# An *in vitro* study of the effects of supplementary dietary fibre in pig diets on forms of faecal N and P

Diego Sosa Argana Campuzano

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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# Abstract

Much of the N and P in pig slurry is in forms that can be readily lost to the environment via volatilisation, surface runoff and leaching with detrimental effects on the environment. This thesis investigated the effect of dietary fibre on the composition of pig faeces and explored the potential for its utilisation to valorise pig slurry. Specifically, it examined how increasing dietary fibre, based on its source and type, affects the forms of nitrogen and phosphorus in pig faeces using an *in vitro* model of the pig's digestive tract. It was hypothesised promotion of fermentative activity in the hindgut of the pig via inclusion of dietary fibre would convert more available inorganic forms of N and P into less available organic forms.

The fermentative phase of the *in vitro* pig gut model was inoculated with pig faeces. Comparisons between the faecal N and P composition of gestating sows and effluent from the *in vitro* model 'fed' the same experimental diets as the gestating sows (control and sugar beet pulp supplemented diets) showed no significant interaction ($P > 0.05$) between source of waste (sows and model) and diet, indicating the model could be used reliably in further experiments.

Subsequent experiments showed that adding 5 % of soluble and insoluble dietary fibres, such as inulin or cellulose respectively, to pig diets significantly ($P < 0.05$) reduced total inorganic nitrogen (TIN) per gram of total nitrogen (TN) by an average of 50%. However, this reduction was not observed when 2% of calcium was added to these fibres. The inclusion of inulin in the diet significantly increased the ratio of inorganic phosphorus (Pi) to total phosphorus (TP) by 142% compared to the control diet ($P < 0.05$).

Then, an increase in fibre content from 15 to 27% was achieved by the inclusion of wheat bran, rice bran or soya hulls resulted in significant changes in the ratios of TIN to TN and Pi to TP, as soya hulls diet decreased by 54.34% the ratio of TIN to TN when compared to the control

diet, while RB showed minimal changes in the ratio of TIN to TN and Pi to TP unlike the other fibre sources used in the experiment.

The experimental results showed that increasing soluble and insoluble fibre in the diets of growing pigs can favourably alter the forms of N and P excreted in an *in vitro* pig model. Soluble fibre, which is more fermentable, increased the availability of different inorganic and organic forms of N and P in the faeces compared to insoluble fibre. As a result, increasing levels of soluble fibre sources, such as inulin or sugar beet pulp, in pig diets may improve faecal quality and promote sustainable nutrient recycling.

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# List of abbreviations

| | |
|---|---|
| AA | Amino acids |
| ADG | Average daily gain |
| ANF | Antinutritional factors |
| ANOVA | Analysis of variance |
| ATTD | Apparent total tract digestibility |
| CP | Crude protein |
| DDGS | Distiller dried grains with solubles |
| DF | Dietary fibre |
| DNA | Deoxyribonucleic acid |
| GHG | Greenhouse gas |
| GIT | Gastrointestinal tract |
| IP6 | Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate |
| LF | Low fibre |
| N | Nitrogen |
| $NH_3$-N | Ammonia nitrogen |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| NSP | Non-starch polysaccharides |
| P | Phosphorus |
| SDF | Soluble dietary fibre |
| TKN | Total Kjeldahl nitrogen |
| TIN | Total inorganic nitrogen |
| TON | Total organic nitrogen |

| | |
|---|---|
| TN | Total nitrogen |
| RNA | Ribonucleic acid |
| VFA | Volatile fatty acid |

# Chapter 1 General overview

## 1.1 Worldwide pig farming

Over the past half-century, the global population has grown significantly due to a number of factors, including advances in healthcare, increased life expectancy, and improvements in living standards. These factors have driven a movement of rural populations toward urbanisation and city living (Fig 1-1). Projections suggest that this trend will continue, with the world population projected to reach an estimated 9 billion inhabitants within the next three decades (Food and Agriculture Organisation, 2021).

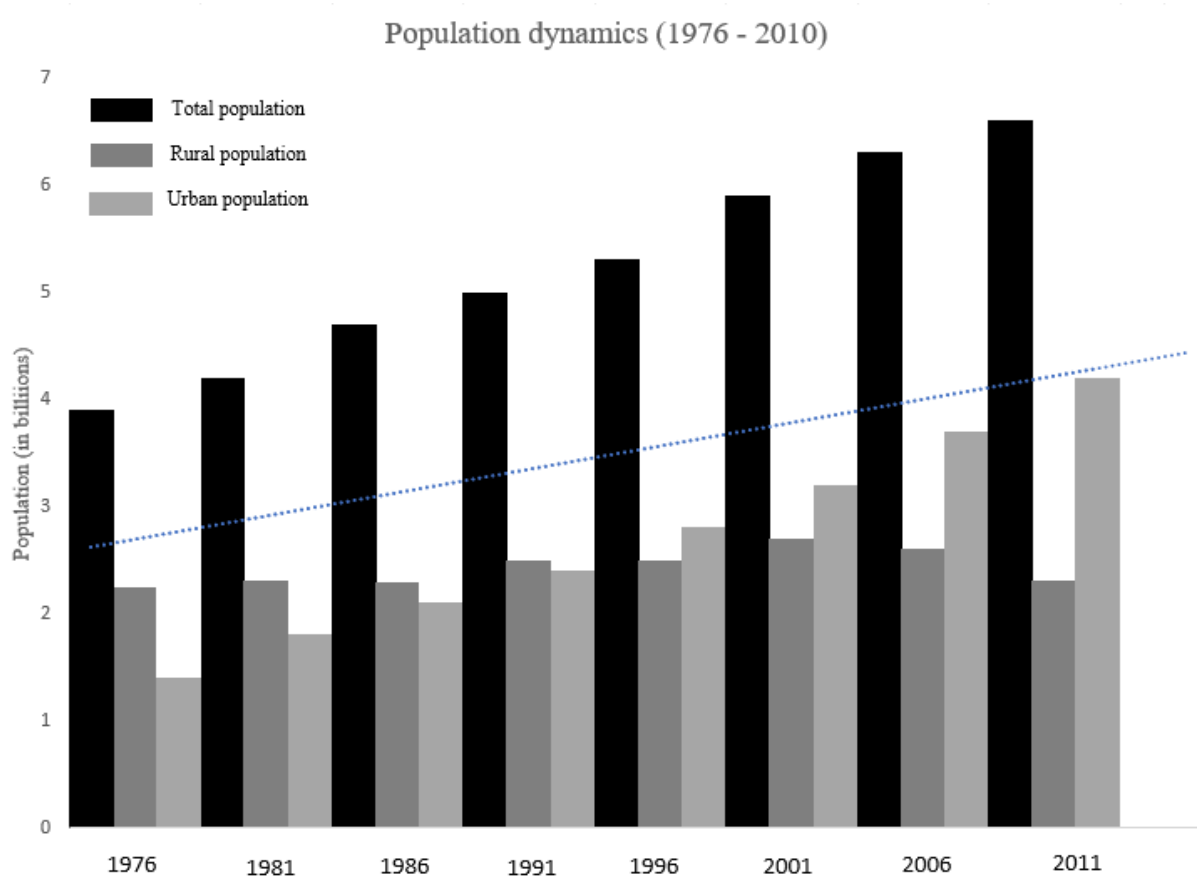


**Figure 1-1 Dynamic of worldwide population during the last 50 years. Modified from FAOSTAT**

Rapid population growth presents both challenges and opportunities, including ensuring food security, access to healthcare, environmental sustainability, and therefore managing the pressure of urbanisation. For example, Salter (2018) reported that in a two-year period, between 2014 to 2016, the global average annual meat consumption per capita was 34.1 kg, with nearly 60% consisting of red meats such as pork, sheep, and beef. As a case point, after chicken meat, pork meat and its derivatives, such as ham or sausage, are the most consumed animal products in both Western and South Asian countries (Nam et al., 2010; Lin-Schilstra et al., 2022). In addition to providing one-third of the animal protein required by humans (Suryawanshi et al., 2017), the production of these livestock products generates significant income and employment across diverse sectors (Collins and Smith, 2022). Consequently, global pig production has risen steadily since the end of World War II, resulting in an output of nearly one billion pigs (Bindelle et al., 2008; DEFRA, 2021; Naimi et al., 2022). This growth illustrates the significant contribution of the pig industry to the global economy, as it exerts considerable influence and exhibits potential for further growth and innovation.

### 1.2 Pig farming in the UK

Over the past decade, there has been a 9% increase in pig production in the United Kingdom. In the preceding two years, an estimated 75,000 tons of pork were produced domestically (DEFRA, 2021). Pig farming in the UK is characterised by a wide range of rearing methods, with the most prevalent being conventional systems, with involve the indoor rearing of pigs on slatted floors. Nevertheless, alternative housing systems are becoming increasingly prevalent (Delsart et al., 2020). In recent decades, there has been a growing emphasis on farming pigs with outdoor access, allowing them to interact with the natural ground in contact with the plants, across both the UK and Europe (Gentry et al., 2008). It is noteworthy that more than

40% of British pig production is now undertaken on those outdoor farms, with a considerable proportion of pigs being reared indoors and then finished in outdoor facilities (AHDB, 2024).

As British farmers have invested in upgrading their pig-rearing facilities, there have been marked improvements in animal health and productivity (Gray et al., 2021). The modern pig farming industry in the UK is increasingly focused on enhancing pig welfare and productivity (Bench et al., 2013), with a particular emphasis on providing environments that foster better health and wellbeing. To maintain this progress, it is essential that the UK not only continues to improve pig housing and rearing systems but also aims to meet or exceed the standards being set across other European countries.

### 1.3 Pig farming in the European Union

It is anticipated that the pig industry in the United Kingdom, as in any other European Union country, will comply with a set of minimum animal welfare standards that are deemed to be appropriate for pig production. It is important to note that these standards are mandatory and must be followed for both indoor and outdoor pig raising systems, as well as welfare recommendations (Pedersen, 2018). Nevertheless, current pig facilities in many European countries, including those situated in the UK, have been subject to criticism for their potential to release nitrogenous compounds, leach phosphorus, and emit odours from their sheds (Jha and Berrocoso, 2016). For example, Canh et al. (1998) stated that a pig excretes nearly a fifth of the total nitrogen intake deriving from digested proteins and half of the nitrogen intake is excreted through urine. Furthermore, the pig industry is responsible for depositing higher quantities of phosphorus (P) in the soil, which poses a significant threat to various ecological environments (Peters et al., 2024). Pigs excrete a greater quantity of P than ruminant species due to their monogastric digestive system and the absence of the enzyme phytase, which is essential for the breakdown of phytate-P ($C_6H_{18}O_{24}P_6$). Phytate, present in the bran and seeds

of cereals, can contain up to 80 % of the phosphorus found in these plants (Golovan et al., 2001; Oonincx and Finke, 2021). Therefore, in contrast to ruminants, pigs are unable to effectively hydrolyse phytate, resulting in elevated phosphorus excretion through manure and urine. These concerns have led to the establishment of measures aimed at reducing the accumulation of such compounds in the environment. Consequently, it is imperative that ongoing efforts be made to mitigate the ecological impact of phosphorus deposition from pig industry.

### 1.4 The environmental impact and ecological consequences of pig farming

The release of a range of undigested nutrients into agroecological environments has led to initiatives aimed at enhancing the properties of pig manure to offset the detrimental effects of land application. It is important to provide context here, as if pig manure is not properly managed, it can negatively affect surface and groundwater through nutrient leaching, leading to increased oxygen demand and eutrophication (Villegas et al., 2011). Moreover, a considerable proportion of the nutrients released by pig manure into the environment significantly increases atmospheric greenhouse gases. In a typical pig farm for instance, pigs emit greenhouse gases such as $CO_2$, $CH_4$ and $N_2O$, which are measure as $CO_2$-equvalents ($CO_2$equiv) (Philippe and Nicks, 2015). Therefore, a gestating sow produces 2.85 kg $CO_2$equiv and 0.81 kg $CO_2$equiv of $CH_4$ and $N_2O$ respectively per day (Costa and Guarino, 2009; Liu et al., 2016). In comparison, a finisher pig generates 3.35 kg $CO_2$equiv and 0.72 kg $CO_2$equiv of $CH_4$ and $N_2O$ respectively per day, according to Costa and Guarino (2009) and Nadine Guingand et al. (2010). Therefore, these gases account for 89 % of the total gases generated in pig facilities. Of these released gases, 69% is methane, and 20 % is nitrous oxide, which flow in the manure (Philippe and Nicks, 2015).

### 1.4.1 Agricultural waste in pig farm

A large amount of agricultural waste is generated worldwide as a consequence of intensive livestock. Moreover, the pig industry also represents a significant risk of environmental contamination due to the presence of hazardous materials, including antibiotics and pathogens (Huang et al., 2017). Since poultry and pig meat are among the most commonly consumed meats worldwide (see section 1.1), it is reasonable to assume that their production will be considerable, resulting in some of the largest outputs within the farm industries (Huang et al., 2017). The release of those agrochemicals and nutrients is one of the inevitable consequences of large-scale pig production in the UK and European countries (Philip Robertson et al., 2014; Bowles et al., 2020). A considerable proportion of the nutrients contained in the manure are released into the surrounding environment. This occurs due to the storage of animal manure, which is not directly disposable due to sanitation limitations. This manure is predominantly stored at or near animal farms for the purpose of fertilisation (Melikoglu and Menekse, 2020).

### 1.4.2 Pig manure as fertiliser

Pig manure is a widely utilised organic fertiliser in sustainable farming practices to enhance agricultural soils with essential nutrients. It contains an elevated nitrogen, phosphorus, and potassium content, which are essential for optimal plant growth and soil fertility. A finisher pig produces nearly 4.5 kg of manure and slurry daily (Schiavon et al., 2009), which may contain around 22.14 g of nitrogen (N), 4.8 g of phosphorus (P), and 11.25 g of potassium (K) (Cavanagh et al., 2011). Despite the potential benefits, approximately one-third of the manure generated in pig farming is not adequately managed or separated for proper land application, which has resulted in significant environmental pollution (Wang et al., 2022). The inadequate disposal of manure can result in the contamination of water bodies through the runoff of nutrients, which in turn contributes to the process of eutrophication and causes harm to aquatic

ecosystems. It can be reasonably deduced that pig manure may also be a source of agricultural pollutants, representing a risk to the environment and public health. To address these concerns, improved manure management strategies must be implemented, including enhanced separation techniques, effective nutrient recovery systems and sustainable application methods. This will aid in reducing the ecological footprint of pig farming and ensure the long-term health of agricultural lands and surrounding ecosystems.

### 1.4.3 The principal compounds that constitute pig manure or slurry

In the preceding section, it was demonstrated that the composition of pig manure is influenced not only by the size of the farm but also by the type of digestive system that pigs possess. Although a small portion of N and P in manure originates from endogenous sources like digestive secretions, the bulk of these nutrients originate from animal feed (Muji et al., 2018). The excess dietary protein beyond the animal requirements is one of the main contributors to the increased nutrient load in manure. While this manure serves as a crop fertiliser, the loss of excess N, P, and minerals such as potassium (K), iron (Fe), and calcium (Ca) through runoff and leaching can lead to the eutrophication of water sources (Van Dijk et al., 2016).

In addition to nutrients, pig manure may contain pollutants such as heavy metals, including zinc (Zn) and copper (Cu). These metals are added to pig diets to promote growth and prevent health complications (Nicholson et al., 2003; Hristov et al., 2011). However, these metals are not fully absorbed by the animals, resulting in their accumulation in manure and the potential for soil contamination when used as fertiliser. Over time, this can impair soil health and lead to groundwater and surface water contamination via leaching. Therefore, the management of both the nutrient and heavy metal content in pig manure is essential to minimise environmental impacts while ensuring sustainable agricultural practices.

### 1.4.3.1 Nitrogen in pig manure

Nitrogen plays a vital role in the body development. Atmospheric or gaseous nitrogen ($N_2$), which makes up to 80% of the Earth atmosphere, is generally unavailable to most organisms (Hardenburger et al., 2005). In living beings, N is a fundamental component of amino acids, which are crucial for the synthesis of proteins necessary for growth and development. Certain anthropogenic activities, such as animal production and agriculture, alter the nitrogen cycle by converting nitrogen into forms accessible to crops through nitrogen fixation. This process optimises nitrogen inputs in areas where its limitation would otherwise impede normal biological processes. According to Van Der Peet-Schwering et al. (1999), the estimated nitrogen excretion, expressed as a percentage of the feed input, was observer to average 38% for weaners, 63% for growing-finishing pigs, and 75% for sows (Table 1-1)

**Table 1-1 Nitrogen chain in growing-finishing pig, Modified from Aarnink and Verstegen (2007)**

| Component | Description | N form |
|---|---|---|
| Dietary Intake | From feed containing protein (amino acids) | Organic N (e.g., crude protein) |
| Digestion | Protein is broken down into AAs in the digestive tract | Absorbed N (body use) / Faecal N |
| Absorption | AAs are absorbed; some N is retained for growth (muscle, tissues) | Retained N |
| Metabolism | Excess amino acids are deaminated; N is converted to urea (liver) | Urinary N (urea, ammonia). |
| Excretion | Undigested N (faeces) and urinary N (urea) are excreted | Faecal N + Urinary N. |
| Slurry storage | Urea hydrolyses to ammonia ($NH_3$); volatilisation occurs | Gaseous N losses ($NH_3$, $N_2O$) |
| Environment | Residual N in slurries may be recycled as fertiliser or lost to air/water | Leached N ($NO_3^-$) or emissions |

In farmyard manures (FYM) nitrogen is present in inorganic forms and organic forms. Inorganic forms of nitrogen include ammonium ($NH_4^-$) and ammonia ($NH_3$). In pig manure, approximately 30-50% of the nitrogen is organic, including mineral N (principally $NH_4^-$) and readily mineralizable N (urea and uric acid) (Bhogal et al., 2016). These forms of nitrogen are determined by the nutrient content of the feed and the stocking density, which together determine the rate at which excreta are added to the land surface (Webb et al., 2014)

#### 1.4.3.2 Nitrogen utilisation and metabolism in pigs

It has been previously mentioned that a pig excretes about 20% of the nitrogen through faeces, urine, and slurry. In pig nutrition, soybean meal is a common ingredient due to its amino acid composition, which closely meets the nutritional requirements of pigs. Cereals also represent a significant source of nitrogen content in pig diets, as they are used extensively in commercial pig diets (Lautrou et al., 2021). Alongside carbohydrates, dietary proteins are one of the main factors regulating the gut microbiota in animals (Holmes et al., 2017). However, a quarter of the protein in a typical fibrous soybean meal diet cannot be utilised by the animal (Aarnink and Verstegen, 2007). Therefore, protease enzymes have been added to fibrous diets as part of enzyme supplements in pig nutrition to improve the digestibility of crude protein (Zuo et al., 2015). While other potential protein sources, including cottonseed meal, rapeseed meal, and distillers dried grains with soluble (DDGS) may offer a viable alternative, their inclusion in the pig diet may lead to a reduction in growth performance. To ascertain the impact of these feedstuffs on feed cost, it is vital to evaluate the true ileal digestible amino acids (AA) present in them. Furthermore, the net energy effect on the true ileal digestible AA values and the influence of various anti-nutritional factors (ANF) on the dietary AA requirements for pigs must be given due consideration (Woyengo et al., 2014).

#### 1.4.3.3 Different forms of dietary nitrogen in the pig hindgut

As the dietary contents reach the hindgut, the levels of fermentable carbohydrates and proteins decrease. As a result, the gut microbiota shifts to using rapidly and slowly fermentable dietary fibre (DF) as an energy source, and eventually turns to resistant dietary proteins and endogenous proteins (Nahm, 2003). Undigested proteins, digestive fluids and microorganisms from the small intestine combine with secretions produced in the hindgut, further modulating microbial activity, and influencing nutrient availability. This environment, characterised by high water content and rich concentrations of nitrogen, energy and minerals, is highly conducive to the proliferation of a robust and active microbial community (Rérat, 1978). In addition, decarboxylation and deamination of unabsorbed amino acids derived from proteins and peptides digested by microbial proteases occurs in the hindgut (Sung et al., 2023). The gut hosts one of the most complex and dynamic microbial ecosystems, with up to $10^{11}$ to $10^{12}$ bacteria per gram of gut content (Liping Zhou et al., 2016). This intricate microbial community plays a vital role in the metabolism of nutrients. The interaction between the composition of the diet and the gut microbiome directly influences the digestibility of faecal crude protein (CP) (Verschuren et al., 2020).

#### 1.4.3.4 Inorganic nitrogen

During hindgut fermentation, inorganic forms of N are transformed via aerobic degradation of organic wastes in pig manure (Dolliver et al., 2008). Microorganisms transform nitrogen through a series of steps: nitrogen fixation ($N_2$ to $NH_3$), assimilation ($NH_3$ to organic nitrogen), ammonification (organic nitrogen to $NH_4^-$), nitrification ($NH_4^-$ to $NO_2$- and then $NO_3$-), and denitrification ($NO_3$- to $NO_2$-, then to NO, $N_2O$, and finally $N_2$) (Kuypers et al., 2018). Therefore, in pig manure significant amounts of nitrogen are lost through $NH_3$ and $N_2O$

emissions due to ammonification and denitrification processes. Various bacteria facilitate nitrogen transformation, thereby increasing nutrient availability (Wang et al., 2022)

#### 1.4.3.5 Organic nitrogen

The predominant forms of nitrogen present in pig manure are organic (Qian and Schoenau, 2002). Organic nitrogen exists in different forms, including amino acids, proteins, nucleic acids, nucleotides, and urea. These forms contribute to the total hydrolysed nitrogen (THN), which includes the following components: hydrolysable $NH_4^+$-N, amino sugars-N, amino acids-N, unidentified-N, and acid-insoluble nitrogen (AIN). THN can be further classified into the following categories: amine nitrogen (AN), amino acid nitrogen (AAN), amino sugar nitrogen (ASN), and hydrolysable unknow nitrogen (HUN) (Chen et al., 2020). On the other hand, the role of soil microorganisms in the conversion of inorganic nitrogen to organically bound nitrogen is of great significance, with the process being influenced by the carbon-to-nitrogen (C:N) ratio (Hjorth et al., 2010; Chen et al., 2020). The C:N ratio is an important factor in the immobilisation of $NH_4^+$-N. For example, Huang et al. (2004) observed that slurries with a low C:N ratio (C:N=15) presented a higher $NH_4^+$-N content compared to those with a higher C:N ratio (C:N=30) ($P < 0.05$). It is crucial to achieve a balanced C:N ratio in manures for optimising their function as soil conditioners and enhancing their efficacy as organic fertilisers (Song et al., 2018).

#### 1.4.3.6 Implications of nitrogen balance in pig production

Given that pigs are monogastric animals with a digestive capacity that allows them to consume a diverse range of ingredients, reducing the crude protein content of their diet has become a common practice. This strategy has the additional benefit of reducing feed costs while enabling the exploration of alternative ingredients. A reduction in dietary protein represents a cost-effective and practical approach to the reduction of nutrient overload, particularly nitrogen (N),

in soil through animal manure. A substantial body of research has been conducted to assess the impact of reducing dietary protein as a means of mitigating its adverse environmental effects (Canh et al., 1998; Portejoie et al., 2004; Hayes et al., 2006). Nevertheless, a reduction in crude protein (CP) necessitates a comprehensive approach that considers factors beyond protein and AA levels. It is equally crucial to optimise the overall feed composition, ensuring adequate levels of fibre, electrolytes, and energy, as these elements may be jeopardised when diets are modified (Alfonso-Avila et al., 2019). This observation is so important because one of the main requisites to feed formulation is to know the exact amount of nutrients that will receive the animal after digestion, to keep with their normal functions of live and produce (Pomar et al., 2021). Balance protein may have some implications in the health, such as the decrease of the abundances hindgut microbiota, the total tract nutrient digestibility of pig feed (Zhou et al., 2022). However, other authors as Cappelaere et al. (2021), suggest that low-protein diets formulated with an adequate AA supply can still maintain growth performance in growing and finishing pigs

#### 1.4.3.7 Phosphorus

Phosphorus is a vital element found in all organisms, and in humans and animals is primarily found in body fluids, bones, and teeth. It plays a crucial role in essential genetic functions such as RNA and DNA, as well as in mechanisms that protect health (Adams et al., 2018). P is involved in maintaining acid-base homeostasis in body fluids and is a key element in energy transfer through its role in adenosine triphosphate (ATP) metabolism (Zhao et al., 2021; Lautrou et al., 2021). Animals not only need P to grow but also for the plant, because plants need a supply of P for photosynthesis and other features of the plants (Abbas, 2016). The bacterial function in the gastrointestinal tract (GIT) of pigs is dependent on an adequate dietary provision of phosphorus (P), as supported by increasing evidence (Heyer et al., 2019).

From the perspective of animal nutrition, an estimated two-thirds of the phosphorus present in cereal grains and oil seeds -the primary components of pig diets- exists in the form of phytate-P, which monogastric animals like pigs are unable to efficiently utilise. However, just one-third of the phosphorus is absorbed and utilised by the animal, with the remainder being excreted (Humer et al., 2015). Phytate-P has the capacity to bind to other nutrients, including minerals, impeding their digestibility and absorption by monogastric animals (Denbow et al., 1995). It thus follows that inorganic P must be supplemented in the diet of pigs to meet their nutritional requirements. The feeding of monogastric animals with phytate-rich feedstuffs leads to interaction between phytate-P (the most myo-abundant inositol hexaquisphosphate) and other nutrients, which further impacts the availability of nutrients and the efficiency of digestion (Humer et al., 2015).

In nature, there are two recognizable P forms: inorganic and organic P. Inorganic P (Pi) is typically found in soil as orthophosphate (Watson et al., 2018), and its availability to plants is highly dependent on soil pH. Pi can exist in various other forms, including octocalcium phosphate, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), or secondary Pi forms bound to oxides, such as aluminium phosphate ($AlPO_4$), iron phosphate ($FePO_4$), trimagnesium phosphate ($Mg3(PO_4)_2$), dicalcium phosphate ($CaHPO_4$), struvite ($Mg(NH_4)PO_4 \cdot 6H_2O$). The interactions between these forms of Pi and metal cations influence soil pH, and contribute to the formation of more labile P, which is more available for plant uptake (Chen et al., 2012). However, the lability of P may also depend upon other soil characteristics, such as its capacity for P retention, which in turn affects phosphorus solubility (Smith et al., 2004; Pizzeghello et al., 2011). Clay surfaces, with a high content of kaolinite, in association with iron and aluminium, exhibit enhanced capacity to bind P, thereby reducing its mobility (Azzoni et al., 2005; Almeida et al., 2019). Meanwhile, most of the organic P fertilisers used for soil amendment are orthophosphate-based. Therefore,

increases in labile P, particularly in biofertilizers such as pig slurry, resulting in the formation of P fractions that are more readily available for plants (Oliveira Filho et al., 2019).

#### 1.4.3.8 Inorganic P

As most of the P present in cereal grains, the major constituent of pig feed, is phytate-P, and the pig cannot hydrolyse completely this antinutritional factor, nutritionists must rely in supplements that contain inorganic P to meet the nutritional requirements of pig (Zimmermann et al., 2003). As was discussed in the previous section, inorganic P. most of the P found in living organisms are in the form of phosphates, a salt or ester of phosphoric acid or calcium phosphates (Humer et al., 2015). According to Adedokun and Adeola (2013), a significant amount of phosphates (Pi) is found either in organic form or binding to other organic compounds such as proteins and lipids. Hence, a little amount of available P is utilised by the animal, P from other inorganic P, including deflourinated or dicalcium phosphate, need to be supplemented in pig diets, resulting in a higher amount of excreted P, being most of poor available PP (Angel et al., 2002).

#### 1.4.3.9 Organic P

The compound Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6) represents the main form of organic phosphorus found in the seeds and grains of most cereals, which are common ingredients in pig diets. This molecule is a phosphorylated cyclic sugar alcohol commonly referred to as phytate-P in its anionic form (Angel et al., 2002). There are other inositol phosphates, including inositol pentaphosphates (IP5) and inositol tetraphosphates (IP4) which are present in very small quantities in these feedstuffs, representing less than 15% of the total inositol phosphates (Grases and Costa-Bauza, 2019). Pigs are very inefficient using the phytate-P because they lack adequate enzymes that allow liberation of the P contained in that molecule (Thacker et al., 2003).

#### 1.4.3.10 Phosphorus digestion and absorption in pigs

The absorption of phosphate occurs in its inorganic form, irrespective of the source, whether inorganic or organic, following hydrolysis (Zhai et al., 2022). In section 1.3 it is explained that the lack of intrinsic phytase in pig results in inadequate breakdown of phytate-P in diets based on cereal grains and oilseed meals. Phytase enzymes can break down IP6 into myo-inositol and inorganic phosphate This enhances the accessibility of mineral elements. The supplementation of phytase has been demonstrated to exert no significant effect on the dry matter, crude protein, or energy digestibility of the diet ($P > 0.05$) (Thacker et al., 2006). Although other authors observed an improvement on those parameters as consequence of the 'neutralising' effect of phytase on the antinutritional factors of Phytate-P. This molecule also interacts with proteins according with the solubility of most proteins. The addition of phytase to the diets of pigs has been demonstrated to improve phosphorus absorption, thereby reducing the necessity for inorganic phosphorus supplements. Furthermore, this practice has been shown to improve weight gain, feed efficiency, feed intake, and bone mineralisation, while also decreasing mortality rates (Assuena et al., 2009).

#### 1.4.3.11 Phosphorus metabolism and homeostasis in pigs

It is well established that the small intestine is the primary site of phosphorus absorption in pigs and other monogastric animals (Partridge, 1978; Breves and Schröder, 1991). Although pigs can obtain sufficient P from their diet, modern commercial pigs rely heavily on inorganic phosphates. However, assuming that the solubility of inorganic phosphorus sources directly reflects their digestibility and absorption is inaccurate. Released phosphate may bind to compounds such as phytate in the digestive tract, reducing its bioavailability (Zhai et al., 2022). Therefore, as pigs are unable to naturally hydrolyse phytate-bound phosphorus, they require the addition of phytase. In monogastric animals, the regulation of systemic P balance is

achieved through the coordination of three key processes: intestinal absorption, bone formation and breakdown, and kidney excretion and reabsorption. A complex network of hormones regulates these processes (Zhai et al., 2022).

#### 1.4.3.12 Factor influencing phosphorus utilisation and excretion

Understanding the nutrient requirements of pigs is crucial for optimising their health, productivity, and environmental impact within intensive pig production systems. The phosphorus utilisation in pigs is influenced by a complex set of factors that include age, and dietary considerations. Genetic advances in pigs have led to more rapid growth and a significant change in the body composition of pigs, and consequently to changes in their nutritional requirements (Varley et al., 2011). On the other hand, as Phytate-P is a polyanionic molecule, has the capacity to chelate to divalent cations, including calcium (Ca), to form mineral–phytate complexes (Selle et al., 2009). This ‘chelating properties of phytate-P’ makes a poor P utilisation by the animal, and poor Ca absorption.

## 1.5 Measures to mitigate or take advantage of the amount of manure produced on a pig farm

In the current agricultural landscape, there is a significant emphasis on improving the quality of pig manure. This is done with the aim of increasing its value for end uses, such as fertilisers to spread over land or for growing insects to feed many animals, such as fish, wild animals or free-range poultry (Sogari et al., 2019; S. Li et al., 2022). The use of organic and waste-based fertilisers in combination with a range of other agronomic practices, has been demonstrated to result in an average reduction of 45.6% in ammonia emissions (T. Li et al., 2022). Although, the feeding of livestock with insect is not yet allowed, the practice of vermicomposting and the utilisation of the resulting material as substrate for black soldier fly larvae (BSFL; *Hermetia illucens* (*L.*)) represents a promising approach. Another approach to the improvement of the

quality of pig manure is the increase of the dietary fibre content in the feed (Canh et al., 1997; Bach Knudsen, 2001; Fledderus et al., 2007). This approach presents a cost-effective solution to enhance pig performance while reducing the risk of nitrogen and phosphorus leaching into the fields. Thus, increasing dietary fibre aims to optimize both animal nutrition and environmental sustainability.

### 1.5.1 Acidification

In the context of pig production, slurry acidification is a widely employed technique that allows farm managers to significantly reduce emissions of ammonia and greenhouse gases by lowering the manure pH to below 6 (Sørensen and Eriksen, 2009). To maintain the desired pH between 5.5 and 6, stored slurry is often treated with strong acids such as sulfuric acid ($H_2SO_4$) or hydrochloric acid (HCl), or alternatively with bio-materials like rice or whey cheese (He et al., 2016; Shin et al., 2019). According to some report acidification of slurry can decrease ammonia emission by third quarter (Sommer and Hutchings, 1995; Kai et al., 2008).

### 1.5.2 Composting

Composting is a widely used process aimed at enhancing the physical and chemical properties of soil using recycled, primarily farm manure. The use of acidified pig manure as ‘composting’ ingredient has been demonstrated to result in a reduction in compost pH, while simultaneously keeping the nitrogen in the form of $NH_4^+$-N (Cao et al., 2020). During the composting process, a number of key reactions occur, the most significant of which is the conversion of $NH_4^+$-N into organic N or the transformation of organic N back into $NH_4^+$-N through biochemical pathway. Furthermore, the close interaction between $NH_4^+$-N and $NH_3$ limits the formation of nitrate. These processes are known as ammonification and denitrification, respectively (Cao et al., 2020).

### 1.5.3 Pyrolysis

Slurry pyrolysis, a key anaerobic thermochemical conversion process, results in the production of an ideal medium (biochar) for land improvement. The biochar produced by this process has the additional benefit of sequestering atmospheric carbon while enhancing soil productivity by increasing the concentrations of P and K (Cantrell et al., 2012). Despite the implementation of diverse treatments aimed to change the physicalchemical properties of farm-produced waste (Van Dijk et al., 2016), to address environmental problems associated to manure accumulation, inconsistencies have been observed among these methods before their application in the field (Chantigny et al., 2007; Makara and Kowalski, 2015). These inconsistencies are mainly attributed to the high cost of the treatments and the release of excess nutrients into the environment.

### 1.5.4 Other strategies to limit or improve nutrient utilisation (nitrogen and phosphorus) compounds in the pig manure

Other measures to control the negative effects from of manure excess on the environment have been examined, including the use of slurry pit covers made with different materials (Misselbrook et al., 2016) coupled with slurry acidification through the addition of sulphuric acid ($H_2SO_4$) (Sørensen and Eriksen, 2009). The covering of manure or slurry storages, such as in building or pits, confers benefits to farmers utilising the farm aqueous biomass. The slurry does not require a previous treatment and can be used on both large and small farms, with a lower cost than other measures (Appels et al., 2011). It is noteworthy that pit covering has been demonstrated to be an effective method for preventing leachate and accelerating the composting process (Cheng et al., 2015). However, the materials utilised to cover the slurry do not have an extended lifespan and can still release considerable amounts of gases (Yagüe et al., 2011). However, an interesting and potentially sustainable approach to optimally use farm

manure as a crop fertiliser, while minimising atmospheric pollution, is through dietary manipulation of the animal (Li et al., 2018; Liu et al., 2019). As previously stated, agricultural waste contains considerable amounts of nutrients (e.g. nitrogen, phosphorous and potassium). These nutrients are produced when undigested food mixed with water used for cleaning and can be harnessed by plants.

### 1.5.5 Diet modification – limiting or improving nutrient utilisation

One of the main strategies, aiming to mitigate the environmental impact of pig production is the manipulation of animal diet. It is well known that many nutritional strategies, as reducing crude protein, include additives and enzyme, or altering the fibre content of the diet, are necessary to monitor feed emissions thus manipulating or customising the properties of manure, either in the houses or field (Cappelaere et al., 2021). The objective of reducing dietary protein or increasing dietary fibre is to optimise both animal nutrition and environmental sustainability. The inclusion of dietary fibre, in particular, the non-starch polysaccharide (NSP) fraction, may exert an influence on variations within the hindgut bacterial community (Passos et al., 2015; Jha and Berrocoso, 2016), thereby enhancing nutrient digestibility in pigs and modifying the forms of nitrogen and phosphorus present in the manure

### 1.5.6 Reduction of protein

As was seen in previous section, reducing crude protein (CP) in pig diets represents a strategy that can be employed to simultaneously reduce production costs and minimise the environmental impact of pig farming. A couple of studies have shown that reducing the CP content in pig diets leads to a decrease in total N excretion. Kerr and Easter (1995) observed an 8% reduction in total N excretion for every 1 percentage unit (pu) decrease in CP content, while Shriver et al. (2003) reported a 10% decrease. Furthermore, irrespective of the dietary

CP content, the nitrogen-to-phosphorus (N:P) ratio in pig manure is higher when fed with low protein diets (LPB) compared to normal protein diets (NB) (Htoo et al., 2007). In addition, the increase of dietary fibre in monogastric diets, without significantly altering the protein content, led to a reduction of approximately 40% in ammonia emissions in pig barns (Ferrer et al., 2021). Because of this, in recent years, there has been increased attention on studies of fibre utilization, driven by its effects on gut health, microbiota composition, and environmental sustainability.

### 1.5.7 Additives and enzymes

The inclusion of various synthetic enzymes on animal feed have been shown to improve pig performance by optimising the utilisation of nutrient, increasing the apparent total tract digestibility (ATTD), and improving the utilisation of phosphorus, thereby reducing o the excretion rate of these compounds in manure (Olukosi et al., 2007; Wang et al., 2020). From a nutritional point of view, the most important exogenous enzymes are the phytases and NSP-degrading enzymes. In this context, extrinsic phytase (*myo*-inositol hexaphosphate phosphohydrolase) plays a role in the sequential breakdown of $InsP_6$ into $InsP_1$ (Humer et al., 2015). Furthermore, phytase inclusion into pig and poultry diets have been demonstrated to have many other positive effects, such as enhanced feed conversion ratios, increase nutrient retention, and optimised bone mineralization, and reduced pollution (Laird et al., 2019). Phytase can be originated from both microbial and plant source. However, some studies according to some reports (Zimmermann et al., 2003), have indicated that microbial phytate is 1.6-2.5 more effective at hydrolysing Phytate-P in the gastrointestinal tract of monogastric animals, even when phytase from microbial and cereal sources are the same. NSP-degrading enzymes may be key enzymes that include, beta-glucanase, xylanase, and glucanase. When

added to the diet of livestock, these enzymes facilitate the partial or complete hydrolysis of the NSP fraction, which is otherwise resistant to degradation prior to hindgut fermentation.

### 1.5.8 Change of fibre level and type

Dietary fibre (DF) is a fundamental and essential component of animal nutrition, playing a decisive role in maintaining overall health and well-being. However, digestion of dietary fibre in monogastric animals is challenging due to the absence of appropriate enzymes that aid in its hydrolysis during its passage through the small intestine (Tiwari et al., 2018; Bai et al., 2022a). DF typically resists digestion by enzymes in the small intestine and becomes partially or fully fermented by the gut microbiota in the large intestine according to their fibre-type (Table 1-2. This fermentation process produces volatile fatty acids (VFAs), including acetate, propionate, and butyrate, along with various gases (Bai et al., 2022).

**Table 1-2 Some predominant fibre-type and their fermentability in the hindgut. Modified from Adams et al. (2018)**

| Fibre-type | Fermentability |
|---|---|
| Cellulose | Partially |
| Methyl and carboxymethyl cellulose | Partially fermentable or non-fermentable |
| Hemicellulose | Partially fermentable |
| Pectin | Highly fermentable |
| Cereal gums | Highly fermentable |

In this respect, the absorption of nutrients is influenced by the physical and chemical properties of dietary fibre, such as its fermentability, ability to add bulk, ability to bind substances, viscosity and gel formation, water retention capability, and solubility (Molist et al., 2014). Therefore, there is increasing interest in modifying or supplementing fibre diets according to its properties in monogastric diets due to its potential functional benefits to the host. The

interest in this topic stems from the recognition of the crucial role of hindgut fermentation in promoting intestinal health and overall welfare. Therefore, hindgut fermentation not only supports digestive function but also enhances nutrient utilization and immune response Luo et al. (2018) highlighting its important role in fostering optimal pig health and well-being.

### 1.5.9 Dietary fibre and its effects of digestive physiology

In monogastric animals, the inclusion of diverse fibre sources in pig diets may offer a potential strategy for modifying growth performance, altering manure composition, and reducing $NH_3$-N and GHG emissions. Thi Bich Ngoc et al. (2020) stated that The LF and HF-DDGS diets resulted in higher ADG and increased $NH_3$-N emissions, while also leading to lower N and P excretion, as well as reduced $CO_2$ and $CH_4$ emissions, in comparison to the HF-BG and HF-CC diets.

On the other hand, fibre tends to reduce the digestibility of various nutrients, thereby enriching manure with fertilised nutrients such as N and P due to reduced utilisation and increased endogenous losses (Lynch et al., 2008)

The passage of digesta and the increase in diet digested in the foregut of monogastric animals can be reduced by viscous and low-fermentable dietary fibres (Hooda et al., 2011). These phenomena in the pig small intestine are due to the ability of viscous fibre to bind water, increasing the viscosity of the digesta and thereby reducing nutrient digestibility in the ileum (Graham et al., 1986; Renteria-Flores et al., 2008). However, the addition of a highly viscous carboxymethylcellulose to the diet of weaned pigs was demonstrated to improve crude protein digestibility (Fledderus et al., 2007). Therefore, the digestibility of fibre by gut microbiota is influenced by the presence of different types of fibre.

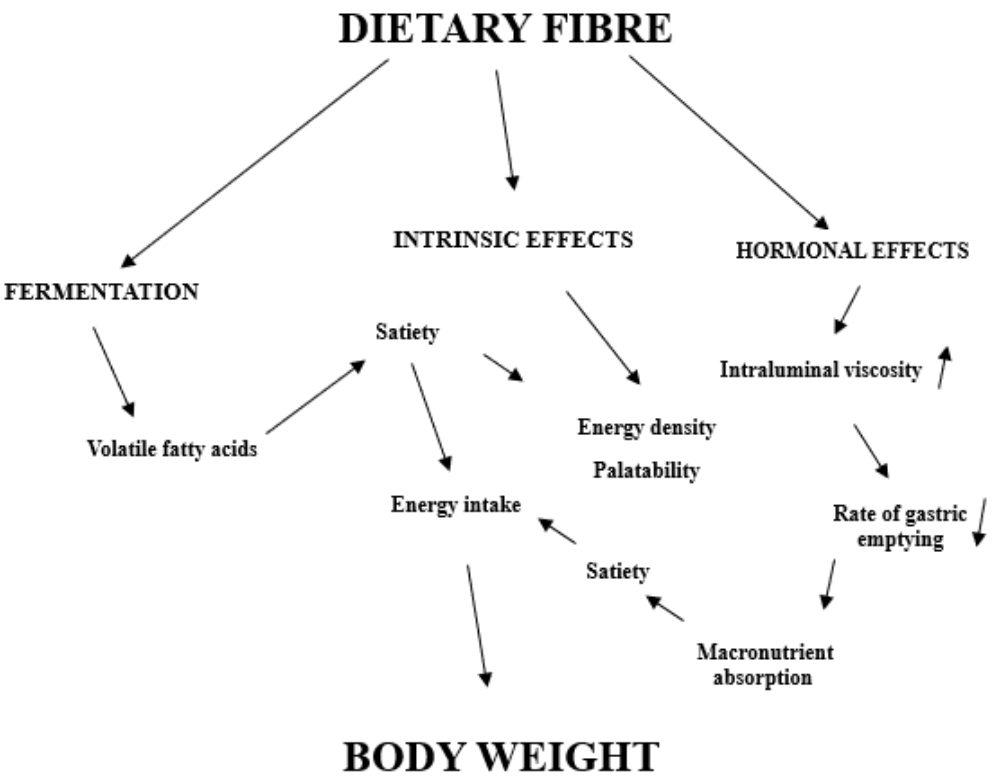


**Figure 1-2 Effects of dietary fibre in the organism. Adapted from Slavin (2013)**

These properties have also an impact on nutrient absorption and therefore, in the gut microbiota composition (Adams et al., 2018). Consuming diets that are low in fibre but high in protein, can trigger a pro-inflammatory response both locally and systemically, increasing susceptibility to disease (Zhao et al., 2015). A diet low in fibres has been demonstrated to facilitate the proliferation of mucin-degrading bacteria, which contribute to the erosion of the protective mucus layer in the gut, thereby compromising its barrier function (Li et al., 2024). However,

at the same time in the upper gastrointestinal tract, dietary fibre increases the duration of gastric emptying and slows the absorption of nutrients. These effects depend largely on the physical characteristics of the fibre, particularly its viscosity (Eastwood, 1992). The degree of viscosity plays a crucial role in regulating both processes, affecting the rate at which nutrients are released and absorbed within the digestive system.

Moreover, the digestibility of different types of dietary fibre varies considerably depending on their specific composition and characteristics, as highlighted by Han et al. (2020). For instance, Chabeauti et al. (1991) reported that wheat straw (*Triticum aestivum*) has a digestibility of 16%, wheat bran, which contains equal amounts of soluble and insoluble fibre, has a digestibility of 44%, and sugar beet pulp (*Beta vulgaris*), which contains more soluble fibre, has a digestibility of 70% in growing pigs. Soybean hulls (*Glycine max*) have the highest digestibility at 79%. The addition of insoluble fibre to post-weaning pig diets appears to be associated with an increase in feed intake and gastrointestinal tract (GIT) development, which reflects significant physiological effects (Molist et al., 2014). The insoluble fibre of particular important in post-weaning diet can be obtained from alfalfa meal, barley hulls, or oat hulls (Freire et al., 2000; Hedemann et al., 2006; Kim et al., 2008; Gerritsen et al., 2012)

### 1.5.10 Types of dietary fibre

Dietary fibre, which is derived primarily from plant-based carbohydrates, exhibits a number of distinctive characteristics. A wide range of dietary fibre is comprised of carbohydrate polymers, which can be categorised into a number of different groups, including soluble polysaccharides and hydrated cellular particles, as well as insoluble lingo-cellulosic material, and resistant starch (Williams et al., 2001). These polysaccharides comprise mainly cellulose, hemicellulose and pectin, which made up to roughly 90 % of the cell primary wall of the plants (Molist et al., 2014). These types of fibres are classified as soluble and insoluble fibre because they resist

endogenous enzyme hydrolysis. Therefore, they act as substrates for microbial fermentation in the animal gut Fig 1-3.

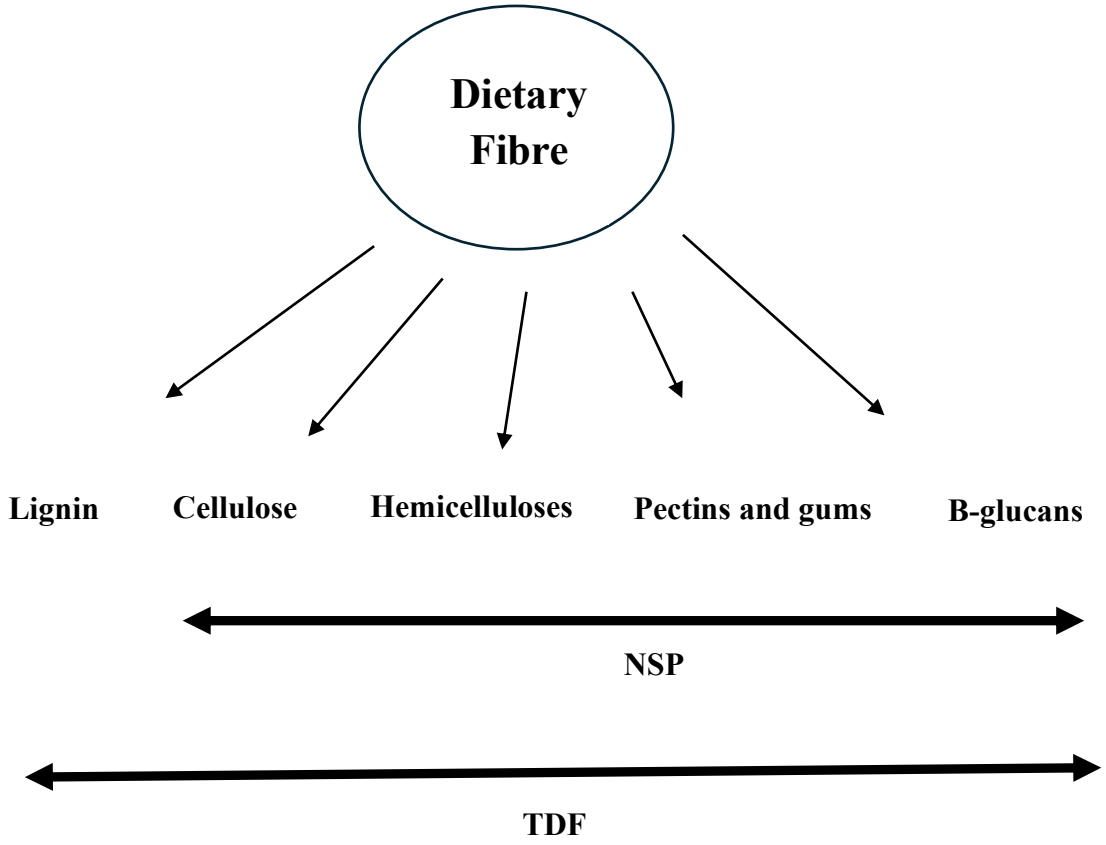


**Figure 1-3 Dietary fibres fractions. Adapted from Chassé et al. (2021)**

### 1.5.10.1 Soluble dietary fibre

The main characteristics of this type of fibre is their highly rate of fermentation in the caecum and colon. According to Makki et al. (2018), SDFs can modulate the gut microbiota, increasing the production of VFAs, which contribute to beneficial health effects. Most of the soluble NSP are viscous. They form a gel in the digestive tract, which affects post-meal metabolism by slowing glucose and lipid absorption (Makki et al., 2018). Plant-derived SDFs can be easily obtained from agricultural by-products such as coffee peel, pear fruit pomace, and soybean hulls (Zheng et al., 2023)

**Pectin**

Pectin is a primary constituent of non-lignocellulosic plant cell walls. It is believed to have co-evolved with the mammalian GIT due to the prolonged consumption of plant-based ingredients across the animal kingdom for millions of years (Wiese et al., 2021). This polysaccharide mostly found in apples, citrus fruits, sugar beets, and potatoes contains linear 1.4-D-galacturonan segments and branched rhamnogalacturonan segments (Beukema et al., 2020; Kara et al., 2023). Pectins are also used in animal nutrition as gelling and stabilising agents. In human health, they are particularly known for their ability to reduce cholesterol and serum glucose levels. These unique functions make pectins fascinating compounds (Mohnen, 2008). Pectins, with their diverse functions, play a crucial role in various contexts and, unlike cellulose, pectins undergo significant degradation within the colon of non-ruminants (Drochner et al., 2004). This process predominantly yields acetate during fermentation. They contribute to plant growth, morphogenesis, and defence signalling.

**Other soluble sources**

Fructans, glucomannans, mucilages, β-glucans and gums are other less abundant oligosaccharides (Kerr and Shurson, 2013). Some of these oligosaccharides are widely used as feed additives due to their recognised prebiotic effects (Bach Knudsen et al., 2012; S. Li et al., 2022). According to several authors, *Lactobacilli* and *Bifidobacteria* are the main targets for these prebiotics (Fuller and Gibson, 1997; Owusu-Asiedu et al., 2006; Parracho et al., 2007; Wylensek et al., 2020). Xylooligosaccharides (XOS) are another notable example of a prebiotic derived from lignocellulosic materials consisting of multiple xylose units linked by β-(1,4)-xylosidic bonds (Amorim et al., 2019). In accordance with the research conducter by Hamaker and Tuncil (2014), prebiotics are defined as “substances that are selectively used by host gut microbiota” thereby promoting overall health and well-being (Han et al., 2020). These specific

types of fibre, frequently categorised as “prebiotic fibres” play a crucial role in stimulating the growth of beneficial bacteria supports digestive health, enhances immune function, and may contribute to the prevention of different diseases.

#### 1.5.10.2 Insoluble dietary fibre

Cellulose is the main structural component of cereal grain cell walls. It is an insoluble fibre made up of a linear homopolymer of β-(1-4) linked glucose units.

As reported by Owusu-Asiedu et al. (2006), the addition of insoluble dietary fibre, such as cellulose, to a diet result in a significant increase ($P < 0.05$) increase in the populations of bifidobacteria and enterobacteria during fermentation. Furthermore, a significant increase in the total anaerobic and enterococcal populations was observed ($P < 0.05$). Despite, the inclusion of a soluble fraction derived from guar gum in these treatments explored by Owusu-Asiedu et al. (2006), the presence of both soluble and insoluble fibres did not influence the levels of total aerobes, lactobacilli, and clostridia. It can therefore be concluded that the combination of both type of fibres into the diet enhances the populations of bifidobacteria and enterobacteria in the ileum ($P < 0.05$), thereby demonstrating the synergistic effect of these fibre types on gut microbiota composition.

There are many affordable options for fibre-rich feed that can be used to increase or improve the dietary fibre content in monogastric diets, including pig and poultry. The increase in dietary fibre content may help to prolong the feeling of satiety and contribute positively to animal welfare (Jørgensen et al., 2007). Furthermore, dietary fibre, depending on its solubility, can enhance the growth rate and improve the body weight of weaned piglet growth rate (Guillemet et al., 2007).

However, fibres that are readily fermentable and viscous, such as pectin and guar gum, tend to reduce protein digestion more than non-viscous and non-fermentable fibres like cellulose (JøRgensen et al., 1996). The consumption of a diet rich in fibre by pigs can result in the fermentation of ingested material in the small intestine. This process can lead to an increase in microbial growth, which in turn can reduce the efficiency of protein digestion (Jha and Berrocoso, 2016). Therefore, consumption of dietary fibre has an impact on nutrient absorption in animal diets. In monogastric nutrition, various physicochemical properties are measured, including solubility, fermentability, cation exchangeability, hydration properties, viscosity, particle size, and organic compound adsorptive properties (Jha and Berrocoso, 2015)

In addition to helping fill the gut, these fibrous compounds contain NSP and lignin. These fibre compounds help fill the gut and are a source of NSPs and lignin.

On the other hand, the increasing production of biofuel by-products such as wheat DDGS or rapeseed meal, rich in fibre and protein, also could provide an attractive alternative to animal feed (Jarret et al., 2012). These by-products can be used in animal feed, replacing cereals and soybean meal. This promotes the use of local resources and reduces reliance on grains in the livestock industry (Jarret et al., 2012).

### 1.5.11 Mechanisms of fibre digestion in monogastric animals

In monogastric animals, the breakdown of the different dietary fibre fractions is highly variable, primarily due to the absence of specific digestive enzymes and their role in increasing viscosity within the intestinal lumen (Józefiak et al., 2004). A considerable proportion of these DF fractions undergo fermentation in the GIT of monogastric species. The digestibility of different types of dietary fibre varies considerably depending on their specific composition and characteristics (Han et al., 2020). Chabeauti et al. (1991) reported that wheat straw (*Triticum aestivum*) has a digestibility of 16%, wheat bran, which contains equal amounts of soluble and

insoluble fibre, has a digestibility of 44%, and sugar beet pulp (*Beta vulgaris*), which contains more soluble fibre, has a digestibility of 70% in growing pigs. Soybean hulls (*Glycine max*) have the highest digestibility at 79%. The addition of insoluble fibre, such as hulls and bran from cereals and legumes to post-weaning pig diets appears to be associated with an increase in feed intake and gastrointestinal tract (GIT) development, which reflects significant physiological effects (Molist et al., 2014).

### 1.5.12 Effects of fibre on gut microbiota composition

Diet digestibility by gut microbiota is influenced by the presence of different types of fibre. Compared with other non-ruminants, such as chicken, the caecal microflora of pigs has been demonstrated to possess a greater capacity for the fermentation of DF fractions (Carré et al., 1990; JøRgensen et al., 1996). In monogastric animals, the fermentation of various fibre sources yields $CO_2$, $CH_4$, and VFAs (Ngalavu et al., 2020). These VFAs serve as the primary products of fermentation by fibrolytic bacteria such as *Prevotella, Lechnopiraceae* among others microorganisms which have carbohydrate functions (Menni et al., 2017; Su et al., 2022). VFAs can function as an energy source for the animal and also act as signalling molecules, regulating various biological processes that contribute to maintaining intestinal health in the host. (Koh et al., 2016; Ngalavu et al., 2020). Moreover, the VFAs produced via fibre fermentation can reduce colonic pH and therefore improve the solubility of minerals, including calcium (Younes et al., 2001). Other products of fermentation include indoles (e.g., indole, 3-methylindole, 2-methylindole, 2,3-methylindole, 2,5-methyindole, and 5-chloroindole), phenols (e.g., phenol, p-cresol, and 4-ethylphenol), and volatile sulfur containing compounds (e.g., dimethyldisulfide, diethydisulfide, di-n-propyland di-nbutyldisulfide) (Nahm, 2003). In mature pigs, the energy derived from the VFAs produced by fibre fermentation in the large intestine can be significant. However, in young pigs during hindgut fermentation of dietary fibre the mean energy supply of VFAs only can provide approximately 7-18% of the net energy

absorbed by the pig need for pig maintenance (Anguita et al., 2006). Besides, the presence of VFAs in digesta and faeces may not accurately reflect the actual production capacity of VFAs by the gut microbiota. This is because they only represent the remnants of VFAs that have been absorbed in the hindgut. VFAs in the hindgut are absorbed by the epithelium and diffuse into the portal vein (Marty and Vernay, 1984).

Only a small portion, approximately 5% of VFA production in the hindgut, is excreted in faeces (Koh et al., 2016). The predominant VFA in the proximal gastrointestinal tract (GIT) is acetate, representing more than 90% of the total VFA generated at this site. The microbiota in the caecum and colon are primarily responsible for the production of acetic acid, propionic acid, and butyric acid through anaerobic fermentation of carbohydrates. Another product of hindgut fermentation is lactic acid. In addition to the fatty acids, these fermentation products facilitate a 'cross-feeding' mechanism among different bacterial groups, which is crucial for their survival (Von Engelhardt et al., 1998; Vermeulen et al., 2018). The VFA are also linked to enterocytes, dendritic cells, type 1 T-helper cells, type 2 T-helper cells and Treg cells to regulate adaptive immunity, exerting either pro-inflammatory or anti-inflammatory effects (Adebowale et al., 2019).

Finally, the extent to which dietary fibre (DF) undergoes microbial fermentation depends on its accessibility to the microbial population in the hindgut (Jha and Berrocoso, 2016). Animals fed a high-fat diet in place of a low-fat grain diet exhibited notable variations in both the chemical composition of their faeces and the release of gases (Trabue et al., 2022).

### 1.5.13 Microbial population responsible for fibre degradation

Fibre fermentation within the pig gut is largely driven by bacterial species such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Bifidobacterium* spp. and *Bacteroides* spp., which can degrade complex polymers, including cellulose, hemicellulose, and pectins (Kaoutari et

al., 2013). The intake of inulin and fructo-oligosaccharides has been demonstrated to enhance the growth of bifidobacteria in the colon (Gibson and Roberfroid, 1995). The types of bacteria that efficiently ferment certain dietary carbohydrates influence the production of VFA, including acetate, propionate, and butyrate. These VFAs have been shown to play a critical role in supporting intestinal health and contributing to the host animal's energy metabolism (Crittenden et al., 2002). The high degree of specialisation of *Bacteroides* spp. in the metabolism of complex polysaccharides can be attributed largely to the extensive repertoire of carbohydrate-active enzymes (CAZymes) encoded within its genome (Raba et al., 2024). The members of the Ruminococcaceae family are important in the process of breaking down resistant starch, which is otherwise difficult to degrade. By liberating these complex carbohydrates, they facilitate their subsequent metabolism by the broader microbial community, thereby contributing to the overall digestive process and energy extraction in the gut ecosystem. Numerous species of the genus *Bifidobacterium* have the ability to proliferate by producing arabionofuranosidases, which are enzymes that facilitate the degradation of arabinoxylan (AX) for utilisation as a carbon source. Understanding the fermentative potential of individual species within the gut microbiota is imperative to elucidate the mechanisms of polysaccharide fermentation in the pig colon and the impact of various non-digestible carbohydrates on the population dynamics of the intestinal microbial community.

VFAs, specifically acetate, propionate, and butyrate, represent the predominant end products of carbohydrate fermentation. These metabolites play a pivotal role in reducing colonic pH, thereby impeding the growth and activity of potentially harmful bacterial organisms. Furthermore, the nitrogen (N) and phosphorus (P) metabolism within the intestinal microbiome of pigs is mediated by various bacterial species, including Clostridium spp., Escherichia coli, and Prevotella spp. These microorganisms facilitate key biochemical processes such as

ammonification and nitrification, converting organic compounds into inorganic forms, including ammonia and nitrates (see Section 1.4.3.4).

Currently, there is a lack of data on the specific bacterial mechanisms involved in phosphorus transformation in the gut. However, certain bacteria, such as *Bifidobacterium spp*., have been shown to produce phytases, which facilitate the release of inorganic phosphorus from phytates. This process enhances the absorption and utilisation of phosphorus by the host organism. Furthermore, a lower gastrointestinal pH, resulting from VF acids, reduces the negative charge of phytic acid. This, in turn, limits the formation of insoluble phytates, which are less susceptible to enzymatic breakdown than phytic acid itself (Vieira et al., 2018).

### 1.5.14 The interaction between fibre and other nutrients in pig gut

Dietary fibre, is an essential component of animal diets, significantly influences various aspects of digestive function and gastrointestinal health. The complex mechanisms by which dietary fibre interacts with the other components of the digestive system reveal its profound effects on nutrient absorption, gut microbiota composition and modulation of faecal characteristics. In contrast, while dietary fibre can have adverse effects, such as reducing the digestibility of certain nutrients and influencing various aspects of digestive physiology, some dietary fibre fractions have historically been considered 'antinutritional factors' (Jha et al., 2019). This perspective is consistent with the pig nutrition paradigm, which considers indigestible carbohydrates to be negative dietary factors due to their fermentation process and adverse effects on overall energy utilisation in the body (Zijlstra et al., 2012).

## 1.6 Effects of fibre on nitrogen and phosphorus utilisation in the pig gut

Some components present in crop by-products such as the case of pectin in sugar beet pulp, which is a soluble fibre, that may increase the viscosity of digesta, reducing the digestibility

of AA in small intestine (Zhao et al., 2020). As was seeing previously, among the different factors that impact the fibre on the utilisation of nutrients are the differences in fibre constituents due to the different processing technologies used (Zhao et al., 2020), the solubility of fibre (Potkins et al., 1991), the water-binding capacity of the fibre (Abdul-Hamid and Luan, 2000), and mainly the type of fibre, which include dose, structure and relative molecular mass (Younes et al., 2001)

### 1.7 Synergistic and antagonistic effects of fibre on nitrogen and phosphorus

There are various synergetic effects of fibre on nitrogen and phosphorus forms. however, how was seen in previous section of this literature review, there are other synergetic effects on water excretion, and manure pH, further reducing emissions. Perhaps, one type of fibre alone per se, cannot exert most effect on nitrogen and phosphorus, however a combination of different carbohydrates has synergistic effect on intestinal nutrient, improving their absorption, and enhancing the transformation of N and P during hindgut fermentation (Younes et al., 2001). Moreover, fibre increase in precision feeding programs contributing to improve environmental performance of pig production, synergy with the reduction of dietary CP (Cappelaere et al., 2021).

### 1.8 Implication for pig health and production efficiency of diet modification

As most of these types of fibre are fermented in the colon, there is a great interest on assess the effects of these dietary fibres, their degree of fermentation and their relationship with the different physiological effects in the animals. The increase of dietary fibre in pig diets, including, the NSP fraction, has been demonstrated to influence the composition of the hindgut bacterial community (Passos et al., 2015; Jha and Berrocoso, 2016). This, in turn, has the potential to enhance nutrient digestibility. As previously discussed, dietary fibre has a great impact on the production of VFA during hindgut fermentation. These VFAs, along with other

compounds, such as ferulic acid (Adebowale et al., 2019) and lactic acid (Gali et al., 2023), serve as an energy source and contribute to improved health and piglet performance. Moreover, the production of VFAs, the addition of these NSPs and other specific oligosaccharides further alters the pathway of nitrogen excretion and reduces odour emission (Sutton et al., 1999). However, that alterations that occur in the animal are dependent upon the type and origin of fibre. On the other hand, for decades the best approach to reducing the manure nitrogen content has been to decrease the amount of protein present in the diet. Even though, in pig production, this strategy may have economics implications, either by having to supplement the diet with synthetic amino acids (Sutton et al., 1999), or having to deal with a poor daily weight gain, particularly in the growing stages (Luo et al., 2015).

Meanwhile, various synthetic enzymes have been shown to improve pig performance, enhancing efficiency of nutrient utilization, increasing nitrogen ATTD and enhancing phosphorus utilization, thereby reducing the excretion rate of these compounds in manure (Olukosi et al., 2007; Wang et al., 2020). The most important exogenous enzymes, from a dietary point of view, are the phytases and carbohydrases. In pig production, the most used mineral supplement in the diet is phosphorus (Van Dijk et al., 2016). This is because, unlike ruminants, pigs cannot process all the P they consume, especially plant P, which is found as phytic acid (myo-Inositol-1,2,3,4,5,6-hexakisphosphate, IP6) (Cowieson et al., 2004). When the gastrointestinal tract of pigs cannot hydrolyze the phytic acid content in the cell wall, phytase enzyme should be added to the rations at levels from 500 up to 750 FTU (Humer et al., 2013). Also, phytase addition has many positive effects in pig diets such as better feed conversion ratios, nutrient retention, and bone mineralization, and reduced pollution (Laird et al., 2019).

## 1.9 Overview of *in vitro* pig gut models

It is challenging and costly to study the fate of nutrients in the digestive system using *in vivo* models, especially when the evaluation involves a wide range of ingredients, such as dietary fibre. Like other living organisms, the pig gut exhibits numerous interactions between physiological components and ingested feed. However, no current assay or model can fully and accurately replicate these complex interactions (Ghiselli et al., 2021). The pig gut model is commonly used to simulate digestion in the stomach and foregut, as well as fermentation in the hindgut. Once *in vitro* pig models are validated, these techniques can be routinely employed to assess the characteristics of fibre fermentation in various feedstuffs. This approach offers a rapid and cost-effective alternative to *in vivo* methods (Williams, Bosch, Boer, et al., 2005). The most common model used for studying the pig gut is batch culture, where a fixed amount of substrate is provided at the beginning of the experiment without further no additional feeding during the process (Williams, Bosch, Boer, et al., 2005).

In contrast, also other *in vitro* models, including continuous or semicontinuous culture protocols, are used to maintain a balanced state by synchronising substrate input and output rates. This methodological approach facilitates the modelling of long-term microbial processes.

### 1.9.1 Batch culture systems

In a batch culture system, a determined amount of substrate is mixed with a suitable inoculum prepared from a fermentation buffer. Gas production is monitored throughout the fermentation process to assess the fermentation kinetics (Cone and Van Gelder, 1999). Typically, the fermentation process lasts between 24 and 72 h, during which time samples are collected for the analysis of various end-products (Williams et al., 2005). After fermentation, key parameters such as dry matter degradation and end-product concentrations, including volatile fatty acids (VFAs) and ammonia, are measured. Due to its simplicity, this technique offers several

advantages, including the ability to independently analyse the behaviour of many ingredients during fermentation while requiring only a minimal substrate. The main disadvantage of batch culture models is their inability to operate for extended periods of time (beyond 72 hours) due to the accumulation of fermentation byproducts, such as VFAs, which affect pH control, and the loss of fermentable substrate.(Bindelle et al., 2007; Tugnoli et al., 2020).

Furthermore, the batch system is unable to allow pH adjustment during the experiments a critical parameter for microbial survival. Maintaining a stable and optimised pH during the *in vitro* fermentation is crucial to ensure maximum microbial activity (Bai et al., 2020). Though these evident and described weaknesses in the batch culture models, there are still strengthens in their use, as could be easily reproducible due to their robustness and simplicity in other labs (Brodkorb et al., 2019).

### 1.9.2 Continuous culture

Continuous models are designed to replicate the critical biological and physicochemical conditions of the pig colon, including temperature, pH, retention time, the input of ileal effluents, and the maintenance of a complex, metabolically active, and sustained anaerobic environment (Fleury et al., 2017). Among these models, the PolyfermS model is recognised as a simple yet highly effective tool for studying gut microbiota under such controlled conditions (Zihler Berner et al., 2013; Tanner et al., 2014). This innovative *in vitro* fermentation system enables researchers the study of ecological shifts within the gut microbiota by facilitating direct comparisons of different treatments on the same microbial community. According to Dostal et al. (2013) this facilitates the simulation of various colon regions using parallel setups inoculated with identical microbiota derived from the inoculum. The utilisation of continuous culture models confers numerous benefits, including precise control over experimental conditions, the capacity to explore extreme dietary variations, maximising microbial activity,

and enhanced reproducibility of results (Zihler Berner et al., 2013; Bai et al., 2020). Therefore, these biological and physicochemical parameters mentioned before are highly controlled when using continuous cultures, which facilitate a steady-state condition

Such models employ a gas production technique designed to measure the volatile fatty acids (VFA) produced and gases generated during fermentation. A key objective of gas production techniques is to assess these fermentation by-products. VFA and lactic acid have been shown to influence total gas production in in vitro models. Intriguingly, in in vitro pig fermentation models, these by-products should reflect the fermentation profile of feed in the hindgut (Palowski et al., 2021). In the field of animal nutrition, in vitro, cumulative gas production methods are widely used to characterise the colonic fermentation of fibre-rich ingredients used in animal diets (Cone and Van Gelder, 1999; Bindelle et al., 2007).

Nevertheless, it is important to remember that current *in vitro* models of the pig gut may have limitations in mimicking all the digestive processes that occur *in vivo*. Most of the models developed thus far do not account for crucial physiological conditions such as pH, temperature, nutrient bioavailability, and metabolism (Jha et al., 2011).

Despite its limitations, the batch culture system, as described by Palowski et al. (2021), was selected for its suitability in assessing N and P forms at the end of fermentation. For this thesis, a modified version of the batch culture system was adopted, as it allowed for easy adaptation to our laboratory setup while maintaining a strong focus on fermentation dynamics. This model provides precise control over experimental conditions, ensuring reproducibility and enabling accurate analysis of nutrient transformations. Although other models, such as continuous, are better suited for simulating long-term and dynamic gut conditions, the batch culture system remains a practical and effective choice for short-term studies aimed at evaluating end-product formation

## 1.10 Limitations and challenges in pig gut research

The diversity of animal physiology poses a unique challenge and creates limitations when performing experiments that aim to simulate complex processes in animals. One notable limitation is the standardization and harmonization of existing protocols (Brodkorb et al., 2019). These limitations are particularly evident when the diet of animals is the main focus of the research, as *in vitro* models cannot accurately replicate the key dynamic processes occurring in the animal gut (Brodkorb et al., 2019). Furthermore, individual variability in the gut microbiota among animals can significantly influence fibre fermentation, leading to model inconsistencies (Li Zhou et al., 2016). Therefore, some methodological aspects of fibre digestion still need to be further investigated before gut models are applied (Bindelle et al., 2007). That means, despite advances in understanding fibre digestion, some methodological gaps persist, indicating the need for further research to enhance experimental methods, improve measurement accuracy and deepen insights into fibre role in digestion and health. Moreover, the process of validating and standardising these methods can require a significant investment of time and financial resources. Furthermore, the quality and characteristics of specific substrates may influence the efficacy of fermentation (Woyengo et al., 2016). Another limitation of pig gut models, particularly those 'static models', is the inability to replicate specific processes throughout the digestive process, such as pH gradients, the gradual addition of enzymes and gastric fluids, or the varying duration of fermentation (Wickham et al., 2009; Ménard et al., 2014)

However, the *in vitro* gut model offers the advantage of observing significant variations when testing different substrates, even with small doses, to assess their effects (El Houari et al., 2022). These characteristics allow us to reduce the cost and time of performing experiments,

including the unnecessary use of animals. Therefore, the main challenge of the *in vitro* animal model will be to harmonise a single model capable of measuring all aspects of digestion.

## 1.11 Project objectives

### 1.11.1 General objectives

The principal aim of this thesis is to examine the impact of varying dietary fibre levels, based on source and type, on the transformation of nitrogen (N) and phosphorus (P) in the excreta within a pig gut model

### 1.11.2 Secondary objectives

Compare the composition of the effluents produced by the *in vitro* model with those produced by living animals will provide a reliable tool for the assessment of different feedstuffs, thus avoiding the use of living animals.

## 1.12 Outline of the thesis

The aim of this thesis was to demonstrate how the addition of dietary fibre in pig diets, using an *in vitro* model of the pig gut, can modify the forms of nitrogen and phosphorus in excreta. This aims to demonstrate the potential for altering the properties of manure in livestock via dietary ingredients to improve its fertiliser value, thereby increasing its utility.

**Chapter 1**

The introduction of the thesis begins by giving a general overview of the challenges associated with nitrogen and phosphorus management in pig production highlighting the central role of dietary fibre in their absorption during their presence in the pig gut. It then explores the complex impacts of dietary fibre on fermentation processes that are driven by hindgut microbiota, thus shaping the composition of the final excreta produced by pigs. Therefore, the

first chapter aims to highlight the importance of dietary manipulation in tailoring faecal characteristics to reduce environmental impact and optimise nutrient utilisation in pig production systems by exploring these interrelationships.

**Chapter 2**

The second chapter of this thesis presents the method section, providing a detailed description of the protocol used in the 'pig gut model', which was based on the models used by Boisen and Fernández (1997) and Palowski et al. (2021) through the different experimental studies, along with an exploration of its output and limitations as discussed in the literature review. This chapter also explains various protocols used to measure different forms of nitrogen and phosphorus, which are the primary focus of this research.

**Chapter 3**

The first results chapter (Chapter 3) aims to evaluate the feasibility of the model by comparing its effluent, hereafter referred to as 'fermentation effluents', with manure samples obtained from gestating sows fed an experimental diet supplemented with sugar beet pulp during their gestation. It is important to note that the substrate used in this experiment was adjusted to represent the same amount of nutrients provided to the gestated sows. The aim of this study is to compare the effect of dietary manipulation on the composition and properties of fermentation effluents and sow faeces to explore how well the *in vitro* model can mimic the digestion and fermentation process occurring in the pig gut. The results shed light on the potential benefits of dietary manipulation to alter the forms of N and P in the effluents of the models, which are comparable to pig manure.

**Chapter 4**

The fourth chapter describes two experiments. The first experiment used an *in vitro* model to study the effects of natural mixed fibre and purified fibre types, such as inulin and cellulose, on the different forms of nitrogen and phosphorus. In the second experiments was explore the interactions of these fibres with calcium nutrients using the same *in vitro* model. The study aims to investigate the impacts of different types and sources of fibre on nutrient dynamics and their effect on tailoring the effluent. The aim of this text is to explore potential dietary strategies for optimizing the use of manure as a fertiliser.

**Chapter 5**

The fifth chapter of this thesis investigates the influence of diverse by-products from the milling and oil industries on various forms of N and P, as well as the fermentation parameters in a pig gut model. The final chapter offers a comprehensive discussion of the thesis's overall findings.

**Chapter 6**

The last chapter discuss the main findings and suggests future research. This chapter integrates the conclusions and suggests areas for further investigation.

# Chapter 2 General methods

## 2.1 Introduction of the methods used

This chapter describes the general methods used in the experiments presented. All the experimental work described in this thesis used an *in vitro* model of the pig gut. The *in vitro* pig gut model used in this study was based on Palowski et al. (2021), which was inspired by the previous work of Boisen and Fernández (1997). The methodology involved a two-stage enzymatic degradation of dry matter, using pepsin and pancreatin sequentially, before advancing to the fermentation stage.

## 2.2 Faecal sample collection for use in the *in vitro* model of the pig gut

The *in vitro* pig gut model includes a fermentation phase using faecal samples as inoculum. Fresh faecal samples were collected from healthy pigs at the National Pig Centre (NPC; University of Leeds). Gestating sows were used as faecal donors in Chapter 3 while growing mixed-sex pigs were used in Chapters 4 and 5. The samples were carefully transferred into airtight plastic bags to minimise air exposure. The samples were stored on ice until being transported back to the laboratory where they were stored at -80°C until required for experimental use.

## 2.3 Dry matter (DM), ash determination in feed, faecal samples, and fermentation effluents

To determine the DM content of the feed and dung samples, a clean pre-weighed crucible was used, with an accuracy of 0.0001 g. Approximately 1 g of each sample was added to the crucible. To ensure the reliability and precision of the results, three replicates of each sample

were used, and recorded as the ′sample fresh′ weight. These samples were then transferred to an oven set up at 95° C, where they were allowed to remain for a minimum of 16 hours. Then, the crucibles containing the ′dry samples′ were allowed to cool in a glass desiccator for 20 minutes before being weighed to determine the DM content in the crucible. For the fermentation samples, a slightly different approach was utilised, which is described in the next section. To avoid mineral contamination, all the crucible and glassware used for P and Ca analysis was subjected to an acid wash with a solution containing 10M HCl. This was then rinsed with distilled water three times and dried at 60°C overnight before use.

To determine the ash content for total P and Ca analysis, a pre-weighed thermostable glass tube approximately 1 g of homogenised dung or 1 ml fermentation sample was accurately weighed. The sample was dried for a minimum 16 hours at 95 °C, and the resulting dry matter in the tube was weighed after allowing to cool to room temperature using a glass desiccator. To determine the ash content, the thermostable glass tube containing the dry matter was placed in a cool muffle furnace and then heated to 500 °C for a minimum of 16 hours.

Following the ashing procedure, the samples were cooled to room temperature for 20 minutes in a glass desiccator. They were then weighed with an accuracy of 0.0001 g, and the ash content of each sample was calculated

### 2.4 Measurement of VFA using Gas Chromatography

A 1.5 ml volume of fermentation effluent was collected from each bottle and stored in a screw-topped tube at -20°C until VFA analysis could be performed. The samples were then thawed and analysed via GC (Jouany, 1982). This involved the addition of 250 µl of a solution containing 2 g/L of mercuric chloride, 20 ml/L of concentrated orthophosphoric acid (85% aqueous solution) and 2 g/L of 4- methylvaleric acid to 1 ml of fermentation effluent in a Nalgene™ Oak Ridge high speed centrifuge tube (Thermo Fisher Scientific, MA, USA). The

4-methylvaleric acid was utilised as an internal standard (IS). The centrifuge tube was capped and thoroughly mixed to ensure uniformity. The samples were subsequently subjected to centrifugation at 20,000 × g for 20 minutes at 10°C. Supernatant was transferred into a GC vial for analysis. Calibration standards, consisting of 5 mM acetate, 5 mM propionate, and 5 mM butyrate, were prepared following the same procedure as that used for the fermentation fluid samples.

A GC with a polyethylene glycol nitroterephthalic acid-treated (BP21) capillary column coupled to a flame ionisation detector (FID) was used to analyse the samples. The injection port temperature was 240°C, the FID temperature 280°C, and the carrier gas was helium (5 ml/min). The concentrations of acetate, propionate, and butyrate were calculated using the peak areas ratio method.

**Table 2-1 Dietary and pharmacological history of the pigs from which faecal samples were collected for the *in vitro* studies**

| Experiment | Chapter | Classification | Sex | Diet | Pharmacological history |
|---|---|---|---|---|---|
| SBP gestating sows | 3 | Gestating sows | Female | Standard gestation + sugar beet pulp | Treated annually with Flubenol™ 5% |
| High fibre diets (purified fibres) | 4 | Growing pigs | Mixed | Standard growing diet | No treatment in this group |
| Bran-based diet | 5 | Growing pigs | Mixed | Standard growing diet | No treatment in this group |

## 2.5 Dietary fibre determination

Total dietary fibre in the dietary samples was determined using the Total Dietary Fibre Assay Kit (TDF-100) from Megazyme International Ireland Ltd. (Bray, Ireland). The analysis included the measurement of soluble dietary fibre (SDF), insoluble dietary fibre (IDF) and total dietary fibre (TDF) according to the gravimetric method developed by Prosky et al. (1988).

## 2.6 Substrates used in the 'pig gut model'

The experimental diets used throughout this thesis consisted of a variety of fibre types, including sugar beet pulp (rich in pectin), inulin, cellulose, wheat bran, rice bran and soya hulls. The source of these fibres is described in the following chapters. The experimental diets were ground to ensure that they could pass through a 1 mm sieve before being used in experiments. The DM and ash content of the experimental diets were determined as described in Section 2.3.

## 2.7 *In vitro* pig gut model

### 2.7.1 Enzymatic hydrolysis

Twenty-four hours prior to commencing each fermentation, the phosphate buffers for enzymatic hydrolysis and the fermentation buffer were prepared and pre-warmed to 39°C in an incubator. The enzymes, pepsin (Sigma P7000, ≤250 units/mg; Sigma-Aldrich Corp., St. Louis, MO) and pancreatin (Sigma P7545; 8 x USP specifications), for the simulated gastric and intestinal hydrolysis steps were freshly prepared on the day of the procedure. Then, the samples underwent a two-step hydrolysis using the procedure described by Palowski et al. (2021).

In essence, approximately 4 g of sample was accurately weighed into 100 ml conical flasks. For the initial step, which involved pepsin hydrolysis, 35 ml phosphate buffer (0.1 M solutions of $KH_2PO_4$ and $Na_2HPO_4$ were prepared and then combined 7:1 respectively and the final pH

adjusted to 6.0 with either 1 M HCl or 1 M NaOH as necessary) and 14 ml of 0.2M HCl were added to each flask. The pH of each flask was adjusted to 2.0, if necessary, with either 1M HCl or 1M NaOH. Then was added 5 ml of 150 mg pepsin/ml 0.2 M HCl solution (Sigma P7000; solids; Sigma-Aldrich Corp., St. Louis, MO) at 39°C was then added to each flask. In addition, to inhibit bacterial growth during hydrolysis, 0.5 ml of an ethanol solution containing 5 mg chloramphenicol per ml (CAS: 56-75-7; Fluorochem Ltd) was added into each flask. Flasks were sealed with suba-seals then incubated in a 39°C incubator for 2 hours with gentle continuous mixing using an orbital shaker set up at 100 rpm.

After incubation, each flask received 14 ml of phosphate buffer (0.2 M solutions of $KH_2PO_4$ and $Na_2HPO_4$ were prepared and then combined 1:1 respectively and the final pH adjusted to 6.8 with either 1 M HCl or 1 M NaOH as necessary) and 7 ml of 0.6 M NaOH. The pH of each flask was adjusted to 6.8 using either 1 M HCl or 1 M NaOH. Five ml of freshly prepared porcine pancreatin solution (100 mg pancreatin/ml 0.2 M phosphate buffer; Sigma P7545; 8 x USP specifications) at 39 °C was added to each flask. The flasks were then sealed with suba-seals and placed in an incubator for 4 hours in a 39°C with gentle continuous mixing using an orbital shaker set up at 100 rpm.

After enzymatic hydrolysis, the residues were collected by filtration using pre-weighed glass filtration crucibles (Porosity 2; Duran 258511208). The collected residues were then washed sequentially with acetone (1 × 20 mL), ethanol (1 × 20 mL, 95%) and distilled water (2 × 20 mL), The residues were then dried at 60 °C for 48 h and weighed to determine the *in vitro* digestibility of dry matter (IVDMD) calculated as follows:

$$IVDMD = \frac{W1 - W2}{W1} X\ 100$$

Where W1 was the weight of sample DM before hydrolysis and W2 was the weight of residue DM after hydrolysis

### 2.7.2 *In vitro* fermentation

After determination of the dry matter (DM) content of the hydrolysed sample, approximately 0.5 g of the sample was accurately weighed into 125 ml serum bottles (Wheaton, USA). The method of cumulative gas production developed by Bindelle et al. (2007) and modified from Palowski et al. (2021) was carried out. To correct for gases produced by the inoculum itself, blank bottles containing only faecal inoculum were included in each batch. Faecal inoculum was prepared in a fermentation buffer at 39 °C under a continuous stream of $CO_2$ (60 g faeces/L fermentation buffer). The fermentation buffer contained macro and micro minerals (Table 2.2; Palowski et al., 2021). Before use, the faecal inoculum pH was checked and adjusted where necessary to ±6.8 with 1 M HCl or 1 M NaOH.

To each fermentation bottle was added 50 ml of faecal inoculum. The bottles were placed on a hot plate (*ca* 39°C) with a continuous stream of $CO_2$ bubbling through them for *ca* one minute. The bottles were then sealed with a rubber stopper secured with an aluminium crimp cap. The bottles were gently swirled to mix the contents and then transferred to a 39°C incubator for a 48-hour incubation period. During the incubation period each bottle was individually removed from the incubator and the accumulation of gas pressure (in kPa) within each fermentation bottle was recorded with a digital manometer (Digitron 2023P, Sifam Instruments Ltd, Torquay, UK). After the pressure recording, the bottle was returned to atmospheric pressure. Before

being placed back into the incubator, each bottle was gently swirled. The recorded gas pressures were converted to volume using the equation described by López et al. (2007) as follows:

$$TVG = \frac{V_h}{P_a} X\ P_m$$

The volume of gas produced in millilitres (ml) is denoted by TVG, while the total headspace volume in the fermentation bottle (110.4 ml) is represented by Vh. The atmospheric pressure (100.52 kPa) is indicated by Pa, and the pressure recorded on the manometer (kPa) is designated by Pm.

At the end of the fermentation, after the final gas pressure reading, the bottles were immersed in ice to cease the fermentation process. The pH of the fermentation effluent was then measured immediately. Samples of the well mixed fermentation effluent were collected for analysis of the VFA, microbial crude protein (MCP), Total Kjeldahl Nitrogen (TKN), nitrate ($NO_3^-$), nitrite ($NO_2^-$), and inorganic P (Pi). For the analysis of $NH_3$-N samples were collected in tubes containing a volume of 0.2 M HCl equal to the volume of sample collected.

### 2.7.3 Chemical analysis

Following the 48-h period of simulated fermentation, the fluids obtained from each replicate were subjected to laboratory analysis including ammonia ($NH_3$-N), TKN, $NO_3^-$, $NO_2^-$, total and inorganic P, VFA and MCP. Calculations were then carried out to determine total nitrogen, total inorganic nitrogen (TIN), total organic nitrogen (TON) and total inorganic P and total organic phosphorus. These calculations were essential in assessing the ratios of inorganic to organic nitrogen and inorganic to organic phosphorus in the fermentation effluent.

**Table 2-2 Chemical composition of the fermentation buffer based on the experiments carried out by Palowski et al. (2021)**

| Original Buffer (Palowski et al., 2021) | Modified buffer (Palowski et al., 2021) |
|---|---|
| Deionised water 474 ml/L | Deionised water 474 ml/L |
| Trace mineral solution 0.10 ml/L | Trace mineral solution 0.10 ml/L |
| 132 g $CaCl_2$/L | 132 g $CaCl_2$/L |
| 100 g $MnCl_2 \cdot 4H_2O$/L | 100 g $MnCl_2 \cdot 4H_2O$/L |
| 10 g of $CoCl_2 \cdot 6H_2O$/L | 10 g of $CoCl_2 \cdot 6H_2O$/L |
| 80 g of $FeCl_3 \cdot 6H_2O$ | 80 g of $FeCl_3 \cdot 6H_2O$ |
| Buffer solution 237 ml/L | Buffer solution 237 ml/L |
| 4 g $NH_4HCO_3$/L | 4 g $NH_4HCO_3$/L |
| 35 g $NaHCO_3$/L | 35 g $NaHCO_3$/L |
| Micromineral solution 237 ml/L | Micromineral solution 237 ml/L |
| 5.7 g $Na_2HPO_4$/L | 5.7 g $Na_2HPO_4$/L |
| 6.2 g $KH_2PO_4$/L | 6.2 g $KH_2PO_4$/L |
| 0.583 g $MgSO_4 \cdot 7H_2O$/L | 0.583 g $MgSO_4 \cdot 7H_2O$/L |
| 2.22 g NaCl/L | 2.22 g NaCl/L |
| Reducing agent | Reducing agent 50 ml/L |
| 47.5 ml/L distilled water | 7.8 g/L Cysteine – HCl |
| 335 mg/L $NaS_2$ | 2 g/L NaOH |
| 2 ml/mL 1M NaOH | Resazurin 1.9 ml/L (1.25 mg/L resazurin) |
| Resazurin 1.9 ml/L (1.0 mg/L resazurin) | |

### 2.7.3.1 Ammonia assay

Ammonia concentration in the acidified fermentation effluent samples were analysed using the method described by Cardozo et al. (2004). Samples were thawed at room temperature. Next, 1.5 ml of the thawed samples were transferred to 1.5 ml Eppendorf tube and centrifuged at 10,000 × g for 20 minutes at room temperature using a microfuge. A volume of the resulting supernatant was diluted 1 to 10 with distilled water.

Calibration standards ranging from 0 to 40 µg $NH_3$/ml were prepared by diluting a stock solution of ammonium sulphate ($(NH_4)$ $2SO_4$; 5.87 mM) with a 0.1 M HCl solution. A volume of 20 µl of both the diluted sample and the calibration standards were added to wells in a 96-well plate (each sample was assayed in triplicate). To each well 80 µl of sodium phenate (2.5% phenol in a 1.25% NaOH solution), 80 µl of sodium nitroprusside (0.01%) and 80 µl of sodium hypochlorite (3%). The wells were then covered with adhesive film, gently mixed, and then incubated at 40°C for 10 minutes. Following the incubation period, the plates were cooled to room temperature before measuring the absorbance of the samples at 630 nm using a SpectraMax 340PC plate reader. The calibration standards were used to calculate the concentration of $NH_3$-N in each diluted sample and corrected for dilution employed (20x).

#### 2.7.3.2 Microbial crude protein

Two ml of fermentation effluent were thawed and centrifuged at 10,000 × g for 5 minutes to remove feed particles and protozoa. Thereafter, the Lowry protein assay, as described by Makkar et al. (1982) was followed where a Folin phenol reagent was used to measure proteins. According to the protocol, the supernatant from the initial centrifugation was further centrifuged at 25,000 × g for 20 minutes at 10 ºC and the subsequent pellet was washed with phosphate buffered saline (PBS) and re-centrifuged. The cells that had been removed from the pellets were then subjected to hydrolysis, which involved the use of 0.25 NaOH at a temperature of 100 ºC for 10 minutes. A final centrifugation step at 25,000 × g for 15 minutes was performed to remove cell debris from the supernatant. A bovine serum albumin (BSA) stock solution (1 mg/ml) was used to prepare the calibration standards. Into a 96 well plate 40 µl of sample supernatant, blank (0.25 M NaOH), and the calibration standards were added. A 200 µl volume of a complex-forming solution (CF) was added to each well, comprising of a 100:1:1 (v/v/v) mix of a 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide, 1% (w/v)

copper (II) sulphate pentahydrate, and 2% (w/v) sodium potassium tartrate respectively, and allowed to stand at room temperature for a minimum of 10 minutes. A 20 µl volume of 1N Folin's reagent was added to each well, mixed, and incubated in darkness at room temperature for 30 minutes. Absorbance (750 nm) of the samples was then measured.

#### 2.7.3.3 Total Kjeldahl nitrogen (TKN)

The titrimetric determination of nitrogen by the Kjeldahl method involved a digestion of 2 ml fermentation effluent in a Kjeldaltherm R block digestion unit with 10 ml of concentrated $H_2SO_4$ and one of Kjeldahl catalyst tablet (5 g, containing 4.875 g $K_2SO_4$,).

Following the Kjeldahl digestion (*ca* 1.5 hours), the digested samples were subjected to steam distillation using a Vapodest R 20 unit. The resulting distillate was collected in an Erlenmeyer flask containing 20 ml of 0.1 M sulphuric acid and two drops of methyl red indicator. This was followed by titration with 0.2 M sodium hydroxide (NaOH).

Calculation for total Kjeldahl nitrogen was based on fact that one atom of N will result in the formation of one mol of $NH_3$ results in the consumption of one mol less of alkali required to neutralise the acid-receiving solution. It can therefore conclude that one mole of NaOH is equivalent to 14 g N, given that one mol of $NH_3$ contains one atom of N with an atomic mass of 14 g.

Therefore, if a solution of alkali solution containing 0.2 mol NaOH/L is required to neutralise an acid-receiving solution (which has been weakened by the $NH_3$ formed from a given sample) the amount of N present in the sample can be calculated accordingly:

A/1000 x 0.2 x 14 g N

And thus

A/1000 x 0.2 x 14 x 6.25 g protein

Or

g protein in sample = A x 0.0028 x 6.25

where A = ((20 – volume of titrant) – B) ml

where B = (20 – volume of titrant for blank) ml

#### 2.7.3.4 Nitrite and nitrate assays

The Griess reaction was used for the measurement of nitrate and nitrite using the method modified by Shand et al. (2008). In this method, the nitrite concentration is measured in samples where nitrate has been reduced to nitrite using hydrazine sulphate. The nitrate concentration is then calculated by difference. Samples were thawed at room temperature and extracted with potassium chloride (0.1 M) 1:10 (ratio of sample to KCl) for two hours at room temperature with end-over-end mixing. The samples were then centrifuged at 10,000 × g for 20 minutes. Supernatant was diluted 1 to 2 with distilled water. The following reagents were used for both methods (nitrate and nitrite), except that hydrazine sulphate (1.71 g/L) was used for the nitrate reaction and distilled water for the nitrite reaction. For the catalyst solution, 35.4 mg of $CuSO_4 \cdot 5H_2O$ and 900 mg of $ZnSO_4 \cdot 7H_2O$ were dissolved in 900 mL of water, which was then diluted to 1 L. Sodium hydroxide was prepared at a concentration of 40 g /L, sulphanilamide at 10 g/L in 3.5 M HCl and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) at 1 g/L. Into a 96 well plate was pipetted 135 µl of each diluted sample and standard, followed by 20 µl additions each of the catalyst, sodium hydroxide and hydrazine sulphate solutions. After a 15-minute incubation at room temperature, 75 µl of sulphanilamide solution was added, followed by 20 µl of the NEDD solution. The optical density was measured at 540 nm, and the concentration of $NO_3$- was determined by comparing it to a range of aqueous

standards (0-1 mg/L $NO_3^-$) prepared from a one solution containing 1 mg/ml $NH_4NO_3$. Then the concentration of $NO_2$- was determined using the same methodology as for $NO_3$-, except that the hydrazine solution was replaced by an equal volume of water. Calibration was established by employing different standards (ranging from 0 to 1 mg/ml $NO_2^-$ prepared from $KNO_2$. The results were presented as mg of nitrate ($NO_3^-$) or nitrite ($NO_2^-$) per gram of DM

#### 2.7.3.5 Extraction of inorganic phosphorus from samples (Johnson and Hill, 2011)

The method described by Johnson and Hill (2011) was then applied. A 1 ml thawed sample, either faecal or fermentation, was extracted with 9 ml of a 0.25 M NaOH and 0.05 M $Na_2$ EDTA solution for 16 hours using end-over-end mixing. Following this, the extracted sample was then centrifuged at 10,000 × g for 20 min and the supernatant samples were diluted either 10 or 40 times, depending on the phosphorus concentration, with 2.5 M acetic acid, 0.4 M acetate buffer (70.5% sodium acetate and 29.5% acetic acid, pH 5.0) and deionised water. The final adjusted extract volume was 1 mL or 4 mL at pH 5.0.

#### 2.7.3.6 Sample solubilisation for Total P and Ca determination

In order to measure total phosphorus and calcium in samples, samples were ′ashed′ as described in Section 2.3. Once ′ashed′ they were solubilized in 5 M HCl. To prevent sample loss due to effervescence the 5 M HCl was added in two 5 ml portions to the glass tube containing the ash. The tubes, containing ash and acid were covered with a watch glass and placed in a cool sand bath. The temperature was gradually increased until the samples were boiling. After reaching boiling point, the sample was left to simmer for 5 minutes. The samples were then removed from the sand bath and allowed to cool. Once cooled, the watch glass was rinsed with 4 ml of hot deionised water collecting the rinsing in the sample tube. The contents of the sample tube were then poured into a 100 ml volumetric flask. The sample tube was rinsed three, 4 ml

volumes of hot deionised water into the 100 ml volumetric flask. The solubilised sample was allowed to cool to room temperature before being adjusted to 100 ml with deionised water. A sample of the solution was filtered through a Whatman 541 filter and stored in 50 ml Falcon tubes until analysis. To analyse the total phosphorus, the solubilised sample was diluted with deionised water based on its anticipated phosphorus concentration (4x for manure and 2x for fermentation effluent).

#### 2.7.3.7 Total phosphorus assay (Dick and Tabatabai, 1977)

Eighty µl of the diluted sample and standards made of 1 mM di-potassium hydrogen phosphate ($K_2HPO_4$, 0-1,000 µM $K_2HPO_4$) were pipetted into wells in a 96-well plate in triplicate. In each well was pipetted 100 µl of solution A (0.1 M ascorbic acid and 0.5 M trichloroacetic acid), 20 µl of solution B (0.01 M ammonium molybdate tetrahydrate). Subsequently, 50 µl of solution C (0.1 M sodium arsenate, 0.2 M sodium arsenate dibasic heptahydrate and 5% acetic acid) was added.

The wells were gently mixed, and the plate was covered with a lid. The plate was then incubated at room temperature for 30 minutes. Finally, the colour intensity was measured at 850 nm on a Spectra Max 340 plate reader. The calibration curve was established using the known concentrations defined in the standards, enabling the calculation of the total phosphorus concentration in the solution. Dilutions were corrected appropriately for in the final determination (mg P/g DM).

#### 2.7.3.8 Calcium determination

The calcium content of each fermentation effluent sample was measured in triplicate using an o-cresolphthalein complexone (o-CPC) reagent as described by Zhu et al. (2014). Solubilised samples (section 2.6.3.5) were diluted 1 to 10 with deionised water ensuring their calcium

concentration fell within the range covered by the calibration standards (0 to 22 mg $CaCO_3$/L). Twenty µl of diluted sample (1:10), calibration standards, and 20 µl of 200 µl of 2-amino-2-methyl-1-propanol (AMP) were added to each of a 96 well microplate. Then, the o-CPC solution (80 µl of o-CPC solution (100 mg $L^{-1}$)) was added to each well in the 96 well microplate. The plate was gently tapped to mix and then incubated for 2 minutes at room temperature before measuring the absorbance at 580 nm. Distilled water (20 microlitres) was used as blank. The calcium concentration in the test samples was then calculated using the standard curve.

### 2.8 Statistical analysis

All *in vitro* fermentation data were expressed as the mean values from triplicate serum bottle experiments. Statistical analyses were performed using SPSS (version 29). The significance of differences in concentration and proportions of Total Nitrogen and Total Phosphorus was analysed using ANOVA (GLM), followed by the Tukey or Sidak post-hoc tests to assess treatment effects or other effect described in each chapter.

# Chapter 3 A comparison of the excreted forms of nitrogen and phosphorus from gestating sows and an *in vitro* pig gut model fed a diet supplemented with sugar beet pulp

## 3.1 Introduction

*In vitro* simulation models can represent a highly effective tool for the evaluation of complex phenomena in a simplified context. They permit the assessment of cell responses under well-controlled and repeatable conditions, which can then be tested *in vivo*. In human and animal nutrition a significant challenge is the development of comprehensive models that accurately perform the multiple physiological functions of the digestive tract's different regions (Boisen and Fernández, 1997; Jonathan et al., 2012; Tanner et al., 2014). The objective of gut models is to eliminate the need for the use of animals in the study of nutrient digestion, which often necessitates the use of cannulated animals. Currently, most *in vitro* gut models are used to assess the effects of dietary fibre on gut microbiota behaviour, as well as study the digestibility of different feedstuffs, and their interaction between those substrates and microbiota. In addition, many of these models utilise faecal microbiota to inoculate discontinuous cultivation system or in one-compartment chambers (Martinez et al., 2013).

Since the development of the first *in vitro* model using rumen inoculum to study fibre degradation more than seventy years ago (Hungate et al., 1966), much attention has been paid in the literature to the beneficial effects of fibre supplementation during gestation, especially in sows. This has been explored using *in vitro* models and *in vivo* animal studies to identify effective methodologies to assess fibre fermentation in the hindgut (Bikker et al., 2017; Li et al., 2019; Yang et al., 2022). Animal nutritionists recommend increasing dietary fibre in gestation diets not only to improve gutfulness but also to reduce constipation and increase the

production of VFA. These VFAs, along with other compounds, such as ferulic acid (Adebowale et al., 2019) and lactic acid (Gali et al., 2023), serve as an energy source and contribute to improved health and piglet performance (Yang et al., 2022).

The efficiency of fibre digestion in sows is influenced by retention time during intestinal transit, which in turn depends on both colonic volume and the amount of feed consumed per unit of live weight (Goff et al., 2002). For this reason, older pigs are more efficient in the digestion of fibre than younger pigs. Consequently, sows exhibit higher efficiency in fibre digestion compared to growing pigs (Jha and Berrocoso, 2015). However, the effect of dietary fibre on energy intake during gestation remains inconsistent and inconclusive, with variations observed in both the type and source of dietary fibre (Li et al., 2019). For example, the supplementation of gestating sow diets with inulin, a soluble fibre, has been demonstrated to increase the number of piglets born alive and improve their birth weight. Similarly, the inclusion of sugar beet pulp (SBP) in sow diets, which is rich in both soluble and insoluble fibre, has been shown to influence piglet performance and health positively (Guillemet et al., 2007; Wang et al., 2016). On the other hand, insoluble fibre derived from cereal brans and other by-products, such as those from the oil industry (e.g., soybean hulls), has been shown to increase faecal volume, thereby reducing the absorption of endotoxins. However, adding cellulose as source of DF to sow gestating diets increase the faecal excretion of N by 400 mg per day, reducing the ATTD (Yang et al., 2022).

Dietary fibre consists of several components, including NSP, resistant starch (RS), fructo-oligosaccharides and non-carbohydrate polysaccharides such as cellulose and lignin, which are barely fermented by gut bacteria in the colon (Adams et al., 2018). While high fibre diets are promising for improving sow performance, they contain components known as antinutritional factors (ANF). The inclusion of these ANFs, such as insoluble NSPs and phytic acid, can

reduce nutrient digestibility and increase endogenous protein losses by promoting intestinal mucus secretion and altering luminal viscosity. These greater endogenous losses may, in turn, result in a reduction in whole-body protein retention (Myrie et al., 2008; Tiwari et al., 2018). Consequently, the increased excretion of nitrogen and phosphorus in pig manure can lead to runoff or leaching into surface and groundwater, which in turn contributes to higher oxygen demand and promotes eutrophication (Carpenter et al., 1998).

For example, ammonia ($NH_3$-N), a common form of inorganic nitrogen volatilised from animal manure, raises environmental concerns due to gaseous emissions that contribute to atmospheric acidification. In addition, other forms of inorganic nitrogen, such as $NO_3^-$ and $NO_2^-$, may pose health risks to humans and animals and may lead to over fertilisation of crops and ecosystems (Lu et al., 2017). Several studies (Canh et al., 1997; Zervas and Zijlstra, 2002; Bindelle et al., 2009; Von Heimendahl et al., 2010) have investigated the effect of low-lignified feedstuffs, such as sugar beet pulp, in pig diets on total nitrogen (TN) excretion. These papers found minimal effects on TN excretion but noted a significant shift of nitrogen from urine to faeces, resulting in reduced faecal ammonia emissions. These authors suggested that the presence of fermentable fibre inhibits the conversion of undigested protein in the ileum to absorbable ammonia, diverting it instead to microbial protein synthesis. As a result, undigested protein is excreted in the faeces or is utilised for microbial protein synthesis rather than being absorbed as ammonia (Galassi et al., 2010).

On the other hand, P does not occur in nature as a free element. Instead, it is typically found in the form of inorganic phosphates or organic phosphorus compounds, as those found present in animal feed and manure (McDowell et al., 2001). These compounds are more stable and mobile in the soil. However, monogastric animals feed with fibrous diets often require inorganic P because the P-content in plant diets is largely bound to phytate-P. Due to low phytase activity in their gastrointestinal tract, pigs are unable to utilise phytate-P efficiently.

Robust *in vitro* methodology for simulating the pigs digestive tract would improve understanding of how different dietary ingredients, such as dietary fibre, effect N and P forms in pig manure (Bindelle et al., 2007), which could inform valorisation strategies for pig manure. Therefore, the aim of this experiment was to compare the forms of excreted N and P from gestating sows fed a diet supplemented with sugar beet pulp with fermentation effluent from the *in vitro* pig gut model described by Palowski et al. (2021) to determine the efficacy of the *in vitro* model for the study how dietary ingredients affect forms of excreted N and P.

The objective of this chapter was to investigate the effects of a diet based on sugar beet pulp on the different forms of N and P in the excreta of gestating sows. Furthermore, we aimed to compare these results with those obtained from an *in vitro* model that replicated the pig gut environment, fed the same diets inoculated with faeces collected from the gestating sows. The purpose was to evaluate the model's capability to predict nutrient output in a simulated pig gut environment.

## 3.2 Materials and methods

An experiment was conducted to assess the feasibility of using an *in vitro* model of the pig gut to investigate the influence of dietary fibre on N and P forms in the final fermentation effluent and to compare the results to those obtained from analysis of the faeces of pigs fed the same diet as the *in vitro* model. The aim was to access the applicability of the model for future studies in pig nutrition.

### 3.2.1 Experimental Setup and Treatments

#### Gestating sow trial

Twelve crossbred gestating sows (Duroc × Landrace × Yorkshire; 241.08 ± 28.83 kg) were randomly allotted to receive either a control diet or an experimental diet, resulting in six

observations per dietary treatment. The control group received a standard gestation diet, while the experimental group was fed a sugar beet pulp diet supplemented with a balancer to ensure nutrient equivalence with the control diet (Table 2-1). All sows received annual Flubenol™ w/w treatments, and both diets were formulated to meet or exceed the National Research Council (NRC, 2012) recommendations for vitamins and minerals. The trial, which included the lactation period, lasted for four months. Fresh feed and faecal samples were collected at weeks 4 and 6, following the procedures described in the General Methods (Section 2.2), for qualitative and quantitative analysis of N and P contents. Additionally, these samples were used in fermentation stages of the *in vitro* model.

### 3.2.2 *In vitro* fermentation model of the pig gut

To conduct the entire experiment, which involved both gastric and small intestine digestion as well as fermentation, 4 g of each experimental diet was tested in triplicate. For further details, refer to the General Methods (2.7). The *in vitro* fermentation phase of the model used faecal samples from gestating sows that had been subjected to the same treatments. This was done in order to simulate the hindgut microbiota that is typically found in the caecal colon of pigs. A detailed description of the *in vitro* pig gut model protocol is presented in Chapter 2. The hydrolysis residues from the replicates were collected and dried in preparation for *in vitro* fermentation. Faecal samples collected during the gestation phase of the trial were frozen immediately after collection and thawed at room temperature prior to each run. The fermentation inoculum was prepared by diluting the thawed faecal samples to a concentration of 0.06 g $ml^{-1}$ in buffer solution as described in the General Methods (Table 2-2). The digestion and fermentation phases were conducted in a single batch. However, two batches of the model were performed, corresponding to the two faecal collection time points from gestating sows

during the trial. Consequently, each *in vitro* model represented faeces collected at the 4-week and 6-week (see Table 3-2).

Each serum bottle, containing approximately 0.5 g of residue from the digestion phase, was inoculated with 50 mL of faecal inoculum. Four different fermentation bottles (experimental units) were prepared for each treatment, with the diets consisting of a control gestation diet and a sugar beet pulp-based diet.

**Table 3-1 Ingredients and nutrient composition analysis of experimental diets**

| Ingredient (g/kg) | Standard gestation diet (control) | Sugar-beet pulp + balancer |
|---|---|---|
| Barley | 25 | 25.3 |
| Wheat | 33.5 | 30 |
| Sugar beet pulp | 0 | 34.23 |
| Wheatfeed | 32.4 | 0 |
| Hipo soya Ext | 0 | 3.2 |
| Rapeseed Ext | 2.4 | 2.4 |
| L-Lysine | 0.25 | 0.135 |
| L-Methionine | 0 | 0.01 |
| Threonine | 0.055 | 0.093 |
| Choline Chloride Solution | 0.03 | 0.03 |
| Limestone | 1.5 | 0 |
| Magnesium Phosphate | 0.382 | 0.6 |
| Rock salt | 0.372 | 0.247 |
| Molasses | 3 | 0 |
| **Nutrient composition (g/kg)** | | |
| DM | 861 | 87.12 |
| Oil | 38 | 39 |
| Crude Protein | 123 | 111 |
| Crude fibre | 46 | 80 |

Table continued…

| **Nutrient composition (g/Kg)** | | |
|---|---|---|
| Crude Ash | 49.7 | 59.8 |
| Salt | 5.5 | 3.5 |
| Calcium (Total) | 7.2 | 9 |
| Calcium (digestible) | 8.9 | 9 |
| Phosphorus | 4.3 | 4.5 |

### 3.2.3 Chemical analysis

The procedures outlined by the Association of Official Analytical Chemists (AOAC, 2006) and described them in General Methods (2. 3) were followed to determine the concentrations of dry matter (DM), total Kjeldahl nitrogen (TKN), and ash in the feed, faeces, and fermentation effluent samples. The procedures to determine the different forms of nitrogen and phosphorus are described in General Methods (2.6)

**Table 3-2 The percentages of feed, sugar beet pulp and balancer included in the models.**

| | **Control diet (%)** | **Sugar beet pulp-based diet (%)** | |
|---|---|---|---|
| | Standard gestation diet | SBP | Balancer |
| 4- week treatment | 100 | 62.57 | 37.43 |
| 6- week treatment | 100 | 62.60 | 37.40 |

### 3.2.4 Data analysis

The experiment followed a 2x2 factorial design. One factor was the diet (treatment), which consisted of a control gestating diet and sugar beet pulp-based diet. The other factor was the type of effluent, which was either the faeces of gestating sows or fermentation effluent from the *in vitro* model.

All the data from the different nitrogen and phosphorus forms were analysed in quadruplicate. A two-factor univariate general linear model (GLM) analysis of variance (ANOVA) was conducted on the concentration of N and P forms using IBM SPSS Statistics to compare the effects of treatment (control diet vs. sugar beet pulp-based diet) and type (gestating sow faeces vs. *in vitro* fermentation effluent). The same model was used to analyse the concentration of total inorganic and organic N as a proportion of total nitrogen, and inorganic and organic phosphorus as a proportion of total phosphorus (mg N or P/g TN or TP). When the results were statistically significant, Tukey post hoc tests was applied for pairwise comparison between the models. The significance of the differences between the treatments was determined based on a $P < 0.05$ or less.

## 3.3 Results

### 3.3.1 Concentration and forms of N in sow gestation faeces and fermentation effluent in a pig gut model at four weeks of treatment

A comparison of the different forms of N in the faeces of gestating sows with fermentation effluent collected from the *in vitro* digestion model simulating the four week four of treatment with a sugar beet pulp-based diet led to significantly ($P < 0.05$) higher levels of ammonia in both faeces and fermentation effluent compared to the control gestation diet (48% and 35% higher respectively; Table 3-3). No significant differences were observed in total Kjeldahl nitrogen (TKN) between the two treatments, either in sow faeces or fermentation effluent ($P > 0.05$). However, an interaction between treatment and type of effluent for TKN was observed, whereby the gestating sow faeces exhibited reduced excretion of TKN when fed the control diet compared to the sugar beet pulp-based diet (18.28 and 41.008 mg TKN/g DM). Conversely, no treatment effect was noted in the fermentation effluent (31.385 vs 33.255 mg/g DM). Furthermore, there were no significant differences in the nitrate and nitrite between the standard gestation diet and the sugar beet pulp-based diet ($P > 0.05$). However, significant differences in the concentration of both forms of nitrogen were observed in the analysed samples of gestating sow faeces and fermentation effluents ($P < 0.05$). There was no significant interaction between the two sample types, sow faeces, and fermentation effluents, in terms of nitrate and nitrite concentrations for the treatments ($P > 0.05$).

The concentration of total inorganic nitrogen (TIN) per gram of total nitrogen (TN) (sum of ammonia, nitrate, and nitrite) in gestating sow faeces was not found to demonstrate a statistically significant difference ($P > 0.05$) from the TIN concentration in fermentation effluent for either dietary treatment (Table 3-3). However, the TIN concentrations in the sow faeces samples were higher, with 66.16 and 54.95 mg TIN/g TN for the control and sugar beet

pulp-based diets, respectively, compared to 4.96 and 6.94 mg TIN/g TN in the fermentation effluent for the same treatments ($P < 0.05$, Table 3-3). Notwithstanding these differences no statistically significant treatment effect or interaction ($P > 0.05$) were found with respect to the TIN concentration per gram of TN across the treatments

**Table 3-3 Concentrations of nitrogen forms and proportions of TIN and TON relative to one gram of total nitrogen in gestating sow faeces and fermentation effluent from an *in vitro* model of the pig gut at four weeks of treatment**

| | | Treatment | | | P values | | |
|---|---|---|---|---|---|---|---|
| | **Type (n)** | **Control** | **SBP** | **SEM[1]** | **Treatment** | **Type** | **Treatment*Type** |
| Ammonia (mg/g DM) | S | **1.112[a]** | **1.642[b]** | 0.061 | <0.05 | <0.05 | NS |
| | F | 0.17 | 0.23 | | | | |
| TKN (mg/g DM) | S | 18.28 | 41.008 | 1.721 | NS | <0.05 | <0.05 |
| | F | 31.385 | 33.255 | | | | |
| Nitrate (mg/g DM) | S | 0.033 | 0.04 | 0.001 | NS | <0.05 | NS |
| | F | 0.028 | 0.026 | | | | |
| Nitrite (mg/g DM) | S | 0.014 | 0.026 | 0.002 | NS | <0.05 | NS |
| | F | 0.035 | 0.031 | | | | |
| Ammonia (mg/g TN) | S | 63.60 | 52.87 | 3.572 | NS | <0.05 | NS |
| | F | 4.36 | 6.96 | | | | |
| TIN (mg/g TN) | S | 66.16 | 54.95 | 3.052 | NS | < 0.05 | NS |
| | F | 4.96 | 6.94 | | | | |
| TON (mg/g TN) | S | 933.04 | 945.05 | 3.052 | NS | <0.05 | NS |
| | F | 995.04 | 993.05 | | | | |

nS= 12, nF= 8, [1]SEM = standard error of the mean, S = sow faeces, F = fermentation effluent NS, not significant. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).

After 4 weeks of treatment with the sugar beet pulp SBP-based diet, there were no significant differences in the concentration of ammonia ($NH_3$-N) to each g of TN between the control gestation diet and the SBP-based diet across both excreta samples (sow faeces and fermentation effluent) ($P > 0.05$). However, the concentration of $NH_3$-N regarding each g of TN was significantly higher in sow faeces than in fermentation effluent (63.60 and 52.87 vs. 4.36 and 6.96 mg TIN/g TN, $P < 0.05$). Like the TIN to TN ratio, there was not a treatment effect for TON:TN ratio in both excreta samples, sow faeces and *in vitro* fermentation effluent, respectively, ($P > 0.05$). Nevertheless, the type of excreta influenced the proportion of TON TN ratio ($P < 0.05$). Fermentation effluents fed both experimental diets had higher concentration of TON per g TN compared with sow faeces from gestating sows fed with the control gestation diet and sugar beet pulp-based diet, respectively ($P < 0.05$). Although control gestation diet tended to have lower concentration of TON per g TN compared with the sugar beet pulp-based diet, there was no interaction between the sow faeces and fermentation effluent for the proportion of ammonia to total nitrogen ($P > 0.05$),

### 3.3.2 Concentration and forms of P in sow gestation faeces and fermentation effluent in a pig gut model at four weeks of treatment

The SBP-based diet significantly reduced total P concentration in the fermentation effluent ($P < 0.05$). Moreover, an interaction was observed between the type of waste (gestating sow faeces and *in vitro* fermentation effluent) and the diet, as total P concentration responded differently depending on the waste source. Specifically, the SBP diet led to an increase in total P concentration in the fermentation effluent compared to the control group. In contrast, in the fermentation effluent, the SBP diet resulted in a reduction of total P concentration (mg/g DM) at 4 weeks of treatment ($P < 0.05$). There were significant treatment and type effects ($P < 0.05$) for both inorganic and organic phosphorus (P) concentrations in the two types of excreta, with

an interaction between effluents, (Table 3-4). A significant treatment effect ($P < 0.05$) and effluent type effects were observed with regards to the concentrations of inorganic and organic P in both type of excreta (Table 3-4). In particular, the fermentation derived from the SBP-based diet exhibited a lower inorganic P concentration compared to the *in vitro* effluent from the control diet (44.709 vs. 29.459 mg/g DM). However, no treatment effect was observed in the faeces of gestating sows consuming the same diets. The concentration of Pi was generally lower in the faeces of gestating sows compared to the fermentation effluent, with more pronounced differences between diets, particularly in the control group (CO = 10.25 mg/g DM and SBP = 8.587 mg/g DM in faeces; CO = 44.709 mg/g DM and SBP = 29.453 mg/g DM in fermentation effluent). A similar trend was observed for Po, with higher concentrations observed in both gestating sow faeces and fermentation effluent. However, the control sample for Po in the fermentation effluent was lost, preventing further analysis of potential interaction (Table 3-4).

Following a four-week of treatment period, the concentration of inorganic P relative to gram of total P was not affected by any dietary treatment, including the control gestation diet, and sugar beet pulp- based diet, respectively ($P > 0.05$; Table 3-4). A significant excreta type effect ($P < 0.05$) was observed, with a higher concentration of Pi per gram of total P in the fermentation effluent compared to sow gestation faeces.

**Table 3-4 Concentrations of phosphorus forms and proportions of Pi and Po relative to one gram of total P in gestating sow faeces and fermentation effluent from an *in vitro* model of the pig gut at four weeks of treatment**

| | | Treatment | | | P value | | |
|---|---|---|---|---|---|---|---|
| | Type (n) | Control | SBP | SEM[1] | Treatment | Type | Treatment*type |
| Total P (mg/g DM) | S | 13.674 | 15.914 | 1.234 | <0.05 | <0.05 | <0.05 |
| | F | **46.821**[b] | **32.165**[a] | | | | |
| Inorganic P (mg/g DM) | S | 10.25 | 8.587 | 0.991 | <0.05 | <0.05 | <0.05 |
| | F | **44.709**[b] | **29.453**[a] | | | | |
| Organic P (mg/g DM) | S | 3.424 | 7.328 | 0.656 | <0.05 | <0.05 | NS |
| | F | - | 2.713 | | | | |
| Pi (mg/g P) | S | 754.78 | 553.30 | 34.72 | NS | <0.05 | NS |
| | F | 892.87 | 913.13 | | | | |
| Po (mg/g P) | S | 245.22 | 446.70 | 33.81 | NS | <0.05 | NS |
| | F | 41.18 | 80.87 | | | | |

nS= 12, nF= 8, [1]SEM = standard error of the mean, S = sow faeces, F = fermentation effluent. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).

However, there was no interaction between excreta type (sow gestation faeces and fermentation effluent) and the two dietary treatments ($P > 0.05$) when analysed, indicating that fibre-type behaviour produced similar results in both faeces and fermentation effluent within the model. Furthermore, nor treatment effect neither interaction was found for Po relative to gram of total P. Control gestation diet exhibited less organic P compared with sugar beet pulp-based diet in the faeces from gestating sows and fermentation effluent of the model, ($P > 0.05$). However, fermentation effluents fed with both dietary, standard gestation diet and sugar beet pulp-based treatment did not show any statistical difference ($P > 0.05$, 41.18 and 80.87 mg Po/g TP, respectively). Organic phosphorus to total P was decreased in the fermentation effluents when were fed with the two dietary treatments compared with sow manure from gestating sow fed both dietary treatments ($P < 0.05$). There was no interaction between both type of excreta, sow manure and fermentation effluents for Po:TP ratio ($P > 0.05$).

### 3.3.3 Concentration and forms of N in sow gestation faeces and fermentation effluent after 6 weeks in a pig gut model at six weeks of treatment

There were significant changes in the concentration of faecal ammonia observed in gestating sows that were fed the two dietary treatments (0.92 and 1.56 mg/g DM for the control diet and the SBP-based diet respectively). Although the sugar beet pulp-based diet resulted in a numerical reduction in ammonia concentration in fermentation effluent, the differences between dietary treatment were not statistically significant ($P > 0.05$). However, a significant interaction was observed when the two types of excreta were compared across treatments, with a greater reduction in ammonia observed in the fermentation effluent than in the faeces ($P < 0.05$; Table 3-5). Furthermore, the study revealed that both the control gestation diet and sugar beet pulp-based diet resulted in a significant increase in ammonia production in fermentation samples compared to gestating sow faecal samples ($P < 0.05$). Additionally, the sugar beet pulp-based diet showed a tendency towards higher faecal ammonia concentrations, leading to a significant difference between the faecal ammonia levels between faeces and fermented effluent ($P < 0.05$)

**Table 3-5 Concentrations of nitrogen forms and proportions of TIN and TON relative to one gram of total nitrogen in gestating sow faeces and fermentation effluent from an *in vitro* model of the pig gut at six weeks of treatments**

| | | Treatment | | | P value | | |
|---|---|---|---|---|---|---|---|
| | **Type (n)** | **Control** | **SBP** | **SEM[1]** | **Treatment** | **Type** | **Treatment*type** |
| Ammonia (mg/g DM) | S | 0.92 | 1.56 | 0.106 | NS | **<0.05** | <0.05 |
| | F | 3.35 | 2.023 | | | | |
| TKN (mg/g DM) | S | **16.09[a]** | **28.86[b]** | 0.928 | **<0.05** | **<0.05** | **<0.05** |
| | F | 11.08 | 13.57 | | | | |
| Nitrate (mg/g DM) | S | 0.089 | 0.119 | 0.01 | NS | **<0.05** | NS |
| | F | 0.192 | 0.187 | | | | |
| Nitrite (mg/g DM) | S | 0.051 | 0.071 | 0.006 | NS | <0.05 | NS |
| | F | 0.065 | 0.059 | | | | |
| Ammonia (mg/g TN) | S | **61.12[b]** | **55.15[a]** | 7.370 | <0.05 | <0.05 | <0.05 |
| | F | **297.30[b]** | **151.85[a]** | | | | |
| TIN (mg/g TN) | S | 70.27 | 61.87 | 8.339 | <0.05 | <0.05 | <0.05 |
| | F | **319.89[b]** | **167.91[a]** | | | | |
| TON (mg/g TN) | S | 929.73 | 938.13 | 8.339 | <0.05 | <0.05 | <0.05 |
| | F | **680.11[a]** | **832.09[b]** | | | | |

nS= 12, nF= 8, [1]SEM = standard error of the mean, S = sow faeces, F = fermentation effluent. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).

Meanwhile, a significant interaction ($P < 0.05$) between treatment and waste type was observed with relation to TKN. The findings revealed that the sugar beet pulp-based diet resulted in elevated total Kjeldahl nitrogen content in the faeces of sows compared to the fermentation effluent. However, the increased TKN content observed in the SBP-based diet did not correspond with a proportional rise in the TKN content of fermentation effluent in the *in vitro* model ($P < 0.05$; 16.09 and 28.86 mg/g DM *vs.* 11.08 and 13.57 mg/g DM) for sow faeces and

fermentation effluent, respectively. Although control gestation diet and sugar beet pulp -based diet tended to have lower faecal TKN in fermentation effluent, there was no significant difference between the control gestation diet and sugar beet pulp-based diet in fermentation effluents ($P > 0.05$).

The study revealed that the fermentation of the control gestation diet resulted in a significantly higher ammonia-to-total nitrogen ratio when compared to the sugar beet pulp-based diet in the pig gut model ($P < 0.05$). Furthermore, the ammonia-to-total nitrogen ratio was elevated in the fermentation effluents of both diets - the control gestation diet and the sugar beet pulp-based diet - compared to the faeces obtained from gestating sows consuming these diets ($P < 0.05$). Moreover, the fermentation of the sugar beet pulp-based diet had an appreciable effect on the ratio of ammonia to total nitrogen ($P < 0.05$). This effect was further compounded by the observation of an interaction between the two excreta samples ($P < 0.05$).

The diet based on sugar beet pulp led to a significant reduction ($P < 0.05$) in the ratio of total inorganic nitrogen to total nitrogen in the fermentation effluent of the pig model. An interaction was observed between treatment and excreta type in the samples where the sugar beet pulp-based diet reduced the TIN to each g of TN (319.89 vs. 167.91 mg/g TN) in the fermentation effluent compared with the gestating sow faeces (70.27 vs. 61.87 mg/g TN for control gestation diet and SBP-based diet respectively). The ratio of total organic nitrogen to total nitrogen was significantly higher ($P < 0.05$) in faeces samples from gestating sows for both dietary treatments compared to fermentation effluents (Table 3-5). subjected to the same diets. Additionally, the fermentation effluent associated with the sugar beet pulp-based diet had a notably higher TON:TN ratio compared to the standard gestation diet (680.11 vs. 832.09 mg/g TN for control gestation diet and SBP-based diet, respectively) while the faeces samples from gestating sows exhibit an equal proportion of TON:TN for both diets.

**Table 3-6 Concentrations of different forms of phosphorus in sow gestating faeces and fermentation effluent from an *in vitro* model of the pig gut at 6 weeks of treatment simulating**

| | | Treatment | | | P value | | |
|---|---|---|---|---|---|---|---|
| | **Type (n)** | **Control** | **SBP** | **SEM[1]** | **Treatment** | **Type** | **Treatment*type** |
| Total P (mg/ g DM) | S | 14.10 | 17.64 | 1.158 | NS | **<0.05** | NS |
| | F | 29.18 | 30.09 | | | | |
| Inorganic P (mg/g DM) | S | 7.91 | 14.51 | 0.654 | NS | **<0.05** | **<0.05** |
| | F | 16.04 | 12.04 | | | | |
| Organic P (mg/g DM) | S | 6.19 | 3.13 | 0.840 | NS | **<0.05** | **<0.05** |
| | F | 13.14 | 18.054 | | | | |
| Pi (mg/g P) | S | 570.03 | 824.04 | 30.031 | NS | **<0.05** | **<0.05** |
| | F | 558.23 | 406.57 | | | | |
| Po (mg/g P) | S | 429.97 | 175.95[a] | 30.031 | NS | **<0.05** | **<0.05** |
| | F | 441.77 | 593.43 | | | | |

nS= 12, nF= 8, [1]SEM = standard error of the mean, S = sow faeces, F = fermentation effluent. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).

### 3.3.4 Comparison of sow gestation faeces and *in vitro* fermentation effluents after 6 weeks of treatment for total P, inorganic P, and organic P

There was significantly more total phosphorus ($P < 0.05$) per gram of DM in the fermentation effluent than in the faecal samples, with values of 29.18 and 30.09 mg/g DM in the gestating control and SBP-based diets for fermentation effluent, versus 29.18 and 30.09 mg/g DM in the respective gestating sow faeces. However, statistical analysis did not reveal any significant differences in total phosphorus concentration, nor did it identify any notable interaction among the factors.

A significant interaction between the inorganic phosphorus content in sow gestating samples and fermentation effluents was observed across both type of samples ($P < 0.05$). Specifically, manure samples obtained from gestating sows fed the control gestation diet displayed lower concentrations of inorganic phosphorus, while fermentation effluents fed the same dietary treatment exhibited the highest concentrations of inorganic phosphorus. There was a significant increase in the concentration of inorganic phosphorus in fermentation effluents from the pig model fed a standard gestation diet compared to the corresponding manure samples from gestating sows after 6 weeks of treatment. The Pi concentrations were found to be 570.03 and 824.04 mg/g TP in the control gestation and SBP-based diets for gestating faeces, while in the fermentation effluent, they were 558.23 and 406.57 mg/g TP, for the control gestation and SBP-based diet, respectively ($P < 0.05$; Table 3-6).

Sows that were fed a diet rich in beet pulp during gestation showed a significant reduction in organic phosphorus concentration in their faeces, with 3.13 mg/g dry matter (DM) compared to the 6.19 mg/g DM observed in the control group ($P < 0.05$). However, statistical analysis did not reveal a significant difference between the two dietary regimens across excreta samples, including both manure and fermentation effluent derived from the *in vitro* model ($P > 0.05$). The study found that both dietary treatments, the standard gestation diet, and the sugar beet pulp-based diet, resulted in lower organic phosphorus concentrations in manure samples compared to the fermentation effluent associated with both treatments in the simulated pig gut model ($P < 0.05$).

## 3.4 Discussion

### 3.4.1 Comparison between the different nutrients present in faeces from gestating sows and fermentation effluents from an in vitro model of the pig model

The findings of a comprehensive laboratory experiment should closely match those obtained directly from animals to ensure consistency and reliability in both controlled and natural environments (Philippe et al., 2015). In this experiment, samples were collected four and six weeks following the start of treatment. Faecal samples collected at these time points were used to facilitate the adaptation of the microbiota of gestating sow to a fibrous diet, while also assessing the concentrations of N and P in the resulting faeces. These samples were taken from sows used in a separate experiment unrelated to this thesis. At four weeks of treatment, excreted ammonia was significantly elevated in gestating sow faeces and effluent from the gut model fed the experimental diet containing sugar beet pulp. The concentration of excreted ammonia was found to be higher in faeces from gestating sows fed a sugar beet pulp-based diet compared to those on a gestating standard diet. During the first four weeks of treatment, the presence of sugar beet pulp in the diet resulted in a 48 % increase in $NH_3$ levels in the manure and a 35 % increase in the fermentation effluents respectively. However, Lynch et al. (2008) and O'Shea et al. (2009) demonstrated that pigs offered diets containing sugar beet pulp exhibited a reduction in faecal ammonia emissions from 0 to 240 hours in comparison to pigs offered diets containing no sugar beet pulp. This contrasts with the findings presented here, where sugar beet pulp-based diets increased faecal ammonia compared to standard gestation diets in both sow manure and fermentation effluent from an *in vitro* model of the pig gut. The discrepancy in faecal ammonia levels observed in this experiment, compared to previous findings, could be attributed to differences in the type of fibre content present in the sugar beet pulp versus the control diet. As stated by Jha et al. (2011), pepsin and pancreatic enzymes are unable to digest

fibre. Consequently, the remaining undigested components of the feed after *in vivo* or *in vitro* digestion are high in fibre. This suggests that variations in type of dietary fibre between the two diets could have contributed to the observed differences in excreted ammonia levels in both models. In the pig gut model and in that from an animal, another factor to be considered is the *in vitro* dry matter digestibility (IVDMD). The *in vitro* dry matter digestibility (IVDMD) of a feed can significantly influence the subsequent fermentation process. This is because the amount of undigested material available for *in vitro* fermentation depends directly on the extent of prior *in vitro* digestion of the feed (Jha et al., 2011). Therefore, the increased faecal ammonia observed in pigs fed a diet consisting of sugar beet pulp may be due to the higher fibre content that remains undigested, thereby influencing the fermentation process. Increased fibre has also been shown to increase the recycling of urea-N back to the gut where it is converted back into ammonia Increased fibre has also been shown to increase the recycling of urea-N back to the gut where it is converted back into ammonia (Morgan and Whittemore, 1988; Canh et al., 1997; Zervas and Zijlstra, 2002). On the other hand, dietary fibre is widely recognised as a ´prebiotic´, providing the necessary energy for microbial fermentation, which facilitates the conversion of ammonia into less harmful N forms. As Gamage et al. (2018) reported, monogastric individuals experience rapid alterations in their gut microbiota within 24 to 48 hours of consuming dietary fibre. As with most prebiotics, dietary fibre promotes the growth of microbial population during hindgut fermentation, and this microbiota plays a crucial role in generating energy and producing key metabolic end products, such as VFA (Li et al., 2022). Moreover, TKN levels were increased at four and six weeks in the two types of effluents (sow faeces and *vs* fermentation effluent) when sugar beet pulp-based diet was present, indicating a re-utilisation of dietary AA present. In one work done by Zhao et al. (2020), the apparent ileal digestibility (AID) of the majority of AA was found to be higher in the other fibrous-based diets (maize bran and soyabean hulls) diets in comparison to the SBP diet. This difference may be attributed

to the high soluble dietary fibre (SDF) content of the SBP diet, particularly pectin, which has been shown to increase digesta viscosity and potentially hinder nutrient absorption. Furthermore, differences in IVDMD between the sugar beet pulp-based diets and the control diet may contribute to the observed variations in ammonia levels. A lower IVDMD would result in more undigested material for fermentation, potentially increasing ammonia production. By considering these factors, it becomes evident that both the type and digestibility of dietary fibre play crucial roles influencing faecal ammonia levels. An understanding of these mechanisms can inform the formulation of diets that minimise ammonia emissions, thereby enhancing the environmental sustainability of pig farming.

It is interesting to note that at four weeks of treatment, the dietary changes did not alter the concentration of other forms of inorganic nitrogen, such as nitrate ($NO_3^-$) and nitrite ($NO_2^-$), in both models ($P > 0.05$). In manure, ammonia nitrogen exists in an acid-base balance between ammonium ($NH_4^+$) and ammonia gas ($NH_3$). The process of nitrification transforms ammonia into nitrogen oxides, which are then converted into nitrites ($NO_2^-$) and nitrates ($NO_3^-$). These forms play a critical role in environmental issues such as eutrophication due to the nitrate leaching (Cappelaere et al., 2021). While the increased dietary fibre from sugar beet pulp raises faecal ammonia levels, it does not appear to significantly impact the concentrations of nitrate and nitrite. This suggests that dietary adjustments can target reductions in ammonia emissions specifically, without necessarily increasing the risk of nitrate leaching. Therefore, a comprehensive understanding of how dietary fibre influences the different forms of nitrogen can help in designing more effective and environmentally sustainable pig diets. By optimising the type and digestibility of dietary fibre, it is possible to mitigate ammonia emissions while maintaining stable levels of other nitrogen compounds, thus protecting both air and water quality.

After four weeks of treatment with a sugar beet pulp-based diet in gestating sows, this study found significant statistical differences in total, inorganic and organic phosphorus between the two treatments in both *in vivo* and *in vitro* models. The intake of dietary P was similar for both treatments in the sow faeces and in the fermentation effluent from models. The results of the current study clearly indicate that a different source of fibre in gestating diets, such as sugar beet pulp, increases total faecal P in both type of excreta: sow gestating faeces, and fermentation effluent from the model. A considerable amount of phosphate (Pi) is present either in its inorganic form or bound to organic compounds such as proteins, lipids, and DNA or RNA (Adedokun and Adeola, 2013). In the case of fermentation effluents from the pig model, concentrations of TP and Pi were higher for both treatments ($P < 0.05$), except for the organic P, which showed negative values.

# Chapter 4 Effect of dietary soluble and insoluble fibre, and addition of extra Ca, on the forms of excreted forms of phosphorus and nitrogen in an in vitro gut model of the pig gut

## 4.1 Introduction

In the literature review (Chapter 1.4.6), we have seen how the nitrogen and phosphorus are essential nutrients required by all living organisms and how dietary fibre may affect the different forms of those nutrients during hindgut fermentation. Chapter 1 reveals that there are ongoing efforts to gain a deeper understanding of the impact of various dietary fibre factors, including fermentation, bulking, binding, viscosity, gel formation, water holding capacity and solubility, on nutrient absorption (Adams et al., 2018).

Meanwhile, other research indicates that dietary fibre consumption enhances the retention and of N and P in pigs due to its prebiotic effects in the hindgut, thereby altering their released forms into the environment (Jarvis and Pain, 1997; Smith, 1998; Lindberg, 2014; S. Li et al., 2022). Fibre components are not completely hydrolysed because pigs lack the enzymes needed to break them down in the small intestine. As a result, these components enter the hindgut as substrates for microbial fermentation and influence the balance of the gut microbiota (Jha and Berrocoso, 2015). Bai et al. (2022) demonstrated that the rate of fermentation in the hindgut is significantly influenced by the source and solubility of dietary fibre (DF); both soluble and insoluble dietary fibres are degraded in the hindgut by fibrolytic bacteria. The process of fermentation produces VFAs, which have significant roles in regulating host metabolism, the immune system, and cell proliferation (Swanson et al., 2002; Koh et al., 2016; Cui et al., 2019). Soluble fibre tends to ferment at an accelerated rate compared to its insoluble counterpart (Noblet and Le Goff, 2001; Luo et al., 2017).

The breakdown of the soluble fraction is also expected to be relatively rapid. However, given that most ingredients contain a higher proportion of insoluble dietary fibre than soluble dietary fibre (Bindelle et al., 2008) it is speculated that the limited presence of soluble dietary fibre (SDF) may reduce its digestibility due to reduced contact with corresponding degradation enzymes (Pu et al., 2020). Typical sources of soluble fermentable fibre included in pig diets are industrial sources such as sugar beet pulp (SBP) or purified sources like inulin. SBP contains a substantial amount of soluble dietary fibre, including pectins, promoting a beneficial shift in the microbiota (Yan et al., 2017). Cellulose, in contrast, represents a purified alternative of fibre, exhibiting the properties of an insoluble fibre that experiences limited fermentation by the gut microbiota.

Beyond fibre composition, another crucial factor influencing digestion and microbial activity in monogastric diets is the balance of P and Ca. These two minerals play an essential role, not only in skeletal development, but also in shaping the microbial community and its fermentative activity (Metzler and Mosenthin, 2008). According to Ten Bruggencate (2004), keeping an optimal ratio of calcium phosphate (Ca:P) ratio within the range of 1:1 to 7:1 in the diet has been demonstrated to enhance intestinal barrier function and promote the proliferation of beneficial lactobacilli in the faeces of monogastric animals, while concomitantly reducing the population of faecal enterobacteria. Furthermore, it has been shown to potentiate the positive effect of inulin on lactobacilli growth. It has been demonstrated by research that extreme variations in the Ca:P ratio can trigger different physiological responses (Prasad et al., 2015). This highlights the need for an optimal balance for effective digestion and microbial function. Moreover, Chaplin et al. (2016) underline that dietary calcium supplementation in high-fibre diets for some animals strengthens the interaction between gut microbiota and host metabolism. This effect mimics a prebiotic action by fostering the growth of beneficial gut bacteria.

*In vivo* studies are challenging to conduct due to their cost and ethical considerations. The rate of fermentation of various substrates in humans and animals has been assessed using *in vitro* gut models employing faecal material as a source of microorganisms (Boisen and Fernández, 1997; Jonathan et al., 2012). In this experiments, two different types of dietary fibre were used: inulin and cellulose, alongside dry sugar beet pulp as a dietary fibre source. Inulin and cellulose represent purified products, with inulin being soluble fibre and cellulose being insoluble fibre. Sugar beet pulp, on the other hand, is an industrialised product, that contains both types of fibre (Bai et al., 2022). Therefore, in first experiment, three different experimental diets were prepared, each characterised by its soluble (DSF) and insoluble (DIF) fibre content. These diets were subsequently combined with a standard commercial grower feed (CO) to make the experimental diets. In the follow-up experiment (Experiment 2, detailed in this chapter), the diets that contained the same type of fibre were supplemented with two levels of additional calcium (0 and 2%). The additional calcium supplied to the pig experimental diets was formulated to provide 120% of the requirement for growing pigs without exceeding it excessively.

It is important to note that Experiment 2 only varied the calcium levels and did not introduce any other diet changes. However, research is scarce investigating the impact of supplemented calcium in high-fibre diets on hindgut fermentation in monogastric animals. Therefore, across this and subsequent experiments, the potential effects of supplemental calcium in fibrous diets were explored. The hypothesis was that the high calcium levels naturally present in fibre ingredients would facilitate the transport of this excess calcium to the hindgut. Consequently, increasing the supplementation of fibre with calcium in pig diets could potentially enhance the production of VFAs in the hindgut, while maintaining a neutral or slightly acidic pH – conditions conducive to the formation of calcium phosphate (Pi) through bacterial activity, such as that of Enterococcus. Therefore, the objective of this study was to investigate the impact of

supplemented pig growing diet with soluble and insoluble fibres derived from both natural sources (sugar beet pulp) and purified (inulin and cellulose) sources and supplemented with Ca to a simulated *in vitro* digestion and fermentation model of the pig gut using faecal samples as the inoculum, on the excreted forms of nitrogen and phosphorus.

## 4.2 Materials and methods

### 4.2.1 Experimental diets

In the first experiment of this chapter, four dietary treatments, including the control diet were used (see Table 4-1). During the second treatment was used a 4×2 factorial design, utilising the same diets used in the first experiments with or without the inclusion of additional calcium. Control diets (CO) was reduced to accommodate the addition of the different fibre-type ingredients (inulin, cellulose and sugar beet pulp). All diets were formulated to have adequate calcium and phosphorus. The additional calcium in the second experiment was used at 2% of the normal calcium content of the diets.

**Table 4-1 Ingredients and calculated nutritional composition of the experimental diets**

| Ingredient (g/kg) | CO | IN | CE | SBP |
|---|---|---|---|---|
| Barley | 400 | 380 | 380 | 360 |
| Wheat | 240 | 228 | 228 | 216 |
| Hi Pro Soya | 210 | 199.5 | 199.5 | 189 |
| Biscuit meal | 100 | 95 | 95 | 90 |
| Rapemeal | 20 | 19 | 19 | 18 |
| Soya oil | 10 | 9.5 | 9.5 | 9 |
| Minerals, amino acid & premix | 20 | 19 | 19 | 18 |
| DM | 902.66 | 898.40 | 900.69 | 906.45 |
| Crude Protein | 180 | 171 | 171 | 164.60 |
| TDF | 141 | 183.95 | 183.95 | 150.1 |
| Calcium (Total) | 7.20 | 6.84 | 6.84 | 6.89 |
| Phosphorus | 5.70 | 5.41 | 5.41 | 5.55 |

CO, control diet; IN, 95% control diet + 5% inulin; CE, 95% control diet + 5% cellulose; SBP 90% control diet + 10% sugar beet pulp.

Faecal samples were collected from animals that had been fed the control diet and were 10-12 weeks old. The animals were housed in mixed-sex groups and were subject to standard farm conditions, as described in General Methods (Table 2-1). No group medications were administered.

### 4.2.2 *In vitro* model of the pig gut (gastric and small intestine digestion, and hindgut fermentation)

The feed samples were subjected to a two-step enzymatic digestion to simulate gastric and small intestine digestion. The methodology used in this model was developed by Palowski et al (2021). A more detailed technical description of this model was described previously in

General Methods (2.6). In triplicate approximately 4 g of both the control and experimental diets were accurately weighed into 100 ml conical flasks. The dry matter content of each diet was determined before the assay (Section 2.3). The procedures for obtaining hydrolysed residues following enzymatic digestion are described in Section 2.6.1. In Experiment 1, the samples were freeze-dried for a period of 15 days. In subsequent experiments, the samples were dried in an oven at a temperature of 60°C for 48 hours. Oven drying was chosen over of freeze-drying to optimise the preparation time the fermentation stage, as both methods yielded the same measurement results.

Samples were subjected to a two-step enzymatic digestion, mimicking gastric and small intestine digestion according to the methodology developed by Palowski et al (2021), a more detail of this technique were described previously in General Methods (2.4). In triplicate approximately 4 g of both the control and experimental diets were accurately weighed into 100 ml conical flasks. The dry matter content of each diet was determined before the assay (Chapter 2.3). Following simulated enzymatic hydrolysis, the residues from each flask were collected by filtration using pre-weighed glass filtration crucibles (Porosity 2) as described in the General Method (2.4.1). The residues were then washed with acetone (1 x 20 ml), ethanol (1 x 20 ml), and deionized water (2 x 20 ml). In Experiment 1, the samples were freeze-dried for a period of 15 days. In subsequent experiments, the samples were dried in an oven at a temperature of 60°C for 48 hours. Oven drying was chosen over of freeze-drying to optimise the preparation time the fermentation stage, as both methods yielded the same measurement results.

In experiment 2, two mL of a calcium stock solution (20 g Ca/L) was added to the samples destined for calcium supplementation during the fermentation phase. To maintain consistent microbial concentrations, 2 mL of deionised water was added to the samples without calcium supplementation to compensate for the volume difference in the inoculum".

### 4.2.3 Analytical methods

Final products of fermentation were analysed for total Kjeldahl nitrogen $NH_3$ -N, nitrate and nitrite (Shand et al., 2008), and total and inorganic phosphorus (Johnson and Hill, 2011) with each serum bottles considered an experimental unit as was described in General Methods (2.5).

Subsequently, the total organic nitrogen (TON) content of each sample was determined by subtracting the ammonia nitrogen content from the total Kjeldahl nitrogen (AOAC, 2006). For the inorganic nitrogen content, the values of nitrate and nitrite, and ammonia in each sample were summed up. To determine the proportions of phosphorus in the final products, it was calculated the organic P in the samples by subtracting the inorganic P content of each sample from the total phosphorus content determined in those samples.

### 4.2.4 Statistical analysis

All *in vitro* fermentation data were expressed as means from triplicate serum bottle experiments. Statistical analyses were conducted using SPSS (Version 29). Differences in proportions among different parameters for the various fibre diets were assessed for significance using the GLM. In Experiment 2, the impact of the 'additional Ca' was analysed using the same approach.

## 4.3 Results

### 4.3.1 Experiment 1

In the first experiment, it was revealed significant differences between the fibre types and the CO diet regarding the TIN per gram of TN, exhibiting an average reduction of 50% ($P < 0.05$, Fig 4-1). This decrease can be attributed to a significant reduction ($P < 0.05$) in $NH_3$-N (Table

4-2) in the samples. Furthermore, there was a significant increase in TON (average 106%) per gram of TN in the samples ($P < 0.05$, Fig 4-2).

**Table 4-2 Nitrogen forms in fermentation effluent at 48 hours of fermentation.**

| | Treatment | | | | SEM | P value |
|---|---|---|---|---|---|---|
| | **CO** | **IN** | **CE** | **SBP** | | |
| **Ammonia (mg/g DM)** | 17.52[b] | 8.2[a] | 8.07[a] | 11.06[a] | 0.734 | <0.05 |
| **Nitrate (mg/g DM)** | 0.087[a] | 0.161[b] | 0.161[b] | 0.173[b] | .004 | <0.05 |
| **Nitrite (mg/g DM)** | 0.257[a] | 0.587[b] | 0.593[b] | 0.563[b] | 0.001 | <0.05 |
| **TKN (mg/g DM)** | 25.810[a] | 24.97[a] | 24.51[a] | 31.30[b] | 0.485 | <0.05 |
| **Total Nitrogen (mg/g DM)** | 25.92[a] | 25.19[a] | 24.73[a] | 31.53[b] | 0.486 | <0.05 |

Data are presented as means ± standard error of the mean (SEM), with **n = 4 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$). CO, control diet; IN, 95% control diet + 5% inulin; CE, 95% control diet + 5% cellulose; SBP 90% control diet + 10% sugar beet pulp.

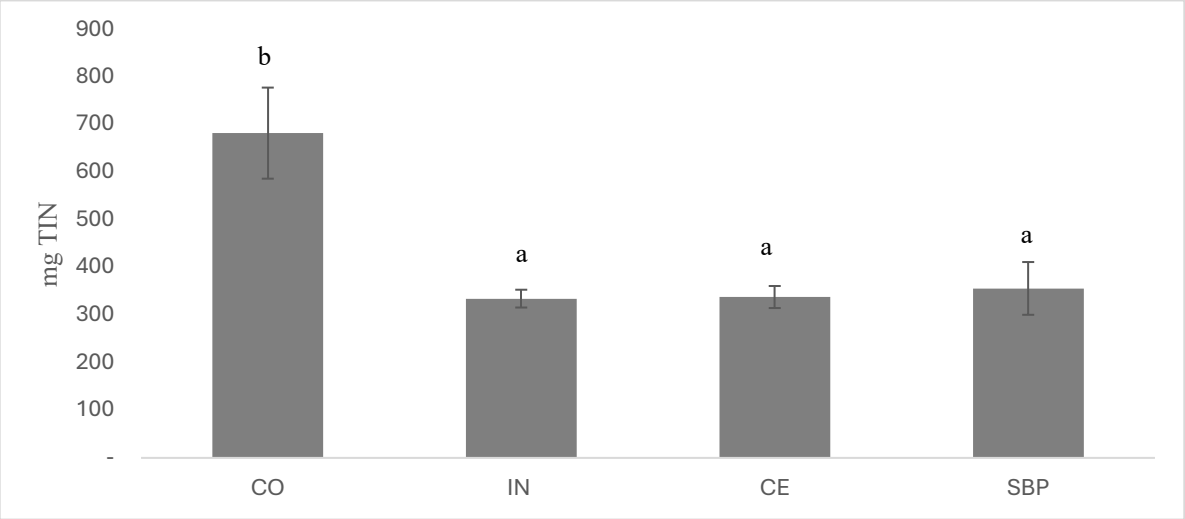


**Figure 4-1 Effects of dietary fibre on total inorganic nitrogen per gram of TN in fermentation effluent (*n* = 4 per treatment). Treatments labelled with different letters are significantly different (*P* < 0.05)**

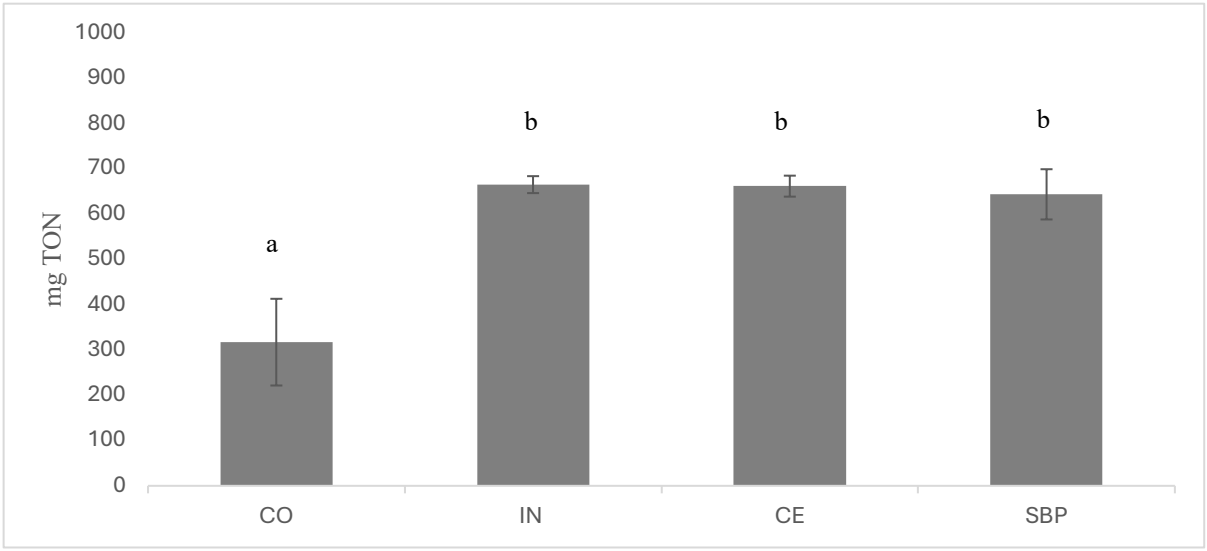


**Figure 4-2 Effects of dietary fibre on total organic nitrogen per gram of TN in fermentation effluent (*n* = 4 per treatment). Treatments labelled with different letters are significantly different (*P* < 0.05)**

The IN diet resulted in a statistically significant increase ($P < 0.05$, Fig 4-3) in Pi per gram of TP in the fermentation effluent, exceeding the CO diet by 142 % and the SBP diet by 43%. Despite the lack of statistically significant differences between the fibre types in relation to Po per gram of TP, all experimental diets showed a significant reduction in Po compared to the CO diet, with an average reduction of 62%, ($P < 0.05$, Fig 4-3).

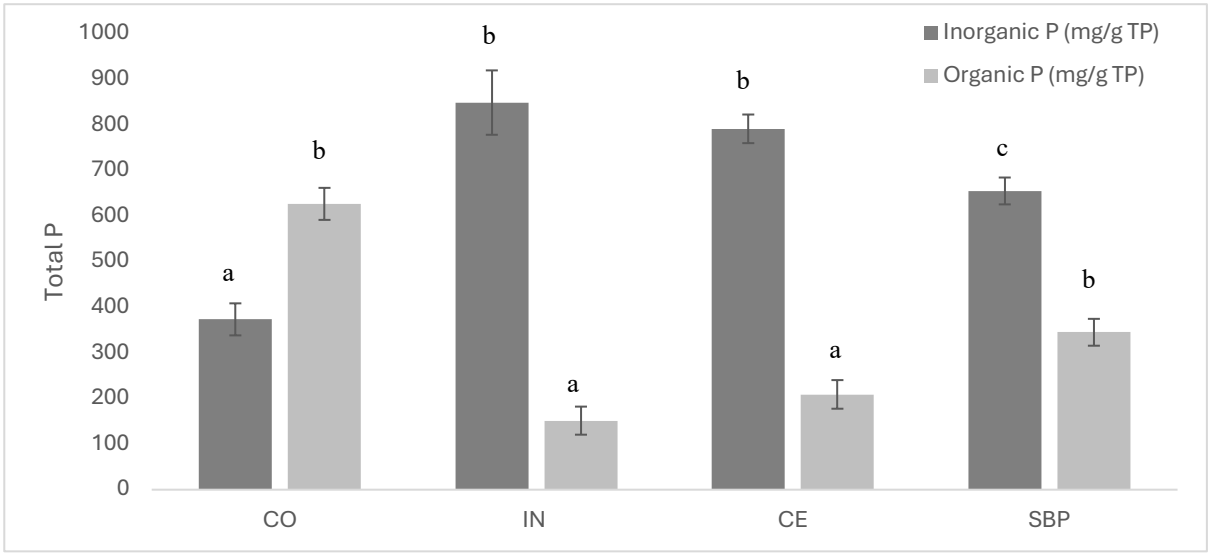


**Figure 4-3 Effect of dietary fibre on inorganic and organic phosphorus per gram of total Phosphorus (TP) (n = 4 per Treatment). The addition of dietary fibre significantly increased the amount of inorganic phosphorus (mg per g of TP) while decreasing organic phosphorus levels**

The different fibre-type diets produced similar amount of gas at 12 h ($P < 0.05$) when compared to each other. However, from 24 to 48 h, the fibre-type diets generated less gas per g DM entering fermentation. The control diet (CO) yielded more gas than the other experimental diets at the end of fermentation (318.83 mL/g DM *vs* 259.39 mL/g DM). Additionally, the SBP diet significantly lowered the pH of the fermentation effluent when compared to the other diets ($P < 0.05$)

**Table 4-1 Volumes of gas (mL/g DM entering fermentation) produced, including the pH of the final fermentation effluent, during the simulated hindgut fermentation of the *in vitro* pig gut model fed. Total Gas Production were calculated as mL/g[1]**

| | Treatments | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CO** | **IN** | **CE** | **SBP** | **SEM** | ***P* value** | |
| **Time** | | | | | | **Treatment** | **Time x treatment** |
| 12 h | 195.83$^{a}$ | 164.79$^{ab}$ | 147.81$^{b}$ | 159.14$^{b}$ | | | |
| 24 h | 273.49$^{c}$ | 223.35$^{d}$ | 206.09$^{d}$ | 225.18$^{d}$ | | | |
| 36 h | 303.25$^{e}$ | 247.28$^{f}$ | 229.83$^{f}$ | 246.31$^{f}$ | 4.154 | **<0.05** | **<0.05** |
| 48 h | 318.83$^{g}$ | 259.39$^{h}$ | 242.26$^{h}$ | 256.32$^{h}$ | | | |
| | | | | | | ***P* value** | |
| **pH** | 6.4$^{i}$ | 6.1$^{ij}$ | 6.1$^{ij}$ | 5.8$^{j}$ | 0.053 | **<0.05** | |

Data are presented as means ± standard error of the mean (SEM), with **n = 4 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$). CO, control diet; IN, 95% control diet + 5% inulin; CE, 95% control diet + 5% cellulose; SBP 90% control diet + 10% sugar beet pulp.

### 4.3.2 Experiment 2

In contrast to Experiment 1, a treatment effect was observed when comparing the fibre-based diets with the control diet in terms of TIN as a proportion of TN, resulting in a significant increase (average 50%, $P < 0.05$) compared with the other diets. There was also an interaction between fibre type and calcium supplementation, with a significant increase in TON per gram

of TN in the cellulose diet without calcium supplementation (average 10%, $P < 0.05$, Fig 4-4) compared with the other fibre types, IN and SBP. For phosphorus, in experiment 2, the addition of supplemental calcium reduced the ratio of Pi to TP in all experimental diets, including the control, by 83% ($P < 0.05$). Conversely, calcium supplementation increased the ratio of Po to TP by 38.25% ($P < 0.05$) in all diets, including the control.

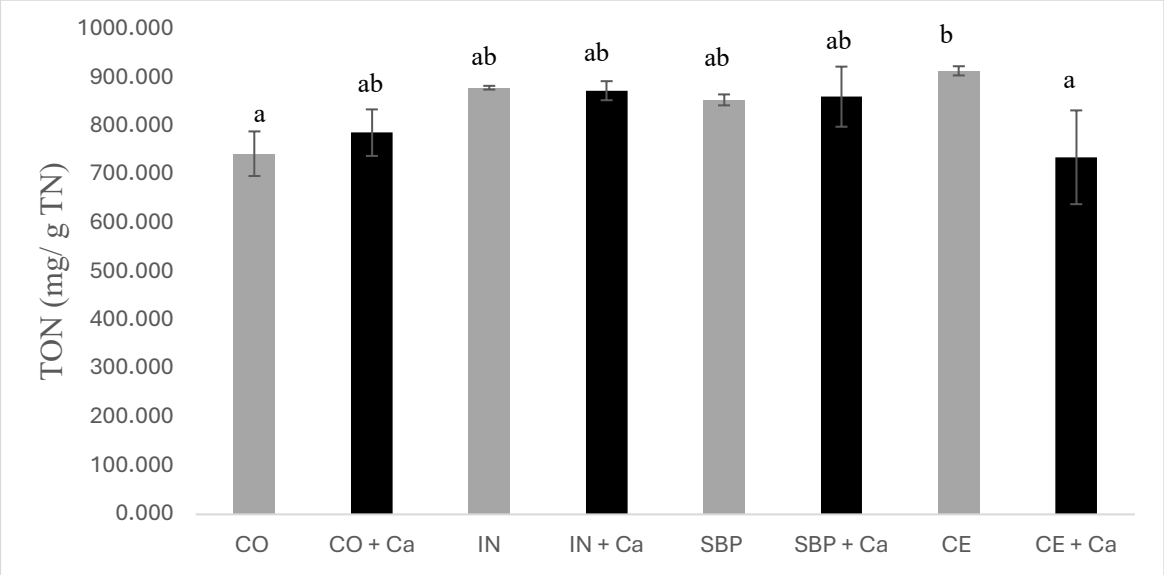


**Figure 4-4 Effect of dietary fibre on organic nitrogen per gram of total nitrogen (TN) across different treatments with and without calcium addition (n = 4 per treatment). Different letters indicate significant differences between treatments (p < 0.05)**

Opposite to Experiment 1, a treatment effect emerged when comparing the fibre-type diets to the control diet with regards to TIN:TN ratio, leading to a significant increase (average 50%, $P < 0.05$) in this parameter for the fibre diets. However, there was no significant effect of Ca on TIN:TN among the diets.

An interaction between fibre type and Ca was observed, with a significant increase in TON:TN in the cellulose diet 'No extra' Ca (average 10%, $P < 0.05$) compared to the other fibre types, IN and SBP.

As in Experiment 1, Experiment 2 monitored cumulative gas production at four time points, including the start and end of fermentation. At 12 h, the cumulative gas production for the fibre diets of SBP and CE was found to be significantly lower than that of CO and IN ($P < 0.05$, see Table 4-2). No effect of calcium (Ca) effect was observed between the different treatments at 12 h ($P > 0.05$). However, from 12 to 48 h, significant ($P < 0.05$) differences in gas production were observed between the different fibre types. At 48 h, the gas production per g of DM for SBP was greater than that for IN and CE (313.2 vs. 248.98 and 265.31 mL/g DM, respectively), irrespective of the presence or absence of additional calcium (Ca). Gas production increased significantly in diets with extra calcium (average 12%, $P < 0.05$).

**Table 4-2 Total Gas Production in Experiment 2 from the different fibre-type treatments of the *in vitro* model at four time point. Total Gas Production were calculated as mL/g[a]**

| | Time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **12 h** | **24 h** | **36 h** | **48 h** | **SEM** | **P value** | | | | | | |
| **Treatment** | | | | | | **Treatment** | **Time** | **Ca** | **Tr*Time** | **Tr*Ca** | **Ca*Time** | **Tr*Time*Ca** |
| CO | 145.00$^{b}$ | 253.08$^{de}$ | 294.43$^{ef}$ | 317.86$^{ef}$ | | | | | | | | |
| CO + Ca | 145.55$^{b}$ | 274.63$^{e}$ | 324.91$^{f}$ | 360.10$^{f}$ | | | | | | | | |
| IN | 137.94$^{ab}$ | 206.28$^{cd}$ | 232.10$^{e}$ | 248.98$^{de}$ | | | | | | | | |
| IN + Ca | 147.00$^{b}$ | 246.50$^{de}$ | 288.71$^{ef}$ | 316.15$^{ef}$ | | | | | | | | |
| CE | 117.38$^{a}$ | 196.18$^{c}$ | 234.79$^{e}$ | 265.31$^{de}$ | 3.618 | **<0.05** | **<0.05** | **<0.05** | **<0.05** | NS | **<0.05** | NS |
| CE + Ca | 127.38$^{ab}$ | 218.85$^{cd}$ | 255.25$^{de}$ | 284.63$^{e}$ | | | | | | | | |
| SBP | 141.32$^{b}$ | 229.48$^{cd}$ | 278.93$^{de}$ | 313.20$^{ef}$ | | | | | | | | |
| SBP + Ca | 129.18$^{b}$ | 242.85$^{cde}$ | 291.79$^{ef}$ | 322.636$^{ef}$ | | | | | | | | |

[a] Data are presented as mean (n=4) ± SEM. Values in the same row with different superscript letters (a-k) indicate a significant difference ($P < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test. CO represents the control diet; IN corresponds to 95% control diet + 5% inulin; CE denotes 95% control diet + 5% cellulose; and SBP refers to 90% control diet + 10% sugar beet pulp.

The SBP and CE treatments, with or without additional calcium addition, showed a significantly ($P < 0.05$) higher acetate to total volatile fatty acid ratio compared to those from the CO and IN groups, with or without additional calcium addition (Figure 4-4).

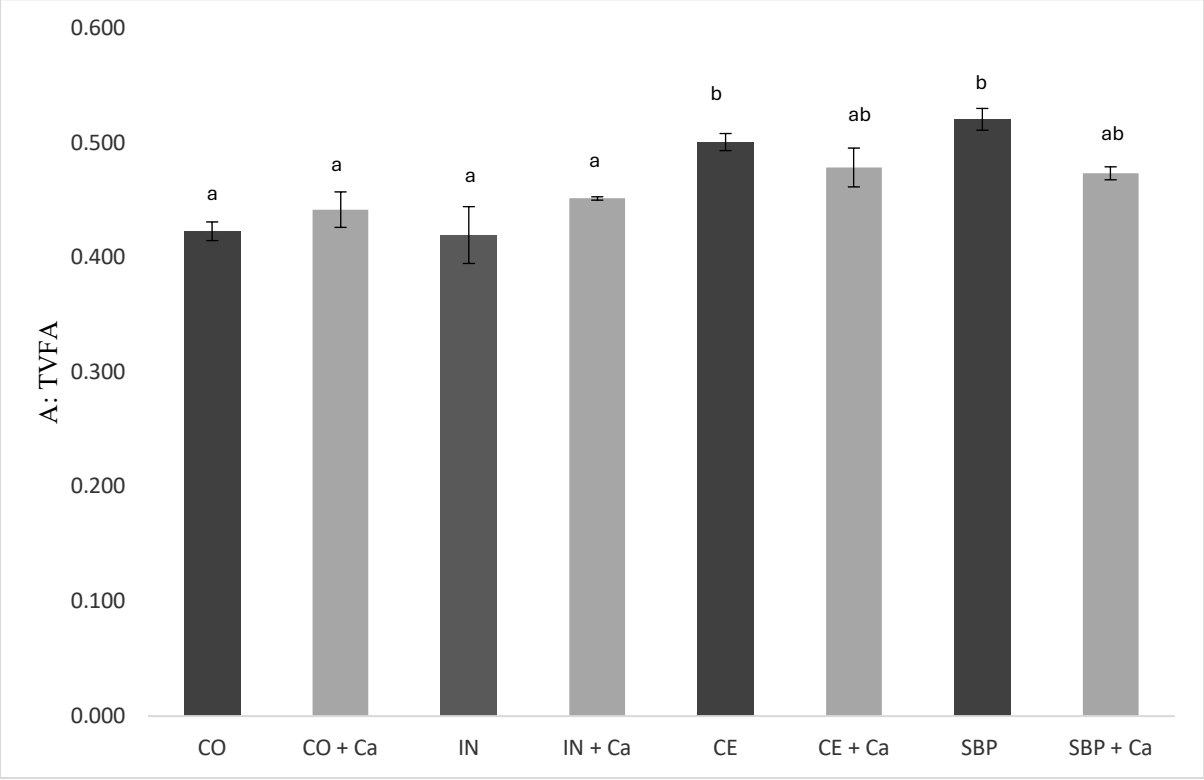


**Figure 4-5 Acetate to total volatile fatty acid ratio observed in fermentation effluent with different fibre diets after 48 hours of fermentation (n=4 per treatment).**

In Experiment 2, similar to the findings with acetate, the IN diet, with and without additional Ca, resulted in a significantly higher propionate production compared to the CO diet, ($P < 0.05$, Fig 4-5). Propionate production was lower ($P < 0.05$) on the CO without additional Ca ($P < 0.05$), but when Ca was added, propionate production on the CO diet became comparable to that on the other fibre diet.

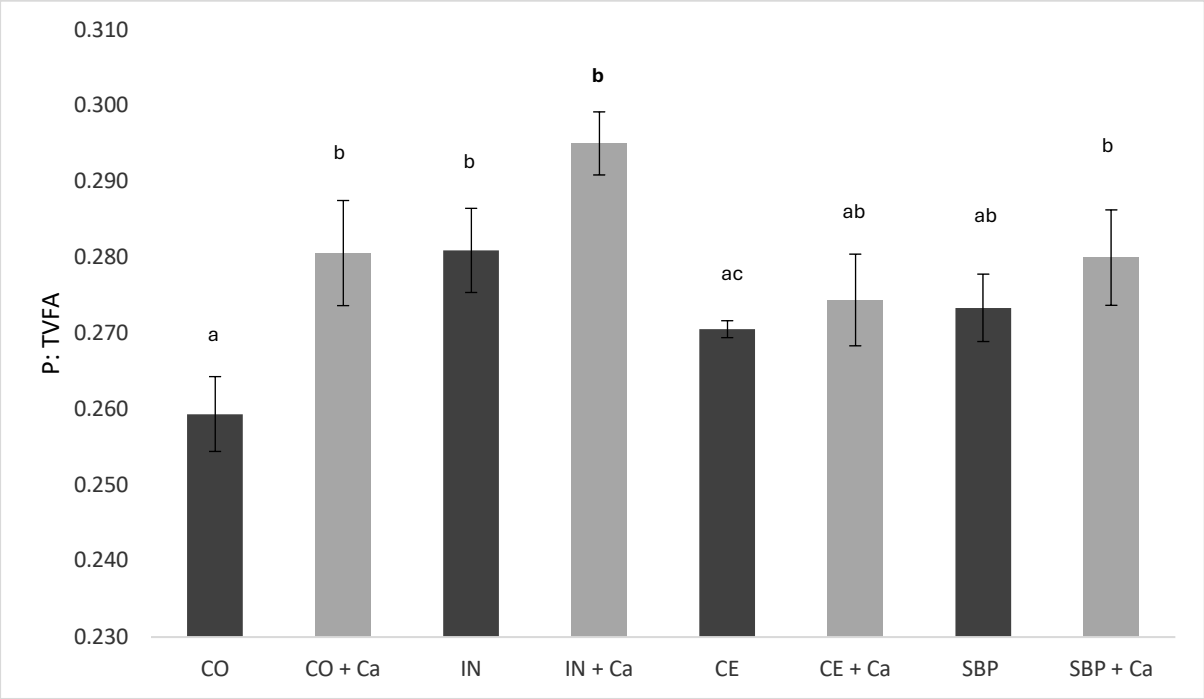


**Figure 4-6 Propionate to total volatile fatty acid ratio observed in fermentation effluent with different fibre diets after 48 hours of fermentation (n=4 per group)**

Regarding Phosphorus, in Experiment 2, the addition of 'supplemented Ca' decreased inorganic P relative to TP in all experimental diets, including CO, by 83% ($P < 0.05$); conversely, 'Extra Ca' increased PO:TP in all experimental diets, including CO, by 38.25% ($P < 0.05$),

## 4.4 Discussion

This study aimed to elucidate the impact of both soluble and insoluble dietary fibre on the faecal behaviour of N and P using an *in vitro* model simulating the pig gut, as well as the influence of calcium within high-fibre diets. The results of these experiments demonstrate that incorporating purified dietary fibre sources into the diet, depending on their respective fibre types, significantly increases total organic nitrogen levels while influencing total inorganic phosphorus to some extent compared to the control group. According to Tian et al. (2020), DF functions as a carbon source, promoting microbial growth. This, in turn, facilitates the gut microbiota utilisation of dietary protein or endogenous nitrogen. Interestingly, gas production was higher in the CO diet compared to the fibre diets in both experiments. The interaction between gas produced per gram of fermented dry matter and propionate to total volatile fatty acid in Experiment 2 appears to be complex, possibly influenced by gases released during diet fermentations and fibre type used in the experiments. Meanwhile, the acetate to total volatile fatty acids ratio during in vitro fermentation was positively correlated with the total gas production, in the fibre diets. This may be partially explained due to the fact the main products during microbial fermentation of pectin are acetic acid (Thakur et al., 1997). It has previously been observed that the ingestion of soluble fibre, such as pectin, was found to be associated with an increased proportion of microbiota from the phylum Bacteroidetes, which are known to ferment starch and fibre (Thakur et al., 1997). Meanwhile, Whisner et al. (2014) have done the first study to demonstrate a diet-induced change in the gut microbiota associated with a physiological benefit of increased calcium uptake in healthy individuals. Returning to the second experiment, adding calcium to high-fibre diets has been shown to enhance VFA production, mainly acetate, as was previously observed in experimental findings (Zhao et al., 2025). It was hypothesised that the high gas volume observed in the control group resulted from the rapid starch fermentation in the serum bottles (Table 4-3). However, it is important to

consider the finding of Knudsen et al. (1993) who reported high starch digestibility in the small intestine of pigs. These findings are consistent with the model used in these experiments, which contains pancreatin, an enzyme mixture of amylase, lipase, and protease. It can therefore be assumed that the largest part of the starch present in the diets has first been digested before entering the fermentation stage (Data not shown).

In an *in vivo* study conducted by Bindelle et al. (2009), the inclusion of different levels of sugar beet pulp (0, 10, 20, and 30) in pig diets was found to be positively correlated with enhanced bacterial N uptake in the hindgut, as measured through an *in vitro* fermentation technique. In our two related experiments, differences in the total inorganic N per gram of total nitrogen were observed across the experimental diets. However, these differences were less pronounced in the second experiment. In the first experiment, diets comprising a high proportion of dietary fibre resulted in an increase in faecal TON. In contrast, in the second experiment, this increase was observed in the fibre-rich diets that did not receive additional calcium. Although, these findings suggest that altering the fibre-to-protein ratio the pig diets influence the forms of faecal nitrogen. This finding may be attributable to the inclusion of additional Ca, in the second experiment, while samples without additional Ca were treated with a placebo contains the same solution that the solution, which the additional Ca was prepared, and this solution may contain a non-protein source. One possible explanation is that the microbiota facilitated the conversion of non-protein nitrogen into amino acids and peptides (Bikker et al., 2017), contributing to overall metabolic processes. In other studies, several microbial species, including *Bacteroides*, *unidentified Ruminococcaceae*, *unidentified Christensenellaceae*, *Faecalibacterium*, *Phascolarctobacterium*, *Oscillibacter*, and *Alloprevotella*, exhibited changes in response to dietary fibre treatments during gestation (Yang et al., 2021) Therefore, further investigation is needed to determine which specific species played a role in these nitrogen utilisation effects.

In Experiment 1, a significant decrease in ammonia levels was observed in the experimental fibre diet groups, likely due to the modification of fibre type in these diets. While extensive research has examined the effects of dietary fibre on gut microbiota, comparatively less attention has been paid to the role of calcium. Furthermore, the combined effects of fibre and calcium remain largely unknown. This implies that fermentable fibre and calcium may interact to influence gut microbial composition in ways that may contribute to improved fermentation characteristics or effluent properties and represent a relatively understudied area of microbiota research.

In Experiment 2, the cellulose diet (CE), without the addition of 'extra Ca', exhibited 10% more organic N, and thus 10% less inorganic N compared to the other diets used in this experiment. *In vivo* studies conducted by Kreuzer et al., (1998) demonstrated an increase in faecal nitrogen, primarily in the form of organic N, in their experimental diet, which consisted mainly of soluble and insoluble fibre. According to the same authors, this corresponding reduction in urinary N led to a decrease in $NH_3$-N, which are less prone to volatilise.

In pigs, dietary fibre is the primary nutrient source for the gut microbiota. Its inclusion in the diet has been shown to promote bacterial proliferation, resulting in increased excretion of amino acids, lipids and minerals, such as phosphorus, of bacterial origin in the faeces (Bikker et al., 2017). In the experimental diets of experiment 1 and within the 'no additional Ca' group of experiment 2, soluble fibre (inulin) tended to increase the proportion of inorganic phosphorus (PI:TP, Figure 4-3). These results suggest that certain bacterial genera, such as lactic acid bacteria, may produce phytase enzymes capable of degrading specific forms of phosphorus (IP6) present in fibre-rich ingredients. This observation was confirmed by Park and Kim, (2025), which indicates that phytates reaching the hindgut undergo near-complete hydrolysis by microbial enzymes. This process enables efficient phosphorus utilisation and transformation in the cecum and colon.

The addition of ‘additional Ca’ in experiment 2 increased the total gas volume in the fibre diets. This increase may indicate the promotion of intestinal lactobacilli bacteria, known for their role in releasing phytase in the hindgut. In monogastric animals, the mechanism by which Ca interacts with a high fibre diet in the hindgut is still unclear. However, some hypotheses regarding these mechanisms and their interactions with the gut microbiota have been proposed by Gomes et al. (2015). For example, calcium may stimulate the production of gastric secretions, leading to increased gastric acidification and modulation of the intestinal bacterial population in monogastric animals. In addition, calcium may promote the precipitation of bile acids and fatty acids, thereby raising the pH of the colon and reducing harmful compounds, particularly non-esterified fatty acids (NEFA) and ionised secondary bile acids. On the other hand, calcium supplementation in the diets increased the proportion of Po in all experimental diets during the second experiment. This effect may be due to calcium reducing the efficiency of microbial phytase in the diets by forming organic complexes with IP6 during hindgut fermentation (Hu et al., 2022). According to James et al. (1978), some constituent of fibre has been observed to bind with calcium, thereby reducing its bioavailability for absorption in the small intestine. The process of fibre breakdown in the colon by microbes has been shown to release that non absorbable calcium, thus restoring its availability for utilisation by the body.

The supplementation of a pig commercial grower feed with insoluble and soluble fibres cellulose and inulin, respectively, resulted in an increase in the ratio of organic faecal nitrogen excretion and a decreased in inorganic nitrogen excretion This enhanced the forms of nitrogen present in the excreta. The inorganic to total phosphorus ratio increased when inulin was included in the diet. In the second experiment, the addition of Ca to the fibrous diet resulted in a greater ratio of organic nitrogen ratio in all diets including the control diet. Based on these findings, it can be concluded that a high dietary fibre diet can be utilised in growing diets using

an *in vitro* model to customise nitrogen and phosphorus forms, even with the inclusion of additional calcium.

# Chapter 5 Utilisation of milling and oil industry by-products to increase *in vitro* insoluble and soluble fibre to alter excreted nitrogen (N) and phosphorus (P) forms in pig feed

## 5.1 Introduction

In Chapter four, two structurally distinct polysaccharides, inulin and cellulose, were subjected to gastric and small intestine digestion, followed by fermentation, using an *in vitro* pig gut model. Following a 48-hour fermentation period, significant alterations in nitrogen and phosphorus forms were observed. Notably, the inclusion of inulin led to a statistically significant decrease in the ratio of organic to total phosphorus ($P < 0.05$) compared to other fibre sources. Furthermore, addition of fibre, irrespective of type, resulted in a significant ($P < 0.05$) decrease in the proportion of inorganic to total nitrogen following 48 hours fermentation.

Inulin, a water-soluble fibre (SF) categorised under the fructans group of non-digestible carbohydrates, undergoes rapid fermentation by the pig gut microbiota. In contrast, cellulose, a water-insoluble fibre (ISF), undergoes minimal fermentation by the same microbiota in the pig hindgut (Yan et al., 2017). The identifiable structural traits and molecular weight of these type of fibres are becoming increasingly acknowledged as fundamental factors determining their fermentability by the microbiota (Holmes et al., 2017). Therefore, the influence of dietary fibre solubility on the excreted N and P forms was examined using an *in vitro* pig gut model. The main objective of the experiments presented in Chapter 4 was to analyse shifts in the excreted nitrogen (N) and phosphorus (P) forms throughout the fermentation process as previously mentioned. However, it is essential to determine whether these changes are attributable to the intrinsic characteristics of the fibre types used or to the experimental model utilised in the experiments. Furthermore, as demonstrated in the second experiment detailed in

the preceding chapter, the addition of supplemental calcium (up to a 2% increase in experimental diets), which also was used in this experiment, led to an increase in the proportion of organic nitrogen and organic phosphorus. This may suggest a positive correlation between microbiota, calcium, and other minerals, as evidenced by fermentation characteristics observed in the preceding experiments.

In light of these findings, it is imperative to investigate further natural sources of both insoluble and soluble fibre, such as cereal brans (including wheat and rice bran) and by-products of the oil industry (such as soybean hulls). The inclusion of such fibres in feed formulations not only provides nutritional benefits but also plays a pivotal role in enhancing livestock health and digestive function by promoting the growth of beneficial microorganisms, such as bifidobacteria. These microbes assist in reducing gut pH, creating an environment that inhibits the overgrowth of pathogenic bacteria (Adams et al., 2018)

Wheat bran, a primary by-product of flour milling, is composed mainly of the epidermis, peel, nucellus layer and aleurone layer (Brouns et al., 2012). Wheat bran is commonly used in humans' cereal-based and bakery products diets (European Flour Millers, 2016) and as growth support for ruminants (Dhakad et al., 2002). However, the high content of cellulose in wheat bran presents a challenge to the fermentability of the microbiota in the gut of most mammals (Deroover et al., 2020). Despite these fermentation characteristics due to its high insoluble fibre content, this cereal bran is resistant to its degradation in growing pig gut. Although, wheat bran is currently employed in the feeding of fattening pigs, further research is required to elucidate its potential for broader use in pig diets.

An alternative option is rice bran, which is an excellent source of nutrients. As stated by Abdul-Hamid and Luan (2000), rice bran is composed of 14.6% protein, 7% minerals, 17% predominantly unsaturated fat, and 27% dietary fibre. The chemical composition of dietary

fibre prepared from defatted rice bran includes 65% total dietary fibre (with 9% being soluble dietary fibre), 17% protein, and 18% ash. It has been demonstrated that the inclusion of cereal brans, along with soybean hulls, a by-product of the oil industry, in pig diets promotes fermentation in the gut, thereby enhancing nutrient utilisation by the host. Furthermore, these by-products have the potential to enhance the properties of manure for plant growth by altering the forms of N and P to optimise nutrient uptake by plants. This optimisation can also facilitate other applications, such as anaerobic fermentation for biogas production, thereby reducing nutrient loss to the environment via leaching and runoff, which can otherwise have detrimental effects. Through fermentation of the fibre present in these by-products, the hindgut microbiota is capable of transforming N and phosphorus P forms, which is a crucial process for improving the quality of pig manure. The aim of the experiment described in this chapter was to determine the impact of a pig *in vitro* digestion and fermentation model using these by-products of milling and oil industry, on the excreted forms of nitrogen and phosphorus. It was hypothesised that the insoluble and soluble fraction of the wheat bran, soyabean hulls, and rice bran would result in an increase in the ratio of organic nitrogen and a decline in the proportion of organic phosphorus. Additionally, it was hypothesised that these dietary sources would significantly influence fermentation characteristics

## 5.2 Methods

### 5.2.1 Design of the experiment and treatments

The experiment included four dietary treatments, one of which was a control diet (CO) that met the nutritional requirements for growing pigs. Three different feedstuffs with varying levels of dietary fibre were used to prepare the other treatments: wheat bran (WB) and rice bran (RB), both of which were sourced from a local provider, and soyabean hulls (SH) obtained from an online store. All these ingredients were high in insoluble fibre and contain different amounts

of soluble dietary fibre. Prior to the preparation of the final diets for each treatment, the fibre content of each feedstuff was determined, thereby enabling the accurate calculation of the dietary fibre content in each experimental diet (Table 5-1)

**Table 5-1 Ingredients and calculated nutrient composition of experimental diets**

| **Ingredient (g kg$^{-1}$)** | **CO** | **WB** | **SH** | **RB** |
|---|---|---|---|---|
| Wheat | 240 | 118.24 | 192.46 | 73.58 |
| Barley | 400 | 198.04 | 320.76 | 122.64 |
| Biscuit meal | 100 | 49.51 | 80.19 | 30.66 |
| Wheat bran | | 504.90 | | |
| Soya hulls | | | 198.10 | |
| Rice bran | | | | 693.40 |
| **Composition (g kg$^{-1}$)** | | | | |
| DM | 921.70 | 905.78 | 916.11 | 954.06 |
| TDF | 141.84 | 263.86 | 267.47 | 249.91 |
| Insoluble F | 135.48 | 142.21 | 137.22 | 187.23 |
| Soluble F | | 121.80 | 130.25 | 62.68 |
| Crude Protein | 180 | 176.48 | 170.07 | 154.11 |
| Phosphorus | 5.70 | 7.63 | 4.47 | 2.72 |

Faecal samples were collected from five growing pigs at the National Pig Centre (NPC) for the purpose of preparing the inoculum for the hindgut fermentation phase of the *in vitro* pig gut model. The pigs were selected from the same batch, and thus were of similar age (approximately 42 days old) and subjected to similar environmental factors, as described in General Methods (Table 2-1). No group medications were administered.

The complete *in vitro* pig gut model, consisting of three distinct phases - gastric digestion, small intestinal digestion and hindgut fermentation - was run for a total of 54 hours, with six bottles allocated to each treatment. After 24 hour, three bottles were removed, while the remaining three bottles continued the fermentation for an additional 24 hours. To guarantee homogeneity when mixing samples with enzymes, the preliminary steps of the model (see Chapter 2, Section 2.4.1), were carried using an orbital shaker incubator due to the size of the sample set (24 bottles). The bottles were positioned at random within the shaker. Gas pressure was measured at regular intervals (2, 4, 8, 12, 24, 36, and 48 hours), following removing the bottle at 24 hours fermentation, which were processes as those taken through to the end of fermentation. Then, samples pH was recorded immediately upon uncapping each bottle. Finally, samples were collected for analysis of TKN, ammonia nitrogen ($NH_3$-N), nitrate ($NO_3^-$), nitrite ($NO_2^-$), total phosphorus (TP), inorganic P (Pi), VFA, and microbial crude protein (MCP). As in the previous experiment, supplemented Ca (2%) was added to each flask, to assess the effect of Ca in high fibrous diets.

### 5.2.2 Statistical Analysis

The data were subjected to statistical analysis as outlined in the General Methods (Chapter 2). As in the previous experiments, each fermentation bottle was considered an experimental unit. Differences in gas production, pH, VFA and MCP were analysed using repeated measures (General Lineal Model Repeated Measures). The general linear model was also applied to data regarding N and P forms concentration.

## 5.3 Results

### 5.3.1 Concentration of the different nitrogen forms

After 48 h fermentation there were no statistically significant reduction in $NH_3$-N among the different treatments, but a numerical decrease was noted in the diets supplemented with WB and RB without additional calcium ($P > 0.05$; 11.84 and 11.66 mg $NH_3$-N/g DM; Table 5-2). Similarly, no statistically significant difference was observed in ammonia concentration between the fibrous diets and the control diet ($P > 0.05$). However, the supplementation of calcium in the experimental diets, including the control, resulted in a statistically significant reduction in ammonia concentration across all diets ($P < 0.05$). Furthermore, the reduction in $NH_3$-N caused by Ca in the RB diet was less pronounced in comparison to the WB and SH diets, with concentrations of 10.70 mg $NH_3$-N/g DM, 8.55 mg $NH_3$-N/g DM, and 7.23 mg $NH_3$-N/g DM, respectively. If we now turn to total Kjeldahl nitrogen, a significant increase in TKN levels in excreta was observed in the SH treatment without calcium supplementation compared to the CO and WB diets ($P < 0.05$). The observed TKN levels were 47.29, 31.44, and 40.81 mg TKN/ g DM for the SH, CO, and WB diets, respectively. While the RB diet evidenced the lowest TKN concentration ($P > 0.05$), there were no significant differences in TKN concentration between the CO, WB, and SH fibrous diets ($P > 0.05$).

As illustrated in Table 5-2, the concentration of nitrate and nitrite increased at the end of fermentation (48 h). There were no significant differences in nitrate ($P > 0.05$). At 48 h among the experimental diets with the control diet. However, the fibrous Ca-unsuplemmented diets exhibited the lowest nitrite concentration in comparison with to the CO diet. The addition of supplementary Ca to the experimental diets resulted in the highest $NO_3$-N concentration in the WB diet compared with the other diets ($P < 0.05$). However, no significant differences in nitrite

**Table 5-2 The concentrations of the nitrogen forms (including total inorganic N and total organic N) in final fermentation excreta[1].**

| | Treatments | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CO** | **CO + Ca** | **WB** | **WB + Ca** | **SH** | **SH + Ca** | **RB** | **RB + Ca** | | ***P* value** | | | | | |
| $NH_3$ (mg/g DM) | | | | | | | | | **SEM** | **Tr** | **Ca** | **time** | **Tx*Ca.** | **Tx*time** | **Tx*Ca** | **Tx*Ca*time** |
| 0 h | 4.46$^{a}$ | | 4.62$^{a}$ | | 5.87$^{ab}$ | | 7.65$^{b}$ | | | | | | | | | |
| 48 h | 12.95$^{d}$ | 6.17$^{b}$ | 11.84$^{d}$ | 8.55$^{bc}$ | 12.34$^{d}$ | 7.23$^{b}$ | 11.66$^{d}$ | 10.70$^{c}$ | 0.26 | NS | <0.05 | <0.05 | <0.05 | NS | - | - |
| TKN (mg/g DM) | | | | | | | | | | | | | | | | |
| 0 h | 58.18$^{bc}$ | | 45.15$^{ab}$ | | 69.25$^{c}$ | | 56.61$^{b}$ | | | | | | | | | |
| 48 h | 31.44$^{a}$ | 50.19$^{b}$ | 40.81$^{ab}$ | 45.48$^{b}$ | 47.29$^{ab}$ | 36.91$^{ab}$ | 36.57$^{a}$ | 27.59$^{a}$ | 1.13 | <0.05 | NS | <0.05 | <0.05 | <0.05 | - | - |
| Nitrate (mg/g DM) | | | | | | | | | | | | | | | | |
| 0 h | 0.02$^{a}$ | | 0.02$^{a}$ | | 0.01$^{a}$ | | 0.008$^{s}$ | | | | | | | | | |
| 48 h | 0.07$^{b}$ | 0.03$^{b}$ | 0.07$^{b}$ | 0.08$^{c}$ | 0.05$^{b}$ | 0.02$^{b}$ | 0.03$^{b}$ | 0.02$^{b}$ | 0.004 | <0.05 | NS | <0.05 | NS | NS | - | - |

[1]Data are presented as means ± standard error of the mean (SEM), with **n = 3 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).CO, control diet; WB, CO supplemented with wheat bran, SH, CO supplemented with soyabean hulls, RB, CO supplemented with rice bran. The diets containing additional Ca (2%) are indicated as 'Tr + Ca′

Table continued…

| | Treatments | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CO** | **CO + Ca** | **WB** | **WB + Ca** | **SH** | **SH + Ca** | **RB** | **RB + Ca** | **P Value** | | | | | | | |
| Nitrite (mg/g DM) | | | | | | | | | **SEM** | **Tx** | **Ca** | **time** | **Tx*Ca.** | **Tx*time** | **Tx*Ca** | **Tx*Ca*time** |
| 0 h | 0.07$^{a}$ | | 0.08$^{a}$ | | 0.10$^{a}$ | | 0.13$^{ab}$ | | | | | | | | | |
| 48 h | 0.30$^{d}$ | 0.12$^{b}$ | 0.25$^{cd}$ | 0.14$^{ab}$ | 0.21$^{c}$ | 0.14$^{ab}$ | 0.21$^{c}$ | 0.13$^{ab}$ | 0.004 | NS | <0.05 | <0.05 | <0.05 | <0.05 | - | - |
| TN (mg/g DM) | | | | | | | | | | | | | | | | |
| 0 h | 58.27$^{b}$ | | 45.25$^{ab}$ | | 69.36$^{c}$ | | 56.75$^{b}$ | | | | | | | | | |
| 48 h | 31.80$^{a}$ | 50.33$^{bc}$ | 41.12$^{a}$ | 45.70$^{ab}$ | 47.29$^{b}$ | 36.91$^{a}$ | 45.66$^{ab}$ | | 1.13 | <0.05 | NS | <0.05 | <0.05 | <0.05 | - | - |
| TIN (mg/g DM) | | | | | | | | | | | | | | | | |
| 0 h | 4.56$^{a}$ | | 4.72$^{a}$ | | 5.98$^{a}$ | | 7.79$^{sb}$ | | 0.26 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | - | - |
| 48 h | 13.31$^{c}$ | 6.32$^{a}$ | 12.16$^{c}$ | 8.78$^{ab}$ | 12.60$^{c}$ | 7.39$^{ab}$ | 11.90$^{c}$ | 10.85$^{b}$ | | | | | | | | |
| TON (mg/g DM) | | | | | | | | | | | | | | | | |
| 0 h | 53.71$^{bc}$ | | 40.53$^{b}$ | | 63.37$^{c}$ | | 48.96$^{bc}$ | | 1.18 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | | |
| 48 h | 18.49$^{a}$ | 44.01$^{b}$ | 28.96$^{ab}$ | 36.93$^{b}$ | 34.96$^{ab}$ | 29.68$^{ab}$ | 24.91$^{ab}$ | 16.89$^{a}$ | | | | | | | | |

[1] Data are presented as means ± standard error of the mean (SEM), with **n = 3 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).CO, control diet; WB, CO supplemented with wheat bran, SH, CO supplemented with soyabean hulls, RB, CO supplemented with rice bran. The diets containing additional Ca (2%) are indicated as 'Tr + C′

concentration was observed among the different diets.

In diets that were not supplemented with Ca (such as WB, SH, and RB), a reduction in the concentration of total inorganic nitrogen (TIN) was observed (Fig 5-1). The reduction in TIN concentration in WB, and SB (299.52 mg TIN/g TN and 272.13 mg TIN/g TN) was not statistically significant when compared to the RB diet (352.97 mg TIN/g TN). However, when compared to the CO diet, only the SH showed a statistically reduction in TIN concentration ($P < 0.05$; 272.13 mg TIN/g TN vs 420 mg TIN/g TN).

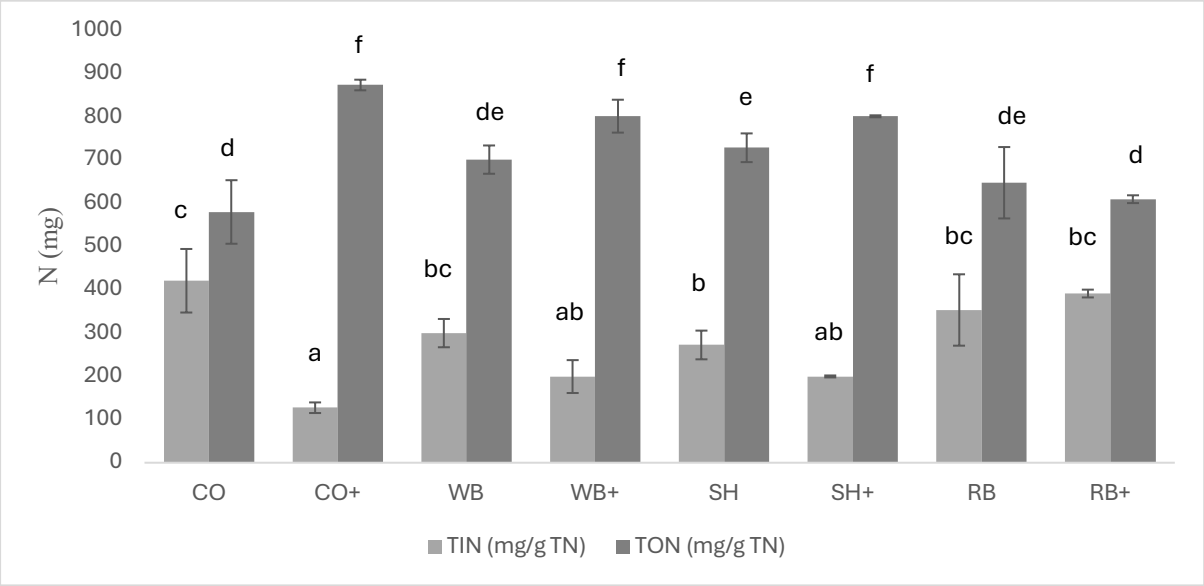


**Figure 5-1 TIN (total inorganic nitrogen) and TON (total organic nitrogen) concentrations (mg/g of Total Nitrogen, TN) with statistical differences indicated by letters (a-f) above error bars. Different letters denote significantly different means (ρ < 0.05).**

The addition of Ca resulted in a statistically significant reduction in TIN concentration across most diets ($P < 0.05$), except for RB. In contrast, RB exhibited a higher TIN concentration of 390.99 mg TIN/ g TN, indicating that the presence of Ca did not elicit the same degree of TIN reduction in the RB diet as it did in the other diets.

Further calculations revealed a numerical, though not significantly increase in total organic nitrogen (TON) in the WB and RB diets (700.48 mg TON/g TN, and 647 mg TON/g TN,

respectively) compared to the CO diet (579.47 mg TON/g TN). This increase was statistically significant in SH, which reached 727.87 mg TON/g TN), compared to both the CO and RB. The addition of Ca to the diets resulted in a further increase in TON concentration: $CO^{+}$: 872.89 mg TON/g TN, $WB^{+}$: 800.88 mg TON/g TN and SH $^{+}$: 800.6 mg TON/g TN. However, the $RB^{+}$ did not show significant change in TON concentrations.

### 5.3.2 Inorganic and organic phosphorus concentrations in the excreta fluid

As illustrated in Table 5-3, there was no difference in total P concentration across all the diets. However, the WB diet ($P < 0.05$) resulted in a significant increase the inorganic P during fermentation with respect to the other experimental diets and the control. As happened to the previous experiment, when purified fibre, such as inulin or cellulose was utilised with additional Ca, a decrease in inorganic P was observed in all experimental diet ($P < 0.05$).

The present study revealed no greater and significant differences in the inorganic and organic phosphorus concentration of P among the different treatments, including supplementation with Ca. however, as illustrated in Table 5-3, there were observed changes in the concentration of inorganic and organic phosphorus across the experiments. For example, when compared to the other by-products, WB exhibited a numerically higher Pi concentration; however, these changes were minimal and not statistically different from the control. In comparison to organic phosphorus, the fibre derived from SH has been observed to result in an increase in Po relative to each gram of total phosphorus (TP). The results revealed that the Po increased in SH ($P < 0.05$; 613.44 mg Po/g TP, Fig 5-2) in comparison with other diets. Similarly, a notable increase in the concentration of Po was observed in RB when Ca is supplemented in the diet ($P < 0.05$; 641.98 mg Po/g TP).

**Table 5-3** The concentration of P forms (including inorganic P, and organic P) in fermentation excreta. All P concentrations are expressed per g of ash[1].

| | Treatments | | | | | | | | P Value | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CO** | **CO + Ca** | **WB** | **WB + Ca** | **SH** | **SH + Ca** | **RB** | **RB + Ca** | **SEM** | **Tr** | **Ca** | **time** | **Tr*Ca.** | **Tr*time** | **Tr*Ca** | **Tr*Ca*time** |
| TP (mg/g ash) | | | | | | | | | | | | | | | | |
| 0 h | 206.32$^{a}$ | | 226.18$^{a}$ | | 254.13$^{a}$ | | 229.24$^{a}$ | | | | | | | | | |
| 48 h | 189.16$^{a}$ | 139.86$^{a}$ | 196.45$^{a}$ | 158.23$^{a}$ | 203.31$^{a}$ | 142.75$^{a}$ | 180.36$^{a}$ | 190.25$^{a}$ | 7.68 | NS | NS | NS | NS | NS | - | - |
| Inorganic P (mg/g ash) | | | | | | | | | | | | | | | | |
| 0 h | 66.73$^{a}$ | | 61.73$^{a}$ | | 68.31$^{a}$ | | 79.48$^{ab}$ | | | | | | | | | |
| 48 h | 92.14$^{bc}$ | 68.12$^{a}$ | 111.69$^{c}$ | 67.94$^{a}$ | 78.67$^{ab}$ | 59.55$^{a}$ | 85.08$^{ab}$ | 67.91$^{a}$ | 1.92 | NS | <0.05 | <0.05 | NS | <0.05 | - | - |
| Organic P (mg/g ash) | | | | | | | | | | | | | | | | |
| 0 h | 139.59$^{ab}$ | | 164.44$^{b}$ | | 185.82$^{b}$ | | 149.75$^{ab}$ | | 0.004 | NS | NS | <0.05 | NS | NS | - | |
| 48 h | 106.01$^{a}$ | 71.73$^{a}$ | 84.77$^{a}$ | 90.29$^{a}$ | 124.64$^{a}$ | 83.20$^{a}$ | 95.28$^{a}$ | 122.34$^{a}$ | | | | | | | | |

Data are presented as means ± standard error of the mean (SEM), with **n = 3 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$).CO, control diet; WB, CO supplemented with wheat bran, SH, CO supplemented with soyabean hulls, RB, CO supplemented with rice bran. The diets containing additional Ca (2%) are labelled with ¨Tr + Ca¨

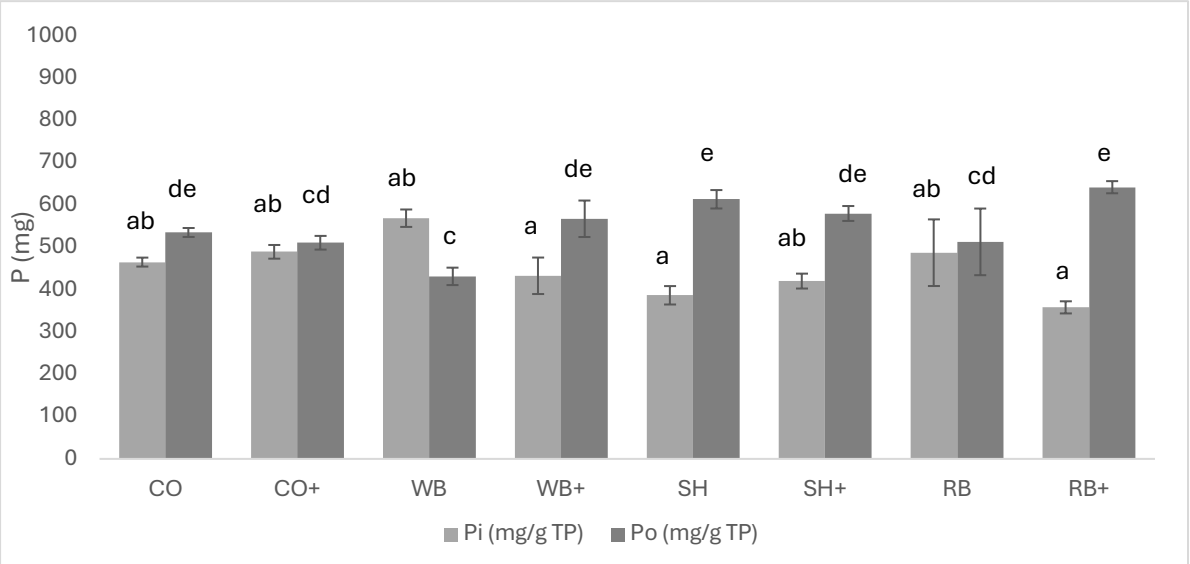


**Figure 5-2 Pi (Inorganic phosphorus) and Po (Organic phosphorus) concentrations (mg/g of Total phosphorus, TP) with statistical differences indicated by letters (a-e) above error bars. Different letters denote significantly different means ($P < 0.05$)**

### 5.3.3 Gas Production and pH

The gas production data at each time point (2 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h), along with the pH values after 48 hours of fermentation, are shown in Table 5-3. Up to 12 hours of fermentation, no significant differences in gas production were observed between the treatments, regardless of fibre or calcium content ($P > 0.05$). However, after 24 hours of fermentation, both fibre and the addition of Ca to the experimental diets significantly influenced the results ($P < 0.05$), as illustrates Figure 5 A and B. The remaining fermentation kinetics, including the production of VFA and microbial crude protein (MCP) are shown in table 5.2. As observed in the preceding experiment, the control diet exhibited the highest numerical gas production at 48 hours. However, the experimental treatments, also yielded high levels of gas, which were not significantly different from each other (P>0.05). It is interesting to note that these values exceeded those observed in both the fibrous diets and the fibrous diet supplemented with additional calcium. Furthermore, the diets containing soy hulls, with or

without additional calcium showed gas production values similar to those of the control diet and the control diet supplemented with additional calcium.

### 5.3.4 VFA production and MCP

The production of acetate, propionate and butyrate in CO, WB, SH and RB, and those diets where supplemental calcium was added during fermentation at 48 h were similar ($P > 0.05$; Table 5-2). Moreover, there were no significant differences in microbial crude protein on the excreta among the different experimental diets. However, it was observed an increase in microbial crude protein when Ca was supplemented in the diets (Ca effect, $P < 0.05$).

**Table 5-4 Volumes of gas (mL/g DM entering fermentation) produced including the pH of the final fermentation excreta, during the simulated hindgut fermentation of the *in vitro* pig gut model fed a pig grower diet supplemented with different fibre treatments[1].**

| | Treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CO** | **CO + Ca** | **WB** | **WB + Ca** | **SH** | **SH + Ca** | **RB** | **RB + Ca** | ***P* Value** | | | |
| **Time** | | | | | | | | | **SEM** | **Tr** | **Ca** | **Tx*Ca** |
| 2 h | 232.50 | 226.21 | 229.13 | 220.97 | 223.23 | 224.77 | 234.89 | 228.34 | | | | |
| 4 h | 212.37 | 214.91 | 214.78 | 215.46 | 211.24 | 218.42 | 216.77 | 219.99 | | | | |
| 8 h | 279.99 | 264.57 | 270.21 | 262.49 | 265.08 | 270.71 | 273.87 | 260.04 | | | | |
| 12 h | 311.30 | 303.65 | 307.79 | 304.26 | 300.59 | 316.02 | 300.90 | 293.96 | 1.584 | <0.05 | <0.05 | <0.05 |
| 24 h | 392.62$^{f}$ | 405.10$^{g}$ | 376.01$^{ef}$ | 388.23$^{fg}$ | 362.61$^{e}$ | 398.37$^{g}$ | 361.80$^{e}$ | 367.43$^{ef}$ | | | | |
| 36 h | 415.14$^{g}$ | 440.99$^{i}$ | 400.31$^{fg}$ | 431.69$^{hi}$ | 391.62$^{fg}$ | 453.16$^{i}$ | 386.16$^{f}$ | 397.12$^{fg}$ | | | | |
| 48 h | 430.02$^{gh}$ | 460.39$^{ij}$ | 412.45$^{g}$ | 442.51$^{i}$ | 406.70$^{g}$ | 479.02$^{j}$ | 400.76$^{g}$ | 408.46$^{g}$ | | | | |
| pH | | | | | | | | | | | | |
| 48 h | 5.6$^{k}$ | 6.1$^{n}$ | 5.7$^{l}$ | 6.1$^{n}$ | 5.7$^{l}$ | 5.9$^{m}$ | 5.9$^{m}$ | 6.3$^{o}$ | 0.008 | <0.05 | <0.05 | <0.05 |

[1] Data are presented as means ± standard error of the mean (SEM), with **n = 3 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$). CO, control diet; WB, CO supplemented with wheat bran, SH, CO supplemented with soyabean hulls, RB, CO supplemented with rice bran. The diets containing additional Ca (2%) are labelled with "+ Ca"

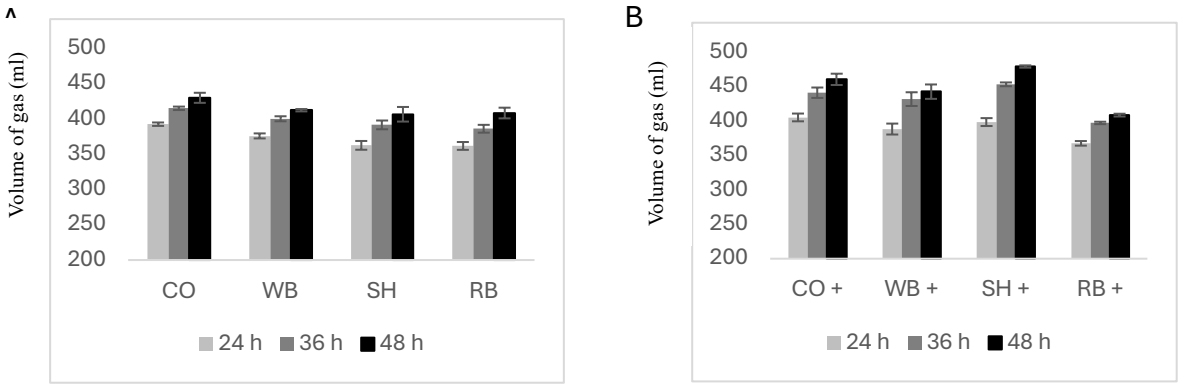


**Figure 5-3 Total gas produced by fermentation in experimental and control diets. Volume of gas produced without supplemental Ca (A), and with supplemental Ca (B) in the experimental diets. P<0.05 with supplementation of Ca between treatments. light barr, 24 h fermentation; gray barr, 36 fermentation; gray barr, 48 h fermentation**

**Table 5-5 Total volatile fatty acid production, including acetate, propionate, and butyrate at 24 and 48 h fermentation from various by-product sources of fibre treatment in the *in vitro* model.**

| | Treatments | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CO** | **CO + Ca** | **WB** | **WB + Ca** | **SH** | **SH + Ca** | **RB** | **RB + Ca** | | ***P* Value** | | |
| VFA (mM) | | | | | | | | | **SEM** | **Tx** | **Ca** | **Tx*Ca** |
| Acetate | 36.80[a] | 37.88[a] | 31.13[a] | 35.71[a] | 36.21[a] | 33.23[a] | 30.23[a] | 31.90[a] | 0.80 | NS | NS | NS |
| Propionate | 20.31[a] | 21.54[a] | 17.20[a] | 20.68[a] | 20.44[a] | 20.84[a] | 19.63[a] | 19.46[a] | 0.44 | NS | NS | NS |
| Butyrate | 18.88[a] | 17.60[a] | 11.07[a] | 15.58[a] | 15.27[a] | 12.47[a] | 13.17[a] | 12.46[a] | 0.73 | NS | NS | NS |
| Total VFA | 76.00[b] | 77.02[b] | 59.40[b] | 71.98[b] | 71.93[b] | 66.53[b] | 63.03[b] | 63.82[b] | 1.87 | NS | NS | NS |
| MCP (mg/ml) | 0.27[a] | 0.36[b] | 0.33[a] | 0.41[b] | 0.31[a] | 0.41[b] | 0.32[a] | 0.36[a] | 0.10 | NS | <0.05 | NS |

Data are presented as means ± standard error of the mean (SEM), with **n = 3 per treatment.** Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values in the same row with different letter superscripts means a significant difference ($P < 0.05$). CO, control diet; WB, CO supplemented with wheat bran, SH, CO supplemented with soyabean hulls, RB, CO supplemented with rice bran. The diets containing additional Ca (2%) are labelled with "+ Ca"

## 5.4 Discussion

The main aim of this experiment was to determine whether increasing the total dietary fibre content of pig feed by including ingredients with varying levels of soluble and insoluble fibre, as those found in by-product of milling and oil industries, results in effects comparable to those observed with purified fibres (such as inulin and cellulose) in altering the forms of N and P excreted in an *in vitro* model of the pig. Figure 5-1 depicts the impact of by-products treatments on the concentration of TIN and TON in the excreta and Figure 5-2 slightly changes in P forms. The addition of Ca, particularly in diets containing wheat bran and soybean hulls, was observed to result in an increase in TON while simultaneously reducing TIN. This suggests that Ca plays a role in promoting the conversion of TN into organic forms of N when fibre-rich ingredients are present in the diets. Moreover, the variation in soluble and insoluble fibre present in the bran from the ingredients used in this experiment may have contributed to shaping the microbiota involved in the transformation of N and phosphorus P. Significant variations between soluble and insoluble non-starch polysaccharides (NSP) have been demonstrated to have a substantial impact on digestive and fermentative processes in growing pigs. The fermentation of dietary fibre by gut microbiota produces VFA, playing a crucial role in microbial activity within the hindgut. This process has important implications for gut health,

influencing nutrient transformation, absorption, intestinal integrity, and overall metabolic function in pigs (Jha and Berrocoso, 2016).

In previous experiments, all material remaining after enzymatic hydrolysis was subjected to a fermentation simulating the pig gut. This resulted in variations in gas production across the experimental diets due to differences in the amount of fermentable material. To evaluate the fermentation characteristics of each diet, which comprises by-products of mill and oil industries, and address more accurately these 'variations', *ca* 0.5 g of the residual material was used for fermentation after enzymatic hydrolysis. Surprisingly, the control diet (CO) produced a higher rate of gas than expected, contradicting the initial assumption that all experimental diets would ferment at a similar rate.

In contrast, the three experimental diets, which contained varying proportions of soluble and insoluble fibre from wheat bran, and soybean hulls, along with the other treatments supplemented with Ca, demonstrated an increase in gas production. Calcium is frequently present in fibrous ingredients. The precise mechanisms through which calcium influences fibre fermentation have yet to be fully elucidated. However, current hypotheses suggest that calcium's influence stems from its ability to modulate the composition of the gut microbiota (Gomes et al., 2015). The less gas produced per g of dry matter fermented compared to the other fibrous experimental diets lacking supplementary calcium may be attributed to diminished microbiota activity within the model. The pig gut model, which was designed to

emulate the conditions of the large intestine, utilised blended faeces. However, as previously noted by Jensen and Jørgensen (1994), the activity of the microbiota is higher in the hindgut than in the faces, even when microbial concentrations are comparable.

The lack of effects in RB for excreted TIN and TON, as well as excreted Pi and Po compared to their excreted forms in the other treatments may be attributable to the fibre composition of the product. Approximately 12% of the TDF content of RB is insoluble, consisting largely of cellulose, hemicellulose, and arabinoxylans, which make up approximately 90% of the TDF present (Oliveira et al., 2011; Daou and Zhang, 2014).

As previously mentioned, a key aspect of this experiment, as well as the previous one, was the inclusion of calcium (Ca) in the experimental diets. Our initial hypothesis was that Ca supplementation in high fibre diets would influence fermentation kinetics by increasing microbial activity during the fermentation process. Notably, the experiment revealed multiple interactions between fibre, Ca, and different nutrients. These interactions were expected, as it is well established that calcium (Ca) increases the pH of the intestinal digesta (Adedokun and Adeola, 2013). Such an increase in pH may be detrimental when phytase is present in the diet, potentially reducing its efficacy, which in turn may reduce phosphorus (P) utilisation and increase organic P excretion in the effluent.

### 5.4.1 Fermentation kinetics, production of gas, and MCP

The results of the current experiment differed from those perfumed in Chapter 4 particularly about fermentation characteristics, VFA and ammonia production. It is worthy of note that the inclusion of up to 27% TDF into standardised growing pig diets exerted a significant effect on the concentration of different forms of N and P in most diets, as observed using an *in vitro* model of the pig gut.

The measurement of gas released during fermentation provides valuable insights into the characteristics of ingredients in the hindgut (Williams, Bosch, Boer, et al., 2005). However, it was also observed that the supplementation of Ca resulted in a higher pH value ($P < 0.05$) in all the experimental diets and in the control. This highlights the importance of Ca in fibrous diets and its potential to modify or alter the different forms of N or P due to their ability to increase the pH of the excreta (Bournazel et al., 2018).

### 5.4.2 Nitrogen and concentration of different N forms

The results demonstrated that the inclusion of insoluble and soluble fibres derived from natural ingredients resulted in a significant increase in the proportion of organic to total nitrogen ($P < 0.05$). This suggests that a higher proportion of fibre can effectively reduce ammonia ($NH_3$-N) levels in an *in vitro* model of the pig gut. As was hypothesized increasing dietary fibre into the diets of growing pig led to a decline in the concentration of TIN for both WB and SH (299.52 mg TIN/g TN and 272.13 mg TIN/g TN). Although, different experiments showed that incorporating different types and sources of fibre can effectively reduce $NH_3$-N levels in

fermentation effluents. In Chapter 5, no reduction in total inorganic nitrogen (TIN) was observed in the RB treatment. Nevertheless, the reduction observed in the other co-products used is significant, as it clearly shows that co-products can reduce TIN in fermentation effluent in an in vitro model, although the effect is less pronounced compared to the use of purified fibre. In Chapter 4, the levels of inulin and cellulose were set at 5%, which is higher than the average levels used in the experiment in Chapter 5 (Metzler-Zebeli et al., 2017). This contrast in fibre levels may account for the differences in the extent of change in excreted TIN observed in fibre-rich diets in this study in comparison to those in studies using purified fibres. The present study demonstrated that diets not supplemented with Ca, which included WB, SH, and RB, resulted in a decrease in the concentration total inorganic nitrogen (TIN) in the excreta fluid.

Bindelle et al. (2009) identified the significant role that dietary fibre plays in altering nitrogen metabolism in pigs, particularly in the partitioning of nitrogen into inorganic and organic forms in excreta. who reported that the presence of dietary fibre can alter nitrogen metabolism in pigs, particularly affecting the partitioning between inorganic and organic forms of nitrogen in excreta. While in this study differs in specific details, this highlights the necessity to investigate the influence of dietary fibre on nutrient metabolism, which remains pivotal in comprehending the environmental and nutritional consequences of pig production. Therefore, was established that diets high in fibre, such as those containing WB and SH, modify gut fermentation processes and microbial activity. This may enhance microbial assimilation of inorganic nitrogen, thereby

reducing TIN in the excreta (Jha and Berrocoso, 2016). The results of the current study are consistent with this observation, particularly with the decrease in TIN observed in the SH diet compared to the CO diet.

Furthermore, the significant decline in TIN concentration with Ca supplementation observed in this study is consistent with the findings of other authors, who demonstrated that Ca supplementation can influence nitrogen metabolism by increasing gut pH and microbial activity. Calcium appears to reduce the availability of inorganic nitrogen in the gut by promoting the formation of insoluble complexes or by enhancing microbial uptake, which leads to a reduction in TIN in excreta. However, the lack of a significant reduction in TIN in the RB diet with Ca supplementation suggests that the interaction between fibre type and Ca is complex and may depend on specific fibre properties or other nutrient interactions within the diet.

Moreover, the study revealed a not significant effect on total organic nitrogen (TON) in the WB and RB diets in comparison to the CO diet, with a statistically significant increase in TON in the SH diet. The addition of Ca to the diets resulted in a further increase in TON concentrations in all diets, with the exception of the RB + Ca diet. These results are consistent with those reported in the meta-analysis done by Metzler-Zebeli and Zebeli (2013) who stated that diets comprising a high proportion of fibrous components, particularly those supplemented with calcium, can facilitate the microbial conversion of inorganic nitrogen to organic forms, as

evidenced by elevated TON concentrations. This increase in TON with calcium supplementation may be attributed to the stimulation of bacterial growth and fermentation processes that utilise inorganic nitrogen, converting it into organic forms such as microbial protein.

The inconsistency observed in the RB diet, where Ca supplementation did not significantly affect TON concentrations, may be attributed to the distinctive composition of RB, which comprises a substantial proportion of insoluble fibre. Insoluble fibres are less fermentable than soluble fibres and may not provide the same substrate quality or quantity for microbial growth and activity, which could explain the reduced impact of Ca on nitrogen metabolism in RB-containing diets (Williams et al., 2011). A common error is the assumption that all materials with a fibrous composition are fermentable (Williams, Bosch, Boer, et al., 2005). The results presented here demonstrate that even when a substrate is highly fermentable, there can still be considerable variation in fermentation rates and the types of end-products produced.

### 5.4.3 Phosphorus and different forms concentration

The present study revealed no statistically significant differences in the concentrations of Pi and Po among the different treatments with by-products, including those with calcium supplementation. Nevertheless, as illustrated in Figure 5-2, some alterations in Pi and Po levels were observable across the diverse diets that were subjected to testing. For example, wheat bran (WB) exhibited a numerically higher inorganic phosphorus (Pi) concentration compared

to other by-products. However, these changes were minimal and not statistically significant when compared to the control diet. In contrast, the study indicated that dietary fibre derived from by-products resulted in an increase in Po, particularly in SH, which demonstrated a statistically significant increase in Po concentration ($P < 0.05$; 613.44 mg Po/g TP). Similarly, there was a significant Treatment/Ca interaction for Po:TP in the RB sample when calcium was supplemented in the diet ($P < 0.05$; 641.98 mg Po/g TP).

These findings are consistent with the conclusions of other researchers, such as Turner and Leytem (2004) who reported that the form and amount of phosphorus in animal diets could be influenced by the dietary fibre content and the availability of calcium. The study demonstrated that diets with high fibre content could modify the digestion and absorption of phosphorus, potentially increasing the proportion of organic phosphorus excreted in the faeces. Moreover, the findings of a couple of studies presented by Rodehutscord (2016) lend support to the notion that the dietary composition, including the inclusion of fibre and mineral supplementation, exerts an influence on the utilisation and excretion of phosphorus in pigs. The results indicated that calcium plays a role in binding phosphorus, influencing the equilibrium between inorganic and organic forms. The observation in the present study that dietary fibre from by-products can elevate organic phosphorus levels in the diet, particularly with the addition of calcium, is consistent with the findings of Bindelle et al. (2009).

## 5.5 Conclusion

In conclusion, by-products from the milling and oil industries, which contain varying proportions of soluble (SDF) and insoluble dietary fibre (IDF), can be utilised to modify different forms of N and, to a lesser extent, P in an *in vitro* model of the pig gut. The kinetic characteristics of these by-products indicate that they do not ferment at the same rate, resulting in differing effects on the excretion of N and P across experimental diets. Furthermore, the inclusion of calcium (Ca) in fibrous diets requires careful consideration, as Ca may interact with fibre and other nutrients, potentially leading to unexpected outcome

# Chapter 6 General discussion

In the hindgut, the breakdown of dietary components follows a stepwise process, with sugar residues and oligosaccharides being degraded first, followed by starch remnants, then soluble NSPs, and finally insoluble NSPs. In the case of pigs, the principal sites of carbohydrate breakdown are the cecum and the proximal colon, where the intensity of microbial activity is particularly pronounced (Bach Knudsen et al., 2017). This thesis primarily aimed to determine the feasibility to alter the excreted forms of N and P during fermentation of commercial pig diets supplemented with both soluble and insoluble fibre using an *in vitro* model of the pig gut. The typical composition of a pig diet includes up to 15% TDF, with the bulk of this derived from natural sources such as cereals and oil-based products. These ingredients are commonly included with the aim of balancing the nutritional value of the feed and reducing costs, primarily due to their protein content. However, pigs are unable to fully digest dietary fibre because lack the specific enzymes required to hydrolyse the fibre present in the feed. As a result, a significant proportion of P present as phytate-P in the fibre becomes less available. Consequently, a considerable amount of the undigested fibre is transported to the hindgut, where it is fermented by the microbiota. However, there is a risk of losing ammonia through volatilisation and P through leaching or run-off, which could have an adverse impact on the environment.

A preliminary study was conducted using faeces from gestating sows to evaluate the effects of a diet containing sugar beet pulp on the excreted forms of nitrogen and phosphorus, and to validate the *in vitro* model for optimising the technique to facilitate in experiments presented in Chapters 4 and 5. SBP is a natural source of both insoluble and soluble fibre that is widely available in the UK and European market, which was included in the diet of gestating sows. However, in Chapter 3 the main aim of the experiment was to compare the concentration and forms of N and P in excreta from gestating sows to that of the fermentation effluent from the *in vitro* model. The experiment was analysed by combining the factors (treatment and type of excreta) with GLM analysis and separately by Ttests. as the results from the analyses were the same, only the results from GLM were presented. The preliminary data demonstrated that there were no interactions between the forms of N and P in manure of the sows or in the fermented excreta of the model. In this experiment, two treatment time points were simulated to compare the effects of sugar beet pulp *in vivo* on sow excreta with those produced by the in *vitro model*. The most compelling data were derived from the four-week gestation period, in which the in *vitro* fermentation effluent was compared with the excreta of a gestating sow. No significant interaction was observed between the excreta types (faeces and fermentation effluent), indicating that the in vitro model is a reliable tool for comparing with in vivo results.

## 6.1 Changes in N forms

In Chapter 3, at six weeks of treatments excreted effluent $NH_3$-N declined, but not in the faeces of those animals, indicating an interaction between treatment and type (source of waste; gestating sow faeces or fermentation effluent). Previously, Bikker et al. (2017) stated that a minimum of ten days is necessary to adapt any animals into a new dietary treatment to assess the nutrient digestibility and the study of the stablish hindgut microbiota. With this in mind, and to replicate the hindgut environment of gestating sows, the faeces used in the experiment performed in Chapter 3, were collected after (at) four weeks of treatment.

Notwithstanding these limitations, inclusion of ingredients with a high total dietary fibre (TDF) content into pig diets is a common practice. In gestating sows, this approach has been demonstrated to reduce feed costs, acts as a ´bulking agent' in the feed, and enhance the feeling of satiety to the animal. In recent decades, a few studies (Galassi et al., 2010; Ball and Magowan, 2012; Ferrer et al., 2021) have demonstrated that increasing the TDF beyond the pig's normal requirements can provide the additional benefit of reducing faecal ammonia and, therefore, ammonia volatilisation, with minimal impact on pig performance. The use of fibrous ingredients and exploration of alternative feeding strategies are important, particularly in the context of sustainable farming and enhancement of manure. The transformation of N and P into more bioavailable forms or improvement of manure properties to serve it as a feed source for insect farming or to produce biogas has the potential to reduce the environmental impact of agricultural practices.

The data presented in this thesis demonstrated significant alterations in nitrogen forms, including a reduction in the amount of total inorganic nitrogen (TIN) and an increase in the concentration of total organic nitrogen (TON) per gram of total nitrogen (TN) in most of the experiments after 48 hours of *in vitro* fermentation. These changes were more evident when the diet for growing pigs was supplemented with TDF in comparison to the diet for gestating sows. This finding is of particular importance given that growing pigs are responsible for just over 50% $NH_3$-N emissions from their facilities (Hayes et al., 2006). These results corroborate the findings of a great deal of the previous work (Bach Knudsen, 2001; Ndegwa et al., 2008; Jha and Berrocoso, 2016; Thi Bich Ngoc et al., 2020; Zheng et al., 2023) in shifting the N forms, from inorganic toward organic forms, after offering to the pigs a diet rich in TDF. However, most of those works (Lynch et al., 2008; O'Shea et al., 2009) have mainly focused on the reduction of ammonia, thereby neglecting other forms of inorganic N, such as nitrate and nitrite, which are beneficial for crop production. Furthermore, these studies tend to ignore other forms of organic nitrogen, which are more stable in the environment and contribute to long-term soil fertility (Van Cleemput and Baert, 1984; Geisseler and Scow, 2014).

The results presented in Chapters 4 and 5 clearly demonstrate changes in the partition of TIN and TON among the treatments partitioning of TIN and TON among the treatments. A significant reduction of approximately 50% in TIN per gram of TN was observed when purified fibres, such as inulin or cellulose, were added. When using by- products, although the decrease was still significant, it was less pronounced (average 36% reduction). In Chapter 5, the

reduction of total inorganic nitrogen per gram of total nitrogen was three times greater than that observed in the control diet, indicating that both type of fibre (soluble and insoluble) is highly effective in reducing the TIN in the samples with increased TDF. According to Jha and Berrocoso (2016), the reduction in the TIN ratio also may be attributed to an increased utilisation of nitrogen for the microbial protein synthesis in the hindgut, which is finally excreted in organic form in the faeces.

One of the main limitations of these experiments was to choose the right fermentation inoculum to best simulate what happens in the pig gut. In the experiment, 60 g of faeces per every litre of fermentation buffer prepared (see Chapter 2) was used to ferment the experimental diets. In most of the experiments, 60 g of faeces per every litre of fermentation buffer prepared (see Chapter 2) was used to ferment the experimental diets. Before the simulation of fermentation, each experimental diet was subjected to a six-hour digestion process in a simulated gastric and small intestine model, using pepsin and pancreatin, respectively. This was done to produce a residue that resembled the *in vivo* ileal chyme. However, as previously observed by Bauer et al. (2010), it could be inaccurate to assume that enzymatic hydrolysis prior to fermentation in the *in vitro* model will yield material with the same fermentability as a pig's ileal chyme. Even though, in this thesis we considered that most of the hydrolysed material may have passed to the fermentation, due to observations done by other authors. This can be attributed to the fact that non-enzymatic processes occurring in the upper digestive tract are not replicated, and microbial fermentation is likely to occur in the distal regions of the small intestine.

Consequently, the observed variation in fermentation characteristics may be attributed to differences in the fermentable material entering the fermentation stage of the model. This observation was noted in Chapter 4, where it is possible that some inulin was lost after enzymatic hydrolysis when samples was being washed to eliminate the chemical used in the processes. The washings were done with ethanol, acetone and deionised water affecting its availability for fermentation. Another interesting finding from the experiments is that it may be erroneous to assume that all the undigested or not hydrolysed material by pepsin or pancreatin undergoes fermentation. The data demonstrate that not all 'fibre-based' treatments were fermented at the same rate. In Experiment presented in Chapter 5, for instance, RB exhibited significantly lower fermentability compared to other fibre sources, despite RB containing a higher fibre content in the mix. However, it should be noted that this experiment did not only focus on fibre fermentability; rather, it assessed fibre within a standard diet. For that reason, some characteristics of fermentation could be not assessed appropriately

In Chapter 4 a significant reduction in total inorganic to total nitrogen ratio was observed when purified fibre was used in the models. In Chapter 5, that reduction was less evident, however, here natural fibre sources with different levels of soluble and insoluble fibre were used. However, the level of total dietary fibre, in the last experiment was double in comparison, which that it was used for experiment in chapter 4, this observation is so important because, we could ascertain that the fermentation ability of the microbiota could vary according to the type of fibre present. For example, in experiment in the three experimental chapters was evident

the reduction of $NH_3$-N when fibre was added to the diets. Furthermore, an increase in $NO_3$-N and $NO_2$ also was observed.

## 6.2 Changes in P forms

The consumption of dietary fibre by pigs is typically limited due to the lack of suitable enzymes required for its digestion in the small intestine and is also limited by the high phytate-P content of diverse fibrous ingredients, which impedes the efficient utilisation of P in monogastric animals.

The effect of supplementing with insoluble and soluble on phosphorus forms varied between the different experiments. In general, as the *in vitro* model is a closed system, it was expected not to find any changes in the total phosphorus content at the end of fermentation than at the beginning. However, as the dry matter was disappearing through the simulated digestion and fermentation, it is expected to find changes in inorganic and organic P through the experiment. One example of this can be seen in Chapter 4, where clearly there is an increase in inorganic P in the diets. However, the supplementation of pig diets with insoluble and soluble fibres has shown minimal effect in those P forms in other experiment in other chapters. A possible explanation for these minimal changes might be related with the microbiota of the faeces. Moreover, given the different levels between phytate-P, non-phytate phosphorus and, phytase present in the diets in the experiments, it was not possible to distinguish between the effects of these factors on the results obtained. In fact, when the fibre content of the diet was increased,

we did not increase the phytase dose of the feed. Therefore, during the experiment the endogenous phytase activity of plant material in the diet and bacteria, including the gut microbiota was relied upon (Zimmermann et al., 2003). During the purified fibres and SBP experiments reported in Chapter 4, the clearest effects of supplementary fibre on phosphorus were described. Here inulin significantly ($P < 0.05$) increased the inorganic to total phosphorus ratio after fermentation. Moreover, a numerical increase in the inorganic P in the diet containing wheat bran in Chapter 5 compared to the control diet and the other fibre sources. According to Vande Ginste and De Schrijver (1998), wheat bran has a considerable phytase activity that stimulates the liberation of phosphate and liberates and the phytic acid-bound calcium in the intestine of pig. It is also imperative to consider that the lack of observed effects in the second experiment of Chapter 4, as well as during the experiment with brans and soyabean hulls in Chapter 5, may be attributed to the static characteristic of the model used in this thesis. In other words, for every experiment done, each digestion process was conducted just one time. In contrast, in vivo animals, the materials can remain in the hindgut to ferment for several days. This observation implies that not all the fibre present in the hindgut is fermented at the same rate and at the same time. For that reason, the findings of this study indicate that purified fibres is more efficient in stimulating phytase activity and enhancing the release of inorganic P, changing this P forms in the fermented excreta. This is presumably due to the fact purified fibre provide a greater amount of fermentable substrate for the microbiota, which are better adapter to recognise and utilise these purified fibres.

## 6.3 Mineral interactions

One aim of this thesis was to investigate the effect of Ca addition, simulating the effect of natural fibre sources with high Ca content during hindgut fermentation. Therefore, we hypothesised that addition of Ca to supplemented growing pig diets will interact with the microbiota of the model, enhancing the transformation of the forms of P and N. Although the exact mechanisms are not fully understood, it is thought that calcium affects the composition and activity of the gut microbiota (Gomes et al., 2015). There was an interaction between, fibre, Ca and nutrients, such as P and N during fermentation, but the findings on those studies have been unable to demonstrate that high fibre diets supplemented with Ca are capable to enhance the transformation of P and N in an *in vitro* model of the pig gut confirming an interaction between Ca and other minerals

## 6.4 Fermentation kinetics, DM, and ash concentration

The evaluation of the fermentability of fibre-rich diets can be done through the analysis of various indicators, such as gas production, VFA concentrations, and microbial crude protein (Bai et al., 2020). The findings of this research showed that, while the fermentability of fibrous ingredients was found to be similar, there were noticeable variations depending on the specific type of fibre and the medium in which they were fermented. The fermentability of fibre appears to be dependent on the source of faeces used for inoculum preparation. For example, SBP had a high fermentation rate as indicated by its VFA concentration after six weeks of treatment (Chapter 3; data not included in this thesis) compared to the diet containing SBP used in the

growing model (Chapter 4). Despite its high content of SDF, SBP also contains a significant amount of lignin, which is not a fermentable fibre (Theander et al., 1989). Therefore, it is possible that differences in the microbiota between gestating sows and growing pig faeces, as well as the presence of other fibrous material in the ingredients, may have influenced the fermentation rates observed in these experiments. However, in Chapter 5 there were no significant changes in VFA production between the fibrous diets and control, which is contrary to the finding of previous studies which suggested that the type of substrate may have a profound influence on the VFA production (Serena et al., 2008; Bai et al., 2022b). However, it should be noted that Yoon and Michels (2021) did not find significant differences in VFA levels between individuals consuming fibre-rich diets and those consuming normal diets. In this study, we found differences in VFA levels in treatments with high-fibre diets. Although these results seem promising, the *in vitro* model, while comparable to live animals, suggests a more immediate response, whereas *in vivo* effects may take longer to manifest

In all the experiments performed in this thesis, the control diet (which contained standard levels of TDF, up to 16%) consistently produced a greater volume of gas than the experimental diets. This may be attributed to the fact that the control diet contained more CP than the experimental diets. It can be reasonably deduced that not all the protein was digested during the gastric and small intestine phases, resulting in a greater amount of fermentable material being present during fermentation. However, as demonstrated in Chapter 5, *ca* 0.5 g of hydrolysed material was used for fermentation after enzymatic hydrolysis, yet comparable results were observed.

It is also possible that the higher gas production in the control diets is attributable to the presence of a considerable amount of 'fermentable material' from non-dietary materials. These findings indicate that a significant proportion of the non-digestible material present in the diets could be fermented during the course of the experiments.

As was explained earlier, the model used in this thesis was designed to simulate gastric and small intestine digestion before fermentation, which occurs in the hindgut of pigs. Therefore, beyond the disadvantages in the fermentation that non-dietary material offers in the measurement of the different parameters, one of the main limitations of these kind of *in vitro* pig models is that it keeps a static digestion process. In other words, the digestion process in this model is conducted with minimal variation in dynamic factors such as pH and enzyme activity, regardless of the type of material used in the model. Brodkorb et al. (2019) suggests this may present a challenge for certain types of experiments, as it might not accurately reflect the physiological conditions within a living pig.

## 6.5 Conclusion

This research set out to determine the effect of increasing the total dietary fibre, and different types of fibre, to pig diets and assess its effects on the excreted forms of N and P using an *in vitro* model based on the work of Palowski et al. (2021). The aim was to determine if dietary fibre could be used to manipulate the N and P composition of the excreta, with a view to producing manure that is a more suitable 'green fertiliser'. The supplementation of dietary fibre

at levels considered to be above standard has shown customising the N and, to some extent, P forms using an *in vitro* pig gut model. However, purified fibres such as inulin and cellulose decreased inorganic N, alongside sugar beet pulp, a natural source of fibre from the processing of sugar beet. While the results obtained from the *in vitro* experiments are promising and showed the efficacy of their ability to simulate the behaviour of different nutrients such as N and P in a controlled environment, it is important to consider the limitations of such models.

The model provides valuable preliminary data and offers both cost and time efficiency. Although it captures many important aspects, the complexity of the monogastric digestive system, especially under significant dietary changes, is best understood through a combination of *in vitro* and *in vivo* approaches. *In vitro* studies are a crucial first step, but validation through *in vivo* studies, which account for the intricate interactions within a living system, enhances the accuracy and relevance of the findings. This integrated approach offers a more comprehensive and reliable understanding of nutrient behaviour and its impact on health and the environment.

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