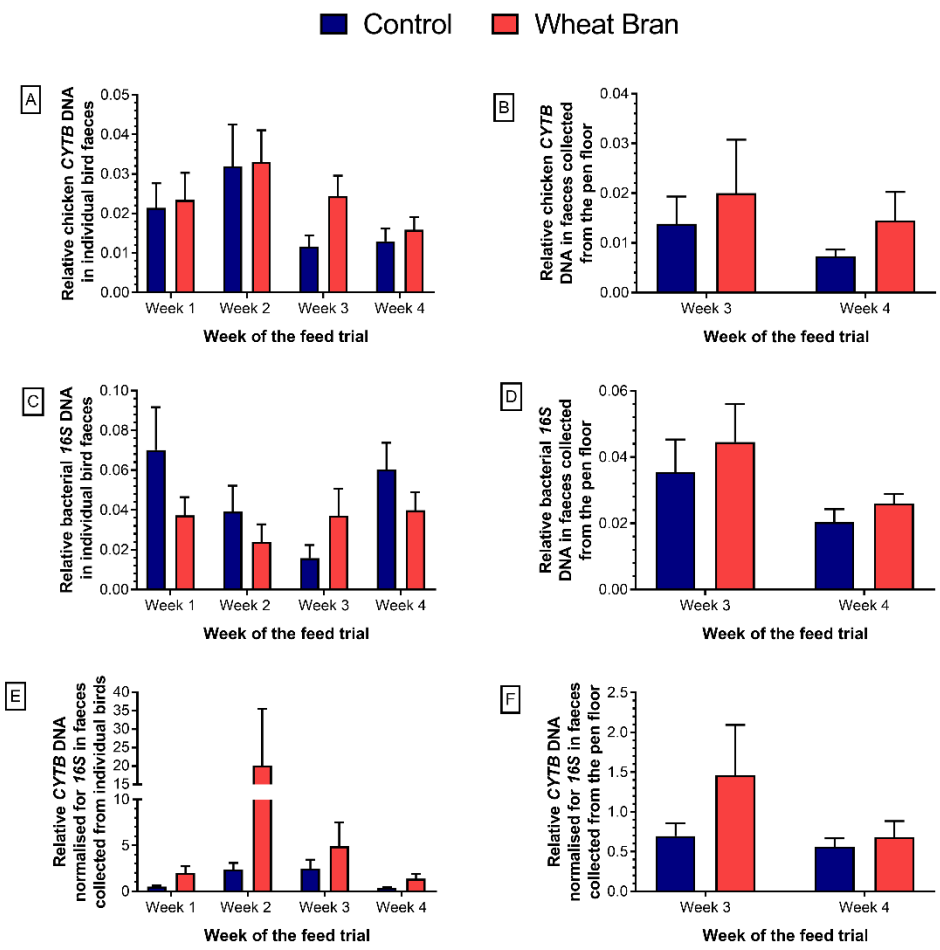


**Figure 5.9** The effect of a wheat bran inclusion diet on [**A**] faecal chicken *CYTB* DNA content in individual bird faeces, [**B**] faecal chicken *CYTB* DNA content in samples collected from the pen floor, [**C**] bacterial *16S* DNA content in individual bird faeces, [**D**] bacterial *16S* DNA content in samples collected from the pen floor, [**E**] *CYTB* DNA normalised for *16S* DNA in individual bird faeces and [**F**] *CYTB* DNA normalised for *16S* DNA in samples collected from the pen floor. Means ± SEM. [**A**, **C** & **E**] n= 15, 16, 15, 12, 10, 13, 14 and 16, respectively. [**B**, **D** & **F**] n=18 for all groups. n numbers = biological replicates. Significant ANOVA results with Bonferroni analyses are illustrated in **Table 5.6**.

Table 5.6 Mean and P values for the effect of time on chicken faecal DNA content.

| DNA content | Mean faecal DNA content | | | | SED | P value for the effect of time |
|---|---|---|---|---|---|---|
| | Week 1 | Week 2 | Week 3 | Week 4 | | |
| Individual bird faeces | | | | | | |
| Chicken *CYTB* DNA | 0.02278$^{ab}$ | 0.03238$^{b}$ | 0.01791$^{ab}$ | 0.01498$^{a}$ | 0.00650 | **0.047** |
| Bacterial *16S* DNA | 0.05184 | 0.03164 | 0.03082 | 0.04798 | 0.01309 | 0.310 |
| Chicken *CYTB* DNA normalised for bacterial *16S* DNA | 1.549 | 11.440 | 2.969 | 1.231 | 4.860 | 0.170 |
| Pen floor faeces | | | | | | |
| Chicken *CYTB* DNA | - | - | 0.0169 | 0.0109 | 0.00671 | 0.369 |
| Bacterial *16S* DNA | - | - | 0.0400$^{a}$ | 0.0232$^{b}$ | 0.00765 | **0.031** |
| Chicken *CYTB* DNA normalised for bacterial *16S* DNA | - | - | 1.0783 | 0.6236 | 0.4858 | 0.190 |

The week number refers to the duration of time the birds were on the experimental diets.
Means differing in superscript letters in the same row differ significantly.

### 5.2.5.4 *Relationship between host faecal DNA and animal performance*

There was no relationship (P>0.05) between chicken *CYTB* DNA content in the faeces, collected from individual birds or from the pen floor, and the FCR at the end of each week of the feed trial (see **Figure 5.10**).

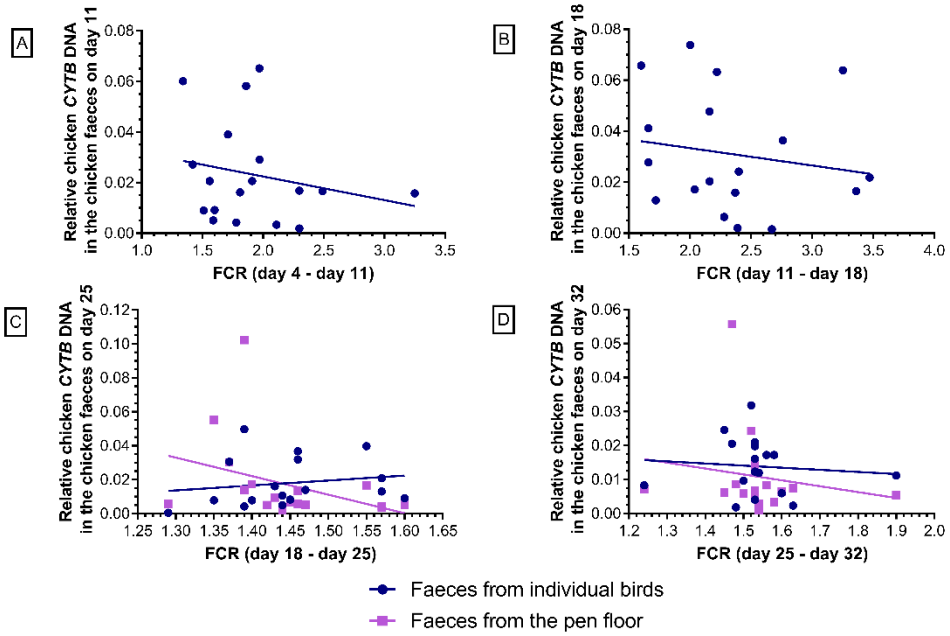


Figure 5.10 The relationship between [A] chicken *CYTB* DNA in the faeces on day 11 compared to FCR (day 4 – day 11), [B] chicken *CYTB* DNA in the faeces on day 18 compared to FCR (day 11 – day 18), [C] chicken *CYTB* DNA in the faeces on day 25 compared to FCR (day 18 – day 25) and [D] chicken *CYTB* DNA in the faeces on day 32 compared to FCR (day 25 – day 32). Relationships were analysed by Pearson's correlation. R values and P values are provided below:

[A] r= -0.2151, P= 0.3913
[B] r= -0.1619, P= 0.5024
[C] individual bird: r= 0.1702, P= 0.5136, pen floor: r=-0.3608, P= 0.1301
[D] individual bird: r= -0.09441, P= 0.7185, pen floor: r= -0.1703, P= 0.4994.

### 5.2.5.5 *Effect of a wheat bran inclusion diet and the section of the gastrointestinal tract on the digesta DNA composition*

There was a significant (P<0.001) effect of gut section on the chicken *CYTB* DNA content in the digesta, however, there was no effect (P=0.767) of diet or an interaction (P=0.292) between the two (**Figure 5.11A**). Bonferroni post hoc analysis of the effect of gut section on the chicken *CYTB* DNA content

in the digesta showed that the levels in the duodenum were significantly higher than in the other regions of the gastrointestinal tract (**Table 5.7**). Similarly, there was a significant (P<0.001) effect of gut section on the bacterial *16S* DNA content in the digesta, with no effect of diet (P=0.975) and no interaction (P=0.971) between the two (**Figure 5.11B**). Post hoc analysis of the effect of gut section on the bacterial *16S* DNA content in the digesta showed that the levels in the colon were significantly higher than in the other regions of the gastrointestinal tract (**Table 5.7**). As there was only a trend for the effect of diet on the bacterial *16S* DNA, chicken *CYTB* DNA was normalised for bacterial *16S* DNA present in the digesta. There was a trend (P=0.054) for an interaction between diet and gut section on the normalised chicken *CYTB* DNA in the digesta, as well as a significant (P<0.001) effect of gut section (**Figure 5.11C**). The trend for the interaction appears to be due to there being a much greater level of *CYTB* DNA in the digesta from the jejunum of wheat bran treated birds, but this difference is not observed in other areas of the GI tract. Post hoc analysis of the effect of gut section showed normalised *CYTB* DNA was significantly higher in the digesta of the duodenum compared with the other three regions of the gastrointestinal tract (**Table 5.7**). In contrast, there was no effect (P=0.100) of diet on normalised *CYTB* DNA in the digesta (**Figure 5.11C**).

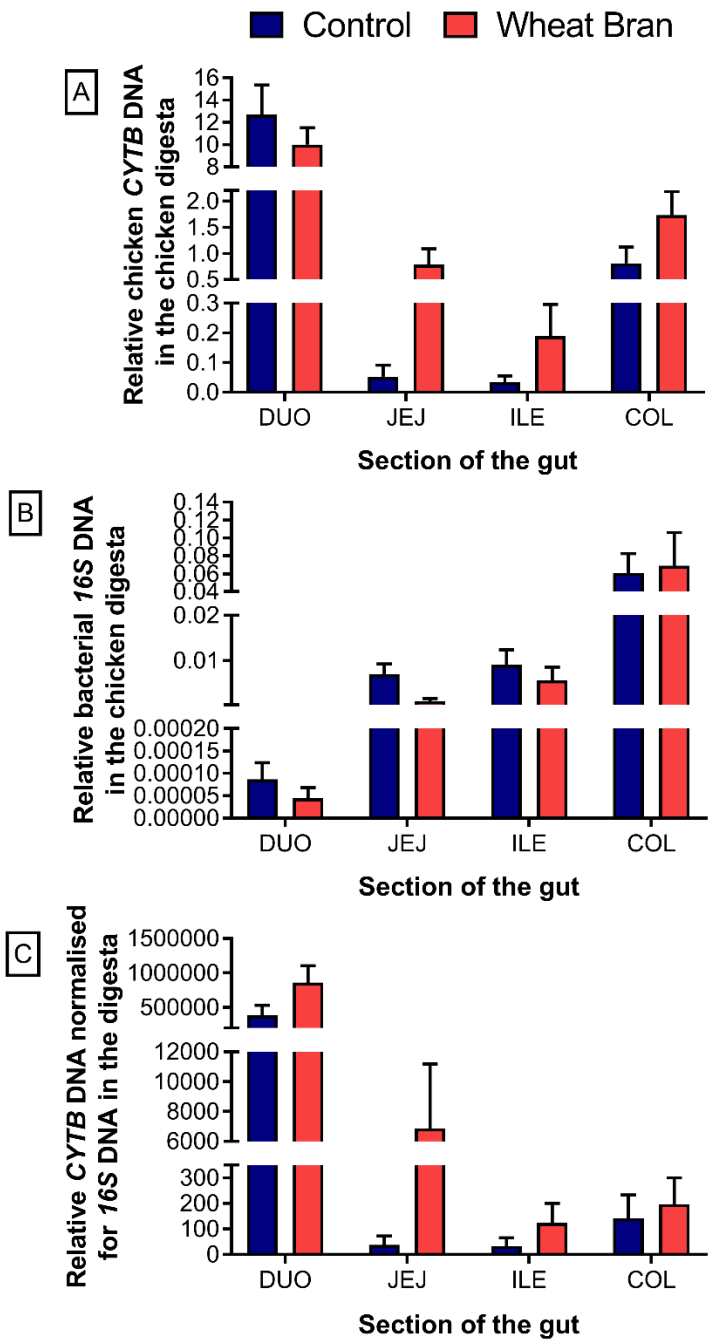


**Figure 5.11** The effect of a wheat bran inclusion diet on [**A**] Relative chicken *CYTB* DNA, [**B**] Relative bacterial *16S* DNA in chicken digesta and [**C**] Relative *CYTB* DNA normalised for *16S* DNA. Means ± SEM, n=9 biological replicates for all samples. There was a significant (P<0.001) effect of gut section on each DNA measurement, post hoc analyses are illustrated in **Table 5.7**. There was also a trend for the interaction (P=0.054) between diet and gut section on normalised *CYTB* DNA.

Table 5.7 Post hoc analysis of the effect of gut section on chicken digesta DNA content.

| Digesta DNA component | Mean digesta DNA content in different sections of the gastrointestinal tract | | | | SED | P value for effect of gut section (ANOVA) |
|---|---|---|---|---|---|---|
| | Duodenum | Jejunum | Ileum | Colon | | |
| **Chicken *CYTB* DNA** | 11.355[b] | 0.418[a] | 0.111[a] | 1.274[a] | 1.053 | **<0.001** |
| **Bacterial *16S* DNA** | 0.00007[a] | 0.00402[a] | 0.00736[a] | 0.06502[b] | 0.01532 | **<0.001** |
| **Chicken *CYTB* DNA normalised for bacterial *16S* DNA** | 622631[b] | 3460[a] | 79[a] | 170[a] | 106602.2 | **<0.001** |

Means differing in superscript letters in the same row differ significantly.

### 5.2.6 Discussion

This study assessed the effect of a 20% wheat bran (WB) inclusion diet on bird performance and the DNA content and composition from faeces, collected from individual birds or the pen floor, and digesta from the three sections of the small intestine and the colon. It also examined whether there was a relationship between FCR and *CYTB* DNA content in the faeces at each week of the study.

WB has a very high insoluble fibre content (Vitaglione *et al.*, 2008) and large amounts of anti-nutritional phytate (Stevenson *et al.*, 2012), making it a relatively poor feed ingredient. In commercial feeding practices if a diet is formulated to include wheat bran the inclusion level is typically low, and formulators must ensure the nutritional content of the diet is appropriately balanced, particularly for energy. Similarly, feed enzyme additives may be added to diets containing WB to improve the nutritional quality to the bird. Therefore, it was surprising that a 20% WB inclusion diet (with no enzyme additive) had no effect on broiler performance, apart from a depressed feed intake between 11 and 18 days of age. An absence of effects of a high fibre diet on FCR and body weight gain is in agreement with Jin *et al.* (1994), although that study was carried out in growing pigs. A study carried out in broilers also saw no effect of a 20% rice bran diet on FCR and feed intake, but there were effects on weight gain (Farrell and Martin, 1998). With regards to the reduction in feed intake in the present study, a high soluble fibre diet is known to increase digesta viscosity which results in increased retention time and gut fill, leading to increased satiety impacting upon feed intake (Jha *et al.*, 2019). If the reduced feed intake was due to soluble fibre then a reduced growth rate may also be expected due to poor nutrient utilisation, which was not the case. However, wheat bran contains largely insoluble fibre which can increase passage rate as it is largely undigested and may therefore typically lead to an increased feed intake which has been observed in other studies where broiler diets have included various levels of wheat bran (Jha *et al.*, 2019; Jørgensen *et al.*, 1996). Hence the significantly reduced feed intake during week 2 alone is unexpected,

and if the soluble fibre content of wheat bran was having a greater effect on the feed intake compared to the insoluble fibre, the effect would be expected throughout the study. The more reasonable explanation for the differences in feed intake at week 2 alone, with no effects on FCR due to no differences in body weight gain, could be the issues with measuring feed intake for the first half of the study where feed troughs were used. It was at the beginning of week 3 when the feed troughs were changed for feed hoppers which led to a visible reduction in feed spillage and contamination with excreta.

The very low faecal DNA yields achieved in both the current study and the chicken phytase study, compared with pig faecal DNA yields achieved in Chapter 4, are in agreement with the results observed in Chapter 3 where comparisons were made between the amount of DNA extracted from chicken and pig faeces, either by crude extraction or by the modified Qiagen kit. This data illustrates further that more research is required to establish why there are these clear differences, although it may likely be due to differences in digestive physiology and resulting faecal composition and consistency as described in Chapter 1. There were no differences in DNA yield associated with the diet, the only effects were due to time. The faeces are not dried prior to DNA extraction and as the consistency of chicken excreta changes over time this may have impacted upon the yield. Due to the problems associated with the DNA extraction procedure from chicken faeces in this study, the significant differences with regards to DNA quality should be taken with caution. The increased DNA yield in the duodenum compared with the other sections of the gastrointestinal tract (GIT) may be due to a dilution effect. The pancreatic and bile ducts are located at the end of the duodenum (Svihus, 2014) and so the levels of these juices may be greater in the GIT region beyond the duodenum leading to a dilution of the digesta contents. In addition, the distal regions of the GIT are visibly larger with a greater luminal diameter than that of the duodenum, therefore the DNA yield results may reflect the greater volume of digesta in the distal gut sections, which likely further dilutes the DNA present in a fixed quantity (~200mg) of digesta. As previously described, pancreatic and

bile ducts are located at the end of the duodenum, the digestive juices may increase the pH in these distal regions and pH impacts upon DNA extraction and the resulting DNA quality of the digesta. Future experiments could measure and record the pH of the digesta from each GIT region, along with the faecal samples at collection, to establish whether the pH does correlate with resulting DNA quality.

There was no significant effect of the 20% WB diet on the chicken *CYTB* DNA present in the faeces or the digesta, which presumably originates from gut cell sloughing. Due to the high fibre content of wheat bran a greater impact on gut cell proliferation and sloughing was expected, which should be measured using the *CYTB* DNA assay. However, previous studies have found that chickens can cope with greater levels of fibre in their diet (Farrell and Martin, 1998), but previous work examining the effects of fibre on gut morphology is conflicting. A study where insoluble fibre was added to broiler diets at 2 or 4% found significant effects on villus height compared to controls, but no effect on other morphology measures such as crypt depth which may be more strongly associated with gut cell sloughing (Rahmatnejad and Saki, 2016). Alternatively, Jin *et al.* (1994) did observe significant effects of 10% wheat straw on pig gut morphology and BrdU labelling of crypt cells, particularly in the jejunum, indicating a greater level of DNA synthesis. In the present study there was more *CYTB* DNA in the faeces of individual birds and from the pen floor in the 20% WB treatment group at week 3, but these differences were not statistically significant. In faecal samples of individual birds there appeared to be more normalised *CYTB* DNA in the faeces of wheat bran treated birds across all time points but particularly week 2, but it has to be recognised that the bacterial DNA used to normalise did have quite a bit of variation. Results were comparable for *CYTB* and normalised *CYTB* DNA between individual birds and pen floor faeces. This indicates that faecal samples from the pen floor, which were contaminated with bedding, could be used for this analysis instead which would make the method of collection much easier, and although the DNA was on average of poorer quality, this did not affect the PCR. The significant effects

of time on the chicken faecal DNA composition likely illustrates the changes in the broiler GIT as the bird develops. Like the phytase trial described earlier in this chapter, there was no relationship between chicken *CYTB* DNA in the faeces and FCR, but again for a relationship to be identified one parameter would at least need to alter.

The chicken digesta DNA results were particularly interesting, with regards to the effect of gut section on both the chicken *CYTB*, bacterial *16S* and normalised *CYTB* DNA content, even though there was no effect of the WB diet. There was significantly more *CYTB* DNA present in the duodenum digesta compared with the digesta originating from the other GIT sections. This should not be confused with the increased DNA yield from the duodenum digesta, as the PCR is carried out on a constant DNA basis, but indicates that there may be more gut cell sloughing in the duodenum, provided the *CYTB* DNA originates only from sloughed epithelial cells. However, the levels of chicken *CYTB* DNA decrease significantly in the jejunum and this reduction continues through to the ileum and colon. The reduction in host DNA could be attributed to an increase in endonuclease activity. Alternatively, there were significantly greater levels of bacterial *16S* DNA present in the colon compared to the 3 proximal sections of the GIT. The increased levels of bacterial DNA present in the colon are understandable as this region is after the caeca, which are where the majority of microbial fermentation occurs in the chicken (Svihus *et al.*, 2013; Svihus, 2014). Normalised *CYTB* DNA was also significantly greater in the duodenum compared with the jejunum, ileum and colon. There was also a trend for an interaction between diet and gut section, but still no singular effect of diet. This was because the normalised *CYTB* DNA only appeared to be greater with wheat bran compared with the control group in the jejunum, indicating a high fibre diet may have the greatest impact on gut cell sloughing in this region of the GIT. These results compare with the Jin *et al.* (1994) study which found a 10% wheat straw diet resulted in significant increases in the proliferation of crypt cells in the jejunum of pigs. Increased crypt cell proliferation rate can lead to increased gut cell sloughing. Taken together with results from the current

study, it can be suggested that the jejunum may be a major site of gut turnover and that the DNA assay is measuring gut cell sloughing.

Overall, the wheat bran inclusion diet had little effect on bird performance. Likewise, there were no significant effects of the wheat bran diet on the faecal chicken *CYTB* DNA and so the absence of a relationship between FCR and faecal host DNA was expected. Digesta DNA composition was affected by gut section the digesta was collected from, illustrating there are clear differences in the interaction between the digesta and intestinal mucosa or microflora at the various points in the GIT. Taken together these results illustrate the need for further work into the sensitivity of the chicken *CYTB* DNA assay when used in chicken studies, but it does provide evidence that the DNA assay measures exfoliated intestinal epithelial cells.

# CHAPTER 6. FINAL DISCUSSION

Despite the extensive research carried out on DNA extraction from faeces (**Appendix 1**), to the best of our knowledge, prior to the work carried out in this thesis there was no work done on extracting host DNA from faeces as a potential marker of gut cell loss in farmed animals. As expected, different faecal DNA extraction methods were required for the 2 species examined in this thesis, pigs and poultry. This was likely due to the different properties of pig and poultry excreta, mainly a pH difference and increased contamination with other substances such as bedding when extracting DNA from chicken faeces. However, mitochondrial *cytochrome b* was an appropriate target gene for host DNA analysis from both species, which is in accordance with numerous studies identifying mitochondrial genes as successful species-specific gene targets in even particularly difficult sample types such as faeces and waste water (Caldwell *et al.*, 2007).

The anticipated mode by which host faecal DNA is a marker of gut cell loss was depicted in **Figure 1.5** and the results of this thesis provide further evidence to support the proposed hypothesis. Firstly, in pigs fed diets thought to positively impact upon gut function, either via increased digestibility or promotion of gut health, there were significant decreases in the levels of host DNA in their faeces. Additionally, there was a weak trend for a relationship with pig faecal host DNA and feed efficiency, although this was a statistically significant relationship when an outlier was removed from the analysis. When measuring host DNA levels through the chicken gastrointestinal tract, gut cell sloughing appeared to be highest at the duodenum, and this DNA was then degraded as it continued down the gastrointestinal tract, likely leading to fragmented DNA being present in the faeces. Equally results for the increased level of host DNA in the chicken jejunum of birds fed 20% wheat bran were in agreement with a previous study where pigs who were fed 10% wheat straw had significantly higher levels of DNA synthesis and crypt cell proliferation in the same region (Jin *et al.*, 1994), further illustrating that the devised assay likely measures gut cell sloughing.

Nevertheless, several questions remain which will require further validation experiments. The first experiment to undertake, as indicated in Chapter 3, is to examine various sample preservation techniques to ensure that at the time of extraction the DNA content within the sample is still representative of the excreted fresh sample. This would involve extracting DNA from fresh faeces and making comparisons with the DNA yield and quality achieved from faeces preserved in a number of different ways such as short term storage at 4˚C, freezing at -20˚C, freezing at a lower temperature such as -70˚C and commercial preservatives such as RNA*later* (Invitrogen). This will be of particular importance when looking to develop the assay for commercial purposes of examining gut cell sloughing in farmed species in a real-world setting and determining the best protocol for the collection and transportation of samples to the laboratory for analysis. The next point requiring further examination is the extractability of DNA from various faecal sample types, also highlighted in Chapter 3. This is because poor extractability could lead to measurements of host DNA which are a misrepresentation of the levels which would be found in the excreted sample.

The next question is whether measuring *cytochrome b* in the faeces enables quantification of all the host DNA in the excreted faecal sample and how variable the mitochondrial number is per cell in intestinal epithelial cells. In terms of whether the *cytochrome b* measurement is measuring all the host DNA in the faecal sample, one method to try could be making comparisons with fluorescence-activated cell sorting (FACS). A group of researchers developed a method for isolating endogenous host DNA from primate faeces using FACS, however, they didn't observe a correlation between the number of sorted cells and total concentration of DNA extracted using a Qiagen method (Orkin *et al.*, 2018). Total DNA will include DNA from a range of sources; therefore, it would be best to attempt FACS from faeces and compare with results from *cytochrome b* DNA assay. Any comparisons would have to be on a relative basis, therefore likely using samples where extremes in gut cell sloughing is expected, as it has already been suggested that residual dyes from FACS could interfere

with accurate quantification using the Qubit quantification method (Orkin *et al.*, 2018). A study examined the number of mitochondria and the amount of mitochondrial DNA between 5 different cell types from cell culture and determined that there was little variability within replicates of the same cell type, but great variability between cell types (Robin and Wong, 1988). However, it was noted that further analysis was required for other cell types (Robin and Wong, 1988). One future experiment could potentially combine FACS from faeces and a protocol for the determination of mitochondrial DNA and mitochondrial number between cells, to provide further information as to whether measuring faecal *cytochrome b* DNA is an appropriate method to measure the level of epithelial cell sloughing from the gastrointestinal tract.

Another question requiring further investigation is whether the DNA measured in the faeces originates only from the gastrointestinal tract and has not survived from an earlier region in the gut. Studies looking at drug delivery highlight the issues with the degradation of ingested DNA in the gastrointestinal tract due to the acidic environment of the stomach and the presence of digestive enzymes (Borges *et al.*, 2005). Equally, the results from the chicken wheat bran study (Chapter 5) illustrate that there is a high level of DNA degradation as it passes through the small intestine. Therefore, it can be assumed that any host DNA present in faeces originates only from sloughed epithelial cells in the intestine, which has been the approach taken in this thesis. However, for validation purposes an experiment could seek to identify whether specially protected DNA could be incorporated into feed for oral delivery and then be measured in excreta, which would identify whether any exogenous DNA was able to survive the gastrointestinal tract. Studies have managed to deliver DNA orally for use as vaccines (Farris *et al.*, 2018). In order to do so, DNA was protected using substances such as zein, which is naturally occurring prolamine containing a high percentage of hydrophobic amino acids and is resistant to degradation in acidic conditions (Farris *et al.*, 2018). Other studies have created nanoparticles using the natural polysaccharide chitosan to protect DNA from degradation by the GI tract prior to reaching the mucosal barrier (Roy

*et al.*, 1999) . However, the protective substances used in DNA vaccine delivery are designed to be removed in the intestine, typically by digestive enzymes (Farris *et al.*, 2018). As a result, it is unclear whether there is an appropriate method of protecting DNA which would survive the whole GI tract. Although these challenges do support the hypothesis that it would be very difficult for any host DNA present in faeces to originate from anywhere other than the intestine.

Finally, and possibly most importantly, speculation remains as to whether the developed DNA assay is measuring gut cell sloughing alone and not another biological process. The results in the thesis provide some validation regarding this question, as faecal calprotectin, a marker of gut inflammation, did not correlate with the results for faecal host DNA, indicating the 2 markers are measuring 2 clearly different processes. Nonetheless, further validation experiments whereby epithelial cells are labelled in some way and compared with faecal DNA measurements are required. The problem with labelling epithelial cells is that it is very difficult to measure gut cell sloughing. In 1970, Clarke attempted to measure the total level of gut cell shedding in the starved rat and compared with the level of cellular production as it is expected that breakdown equals synthesis at steady state (**Figure 1.1**). However, the study required highly invasive procedures and relied on 3 assumptions, i) that the shed cells remained intact and identifiable within the mucus; ii) no shed cells had escaped beyond the mucus layer and iii) that the experimental conditions did not affect the rate of cell shedding (Clarke, 1970). More commonly epithelial cells are stained, often using a pulse-chase injection of 5-bromo-2-deoxyuridine (BrdU), to look at epithelial cell proliferation (Cortés *et al.*, 2015). The problems with this method are that it examines cell proliferation alone and does not quantify the biological process of gut cell sloughing, plus the fact it is an invasive procedure. A less expensive technique of examining gut mucosa morphology, which is thought to alter during disturbed intestinal homeostasis, is haemotoxylin and eosin staining, although this is also invasive and does not provide a true estimation of gut cell shedding. More recently, studies have tried

to use other less invasive techniques to measure gut turnover such as stable isotope-labelled amino acid infusion (Buttery, 1981) or deuterium labelled water (Gasier *et al.*, 2010), but unfortunately these methods are i) either incapable of, or not routinely used for, measuring DNA; ii) expensive and iii) will not provide clear validation of the developed DNA assay.

Hence, I would suggest a future experiment to be clearly structured and aim to combine a range of different techniques in order to establish the wider picture as there is no clear definitive validation method to compare the *cytochrome b* DNA assay to. An experiment would have to be well thought out to reduce the number of animals required, rather than conducting a number of smaller experiments. Although, any study involving the labelling of epithelial cells would require a pilot study to establish the optimal time window between infusion and sample collection. The time window would likely be as little as 10-15 minutes due to the quick rate of cellular turnover at the intestine.

## 6.1 MAIN FINDINGS OF THE THESIS

Under the specific outlined conditions in each chapter of this thesis, the main findings were as follows:

1. Different DNA extraction protocols are required when extracting DNA from pig or chicken faeces. Specifically, it is best to employ bead-beating when extracting DNA from chicken faeces to increase DNA yields and PCR ability.
2. *Cytochrome b* (*CYTB*) is an appropriate gene target for detecting host DNA in pig and poultry faeces.
3. A xylanase inclusion diet leads to significantly less *CYTB* DNA present in the faeces of pigs, potentially indicating that xylanase reduces gut cell sloughing.
4. A yeast-enriched protein concentrate (YPC) inclusion diet (particularly at a 2.5% inclusion) leads to significantly less *CYTB* DNA present in the faeces of pigs, while also resulting in significant improvements to FCR.

Therefore, a YPC inclusion diet may be beneficial in reducing gut cell sloughing which is an energy demanding process.

5. The amount of time post-weaning is the main driver of the levels of intestinal inflammation, as measured by faecal calprotectin, in newly weaned pigs. The closer to weaning, the greater the levels of intestinal inflammation in growing pigs.
6. A phytase inclusion diet (2500FTU) has no effect on performance of broiler chickens and equally does not alter faecal *CYTB* DNA content.
7. A 20% wheat bran diet does not negatively impact upon bird performance in broiler chickens.
8. Levels of *CYTB* DNA are highest in the digesta present in the duodenum in the broiler chicken gastrointestinal tract, suggesting this region of the GI tract is where the greatest level of gut cell sloughing occurs. However, a 20% wheat bran diet has the greatest influence in levels of host DNA at the jejunum, in accordance with the literature examining the effects of a high fibre diet on gut cell sloughing.
9. Measuring faecal *CYTB* DNA may be a non-invasive measure of gut cell sloughing, but further validation studies are required.

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# APPENDICES

Appendix 1 Review of published articles utilising or comparing various DNA extraction protocols from faeces. Lysis methods: C – chemical, E – enzymatic, H – heat, M – mechanical.

| DNA Extraction Method | Lysis method | Article which utilised it | Article which suggested it was the optimal DNA extraction protocol in comparative studies |
|---|---|---|---|
| **Commercial Kits** | | | |
| QIAamp® DNA Stool Mini Kit (Qiagen)[1] | C, H, E | (Ariefdjohan *et al.*, 2010; Gorzelak *et al.*, 2015; Jiang *et al.*, 2005; Josefsen *et al.*, 2015; Kikuchi *et al.*, 2010; Kolodziej *et al.*, 2013; Li *et al.*, 2007; Li *et al.*, 2003) (McOrist *et al.*, 2002) (Nechvatal *et al.*, 2008)(Peng *et al.*, 2013) (Ramón-Laca *et al.*, 2015) (Roperch *et al.*, 2015) (Scupham *et al.*, 2007) (Yu and Morrison, 2004) (Burbach *et al.*, 2016) (Dimsoski, 2017) (Tang *et al.*, 2008) (Grześkowiak *et al.*, 2016) (Smith *et al.*, 2011) | (Li *et al.*, 2003; Maksimov *et al.*, 2017; McOrist *et al.*, 2002; Nechvatal *et al.*, 2008) |
| TIANamp Stool DNA Kit (Tiangen, China)***** | C, H, E | (Peng *et al.*, 2013) | |
| Bead-beating + QIAamp® DNA Stool Mini Kit (Qiagen) | M, C, H, E | (Li *et al.*, 2007) (Salonen *et al.*, 2010) (Grześkowiak *et al.*, 2016) (Smith *et al.*, 2011) | (Grześkowiak *et al.*, 2016; Li *et al.*, 2007; Smith *et al.*, 2011) |

| | | | |
|---|---|---|---|
| Modified QIAamp® DNA Stool Mini Kit (Qiagen) | C, H, E | (Li *et al.*, 2007; Lindquist and Wictum, 2016) (Dimsoski, 2017) (Ferrand *et al.*, 2014) (Claassen *et al.*, 2013) | (Lindquist and Wictum, 2016; Dimsoski, 2017)[2,6] |
| QIAamp® Fast DNA Stool Mini Kit (Qiagen) | C, H, E | (Josefsen *et al.*, 2015; Maksimov *et al.*, 2017) | |
| QIAamp® DNA/RNA Mini Kit (Qiagen) | C, H, E | (Jiang *et al.*, 2005; Nechvatal *et al.*, 2008) | |
| Lysozymes with RNA/DNA Mini Kit (Qiagen) | C, H, E | (Nechvatal *et al.*, 2008) | |
| QIAsymphony® Virus/Bacteria Midi Kit (Qiagen) | M, C, E | (Claassen *et al.*, 2013) | (Claassen *et al.*, 2013) |
| Swabbing outer layer of faeces, incubating with enzymes overnight at 56˚C and then automatic extraction using QIAxtractor with QIAxtractor reagents (Qiagen) or manually using DNeasy Blood and Tissue Kit (Qiagen) | C, H, E | (Ramón-Laca *et al.*, 2015) | (Ramón-Laca *et al.*, 2015) |
| FastDNA® SPIN Kit for Soil (MP Biomedicals) | M, C | (Ariefdjohan *et al.*, 2010; Li *et al.*, 2007; Lindquist and Wictum, 2016) (Jiang *et al.*, 2005) (Josefsen *et al.*, 2015) (Burbach *et al.*, 2016) (Maksimov *et al.*, 2017)(Ferrand *et al.*, 2014) | (Ariefdjohan *et al.*, 2010; Burbach *et al.*, 2016; Ferrand *et al.*, 2014; Jiang *et al.*, 2005)[3] |
| FastDNA® SPIN Kit for Feces (MP Biomedicals) | M, C | (Ariefdjohan *et al.*, 2010; Lindquist and Wictum, 2016; McOrist *et al.*, 2002; Yu and Morrison, 2004) (Josefsen *et al.*, 2015) (Burbach *et al.*, 2016)(Ferrand *et al.*, 2014) | (Ariefdjohan *et al.*, 2010; Josefsen *et al.*, 2015)[4] |
| FastDNA® Kit (when manufactured by Qbiogene) | M, C | (Scupham *et al.*, 2007) | |
| Bioeasy Fecal Magnetic Isolation Kit (Bioeasy, China) | M, C | (Peng *et al.*, 2013) | |
| Bioeasy Animal Column Isolation Kit (Bioeasy, China) | Unknown | (Peng *et al.*, 2013) | |

| | | | |
|---|---|---|---|
| MO BIO Fecal (protocol unspecified) | Unknown | (Nechvatal *et al.*, 2008) | |
| UltraClean® DNA Kit for Feces (MO BIO) | M, C, Optional H | (Ariefdjohan *et al.*, 2010; Claassen *et al.*, 2013; Peng *et al.*, 2013; Scupham *et al.*, 2007) | |
| UltraClean® DNA Kit for Soil (MO BIO) | M, C, Optional H | (Jiang *et al.*, 2005; Scupham *et al.*, 2007) | |
| PowerSoil® DNA Isolation Kit (MO BIO) | M, C, Optional H | (Burbach *et al.*, 2016; Claassen *et al.*, 2013; Josefsen *et al.*, 2015) | |
| PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO) | M, C, Optional H | (Josefsen *et al.*, 2015) (Burbach *et al.*, 2016) | (Josefsen *et al.*, 2015)[4] |
| PowerFecal® DNA Isolation Kit (MO BIO) | M, H, C | (Burbach *et al.*, 2016; Janabi *et al.*, 2016; Josefsen *et al.*, 2015) | |
| High Pure PCR Template Preparation Kit (Roche) | C, E, H | (Scupham *et al.*, 2007) | |
| Easy-DNA™ gDNA Purification Kit (Invitrogen) | C, H, E | (Scupham *et al.*, 2007) | (Scupham *et al.*, 2007) |
| Quantum Prep AquaPure™ Genomic DNA Isolation Kit (Bio-Rad) | C, H, E | (McOrist *et al.*, 2002; Scupham *et al.*, 2007) | |
| SoilMaster™ DNA Extraction Kit (Epicentre) | C, E, H | (Scupham *et al.*, 2007) | |
| MasterPure™ Gram Positive DNA Purification Kit (Epicentre) | C, E, H | (Ferrand *et al.*, 2014) | |

| | | | |
|---|---|---|---|
| ZR Fecal DNA MiniPrep™ (Zymo Research Corp)** | M, C | (Josefsen *et al.*, 2015; Maksimov *et al.*, 2017) (Claassen *et al.*, 2013) (Ferrand *et al.*, 2014) | (Claassen *et al.*, 2013; Maksimov *et al.*, 2017) |
| Wizard® Genomic DNA Purification Kit (Promega) | C, E, H | (Josefsen *et al.*, 2015; Salonen *et al.*, 2010) | |
| Maxwell® 16 DNA Purification Kit (Promega) | C | (Burbach *et al.*, 2016) | |
| NucliSens® miniMAG® Nucleic Acid Purification (BIOMÉRIEUX) | C | (Ferrand *et al.*, 2014; Josefsen *et al.*, 2015) | |
| Stool-E-Sphere (Phthisis Diagnostics) | Unknown | (Lindquist and Wictum, 2016) | |
| Nucleospin C+T Kit (Macherey-Nagal) | Unknown | (McOrist *et al.*, 2002) | |
| NucleoSpin® Tissue Kit (Macherey-Nagal) | C, E, H | (Kolodziej *et al.*, 2013) | (Kolodziej *et al.*, 2013)[5] |
| NucleoSpin® Soil Kit (Macherey-Nagal) | M, C | (Burbach *et al.*, 2016; Maksimov *et al.*, 2017) | |
| Precellys Soil DNA Kit (PEQLAB – part of VWR) | M, C, H | (Burbach *et al.*, 2016) | |
| NukExPure DNA/RNA (Gerbion) | E, C, H | (Burbach *et al.*, 2016) | |
| innuPREP Stool DNA Kit (Analytik Jena) | C, H, E – depending on protocol | (Burbach *et al.*, 2016) | |
| innuSPEED Stool DNA Kit (Analytik Jena) | C, H | (Burbach *et al.*, 2016) | |
| innuSPEED Soil DNA Kit (Analytik Jena) | C, H | (Burbach *et al.*, 2016) | |

| | | | |
|---|---|---|---|
| **Non-commercial methods** | | | |
| Traditional Phenol Method | C | (Janabi *et al.*, 2016; Jiang *et al.*, 2005) | |
| Phenol-free DNA extraction procedure | C, H | (Tang *et al.*, 2008) | (Tang *et al.*, 2008) |
| Phenol-free bead-beating followed by phenol extraction | M, C | (Li *et al.*, 2003; Yu and Morrison, 2004) | |
| Phenol dependent bead-beating followed by phenol extraction | M, C | (Whitford *et al.*, 1998) | |
| Non-commercial guanidium isothiocyanate/silica matrix method | C | (McOrist *et al.*, 2002) | |
| Modified phenol-free bead-beating method including QIAamp® DNA Stool Mini Kit purification columns and reagents | M, C, H | (Salonen *et al.*, 2010) (Yu and Morrison, 2004) | (Salonen *et al.*, 2010; Yu and Morrison, 2004) |
| Boiling faecal samples (1, 3, 5, 10, 30mg) | H | (Peng *et al.*, 2013) | (Peng *et al.*, 2013) |

*****Protocol is very similar to the QIAamp DNA Stool Mini Kit – Tiangen (China) is a subsidiary of Qiagen (Germany).

******Now being sold under the commercial name '*Quick*-DNA Fecal/Soil Microbe Miniprep Kit'.

[1](Ramón-Laca *et al.*, 2015) Involves incubating the samples with the InhibitEX matrix overnight at 56˚C.

[2](Lindquist and Wictum, 2016) Optimal method included a swab sampling method from the external area of fresh faeces that had been dried for 24hr, as well as a step that concentrated the DNA.

[3](Jiang *et al.*, 2005) Optimal direct kit based DNA extraction procedure in this experiment, when Bovine Serum Albumin was included as a PCR facilitator.

[4](Josefsen *et al.*, 2015) Highest DNA yield was achieved with PowerLyzer PowerSoil DNA Isolation Kit, but the FastDNA Spin Kit for Feces resulted in the highest PCR-amplifiable *C. jejuni* DNA which was the amplicon of interest in the study.

[5](Kolodziej *et al.*, 2013) Optimal method following 4 examined modifications was to carry out the wash steps 3 times.

[6](Dimsoski, 2017) A number of modifications were included together. Modifications used were from previous literature.

Appendix 2 Diet composition for the pig YPC trial (Chapter 4), formulated by AB Agri Ltd.

| % Ingredient unless otherwise specified | Diet 1 (wean-day 14) | | | | Diet 2 (day 14-day 28) | | | |
|---|---|---|---|---|---|---|---|---|
| | Control 0% YPC | 2.5% YPC | 5.0% YPC | 7.5% YPC | Control 0% YPC | 2.5% YPC | 5.0% YPC | 7.5% YPC |
| Raw wheat | 38.3 | 37.4 | 35.6 | 33.9 | 53.8 | 52.0 | 49.8 | 47.6 |
| Micronised barley | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Micronised oats | 5.0 | 5.0 | 5.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| HiPro soya | 10.0 | 10.0 | 10.0 | 10.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Soya oil | 5.1 | 5.3 | 5.5 | 5.7 | 1.1 | 1.3 | 1.6 | 1.8 |
| YPC | 0.0 | 2.5 | 5.0 | 7.5 | 0.0 | 2.5 | 5.0 | 7.5 |
| AlphaSoy 530 | 7.5 | 7.5 | 7.5 | 7.5 | 5 | 5 | 5 | 5 |
| Vitamin and mineral premix | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Whey powder | 10.4 | 10.4 | 10.4 | 10.4 | 3.5 | 3.5 | 3.5 | 3.5 |
| Potato protein | 4.0 | 2.0 | 1.0 | 0.0 | 2.0 | 1.0 | 0.5 | 0.0 |
| L-Lysine HCl | 0.57 | 0.66 | 0.68 | 0.70 | 0.59 | 0.61 | 0.60 | 0.59 |
| DL-Methionine | 0.27 | 0.29 | 0.28 | 0.28 | 0.26 | 0.26 | 0.25 | 0.24 |
| L-Threonine | 0.27 | 0.31 | 0.31 | 0.31 | 0.28 | 0.28 | 0.26 | 0.25 |
| L-Tryptophan | 0.06 | 0.07 | 0.07 | 0.08 | 0.05 | 0.05 | 0.05 | 0.05 |
| L-Valine | 0.15 | 0.20 | 0.21 | 0.22 | 0.14 | 0.15 | 0.14 | 0.13 |
| Limestone flour | 0.32 | 0.38 | 0.44 | 0.50 | 0.08 | 0.14 | 0.19 | 0.25 |
| Dicalcium phosphate | 1.87 | 1.86 | 1.84 | 1.82 | 2.04 | 2.02 | 2.00 | 1.98 |
| Salt | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.03 | 0.04 | 0.04 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sodium bicarbonate | 0.58 | 0.61 | 0.60 | 0.60 | 0.62 | 0.61 | 0.58 | 0.55 |
| Calculated nutritive values | | | | | | | | |
| Protein | 19.7 | 19.5 | 19.9 | 20.2 | 19.3 | 19.7 | 20.3 | 20.9 |
| Digestible energy MJ/kg | 15.3 | 15.2 | 15.2 | 15.2 | 14.1 | 14.2 | 14.2 | 14.2 |
| Oil | 7.6 | 7.7 | 7.9 | 8.2 | 3.3 | 3.6 | 3.8 | 4.1 |
| Fibre | 2.2 | 2.3 | 2.4 | 2.5 | 2.4 | 2.5 | 2.6 | 2.7 |
| Calcium | 0.77 | 0.79 | 0.81 | 0.83 | 0.77 | 0.79 | 0.80 | 0.82 |
| Digestible phosphorus | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Lysine[a] | 1.35 | 1.35 | 1.35 | 1.35 | 1.28 | 1.28 | 1.28 | 1.28 |
| Methionine[a] | 0.54 | 0.54 | 0.54 | 0.54 | 0.51 | 0.51 | 0.51 | 0.51 |
| Methionine + Cysteine[a] | 0.81 | 0.81 | 0.81 | 0.82 | 0.78 | 0.79 | 0.79 | 0.80 |
| Threonine[a] | 0.90 | 0.90 | 0.90 | 0.90 | 0.86 | 0.86 | 0.86 | 0.86 |
| Isoleucine[a] | 0.72 | 0.68 | 0.67 | 0.66 | 0.69 | 0.67 | 0.68 | 0.69 |
| Tyrosine[a] | 0.57 | 0.53 | 0.52 | 0.52 | 0.54 | 0.54 | 0.55 | 0.57 |
| Valine[a] | 0.95 | 0.95 | 0.95 | 0.95 | 0.90 | 0.90 | 0.90 | 0.90 |

[a] Amino acid levels are expressed as standardised ileal digestible content.

Appendix 3 Nutrient composition of Yeast-enriched Protein Concentrate (YPC), see Chapter 4.

| **Ingredient** | **YPC** |
|---|---|
| Crude Protein % | 49.0 |
| Dry Matter % | 95.0 |
| Gross energy MJ/kg | 20.2 |
| Ether extract | 3.80 |
| Ash % | 5.0 |
| Calcium % | 0.02 |
| Phosphorus % | 0.92 |
| Neutral detergent fibre (NDF) | 2.9 |
| Acid detergent fibre (ADF) | 8.5 |
| Starch | 3.0 |
| Sugars | 0.5 |

Appendix 4 Diet compositions for the broiler phytase trial (see Chapter 5), formulated by Target Feeds.

| % Ingredient | Broiler starter | Broiler finisher |
|---|---|---|
| Corn | 59.66 | 62.26 |
| Soybean meal 48 | 35.91 | 32.52 |
| Soy oil | 0.59 | 2.32 |
| Salt | 0.35 | 0.35 |
| Sodium bicarbonate | 0.12 | 0.01 |
| DL Methionine | 0.21 | 0.17 |
| Lysine HCl | 0.08 | 0.00 |
| Limestone mono calcium | 1.22 | 0.85 |
| Phosphorus | 1.36 | 1.03 |
| Vitamin premix | 0.50 | 0.50 |
| Calculated nutritive values, given as a % unless otherwise stated | | |
| Crude protein | 21.93 | 20.41 |
| ME MJ/kg | 12.45 | 13.07 |
| Calcium | 0.90 | 0.70 |
| Phosphorus | 0.71 | 0.61 |
| Available phosphorus | 0.42 | 0.34 |
| Fat | 3.38 | 5.09 |
| Fibre | 2.65 | 2.60 |
| Methionine | 0.55 | 0.49 |
| Cysteine | 0.37 | 0.35 |
| Methionine + Cysteine | 0.92 | 0.84 |
| Lysine | 1.28 | 1.12 |
| Histidine | 0.60 | 0.57 |
| Tryptophan | 0.26 | 0.24 |
| Threonine | 0.85 | 0.79 |
| Arginine | 1.50 | 1.39 |
| Isoleucine | 0.94 | 0.87 |
| Leucine | 1.88 | 1.77 |
| Phenylalanine | 1.10 | 1.03 |
| Tyrosine | 0.82 | 0.77 |
| Valine | 1.03 | 0.96 |
| Glysine | 0.92 | 0.86 |
| Serine | 1.07 | 1.00 |
| Phenylalanine + Tyrosine | 1.92 | 1.80 |
| Phytate Phosphorus | 0.23 | 0.23 |
| Sodium | 0.19 | 0.16 |
| Chlorine | 0.27 | 0.25 |
| Potassium | 0.96 | 0.90 |
| Linoleic acid | 1.42 | 2.15 |
| Magnesium | 0.19 | 0.18 |

Appendix 5 Diet composition for the broiler wheat bran trial (see Chapter 5), formulated by Target Feeds Ltd.

| % Ingredient | Control diet | 20% Wheat bran diet |
|---|---|---|
| Raw ground barley | 8.9 | 0.0 |
| Raw ground wheat | 55.0 | 39.8 |
| Wheatbran | 0.0 | 20.0 |
| HiPro soya | 23.0 | 23.0 |
| Full fat soya (Cherwell) | 5.0 | 5.0 |
| L-Lysine HCl | 0.30 | 0.25 |
| DL-Methionine | 0.35 | 0.35 |
| L-Threonine | 0.15 | 0.15 |
| Soya oil | 4.0 | 8.4 |
| Limestone | 1.25 | 1.50 |
| Monocalcium phosphate | 1.25 | 0.75 |
| Salt | 0.25 | 0.25 |
| Sodium bicarbonate | 0.15 | 0.15 |
| Vitamin and mineral premix | 0.4 | 0.4 |
| Calculated nutritive values | | |
| Oil | 6.361 | 11.036 |
| Protein | 20.252 | 20.627 |
| Fibre | 3.051 | 4.409 |
| Ash | 5.693 | 6.168 |
| Metabolisable energy | 12.927 | 12.928 |
| Total lysine | 1.270 | 1.274 |
| Digestible lysine | 1.185 | 1.186 |
| Methionine | 0.627 | 0.639 |
| Methionine + Cysteine | 0.939 | 0.967 |
| Threonine | 0.851 | 0.875 |
| Tryptophan | 0.237 | 0.247 |
| Calcium | 0.931 | 0.951 |
| Phosphorus | 0.659 | 0.704 |
| Digestible phosphorus | 0.424 | 0.360 |
| Salt | 0.308 | 0.326 |
| Sodium | 0.177 | 0.170 |
| Vitamin A | 10.0 | 10.0 |
| Vitamin D3 | 5.0 | 5.0 |
| Vitamin E | 100.0 | 100.0 |