Tenebrio molitor (yellow mealworms) as potential 'nutrient concentrators' for sustainable animal feeds

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Dedicated to my late Gramps

'A good sword and a trusty hand, a merry heart and true.

King James's men shall understand what Cornish lads can do!'

Trelawny, Cornish National Anthem

Abstract

Alternative protein sources are urgently needed to replace the traditional sources for animal feeds (e.g. soybean meal). Mealworms are being investigated as one such source, due to similar nutrient composition to soybean meal, but production methods and feed sources need to be sustainable for it to be an effective replacement feed ingredient. There is large variation in production methods and already available technologies in conventional livestock production, such as commercial enzymes have not been utilised in insect production.

The aim of this work was to investigate mealworm production methods such as water supply, feed sources and production environment, to manipulate mealworm growth and proximate composition using exogenous enzymes and finally, to utilise mealworms and potentially improve mealworms as a partial replacement for soybean meal in broiler feeds.

Results found that feeding mealworms rehydrated wheat bran significantly reduced growth compared to a control feed of wheat bran and carrot (P<0.001), water alone can replace vegetable sources, such as carrots, with no negative effects on growth (P>0.05) and increased crude protein (%DM) (P>0.05). Mealworms could be fed on either high quality chick crumb or low-quality wheat bran in either an uncontrolled room or a controlled incubator with no significant differences in initial or final group live weights, dry matter, crude protein, total fat or total energy content of mealworms (P>0.05). Total feed intake was significantly lower in the groups fed in the room (P<0.001), with those on wheat bran the lowest suggesting these were the most feed efficient.

There was no effect of phytase inclusion in the feed or water on mealworm growth or proximate composition (P>0.05). Pre-treatment of wheat bran with water (with or without exogenous phytase), decreased mealworm growth (P=0.02) and crude protein content

(P=0.002) while increasing total fat content (P=0.044). This suggests that endogenous phytase within the wheat bran was activated by water soaking. Varying effects of high dose phytase were seen on mineral content of mealworms. Xylanase inclusion significantly reduced mealworm growth (P=0.033) and dry matter (P=0.004). In the absence of phytase, xylanase reduced crude protein (P=0.002) and increased total fat content (P=0.007).

Replacing 10% of soybean meal with mealworms had no effect on broiler growth or apparent ileal digestibility but did increase feed intake (P=0.01) resulting in a negative increase in feed conversion ratio (P=0.002). There was also a significant effect on the caecal microbiota at both a phyla and genus level, with increases in *Romboutsia timonensis* (P=0.006) and *Sellimonas intestinalis* (P<0.001) associated with healthy gut. Exogenous chitinase successfully hydrolysed chitin releasing D glucosamine *in vitro* (P<0.001) but was inconclusive with mealworms. Chitinase inclusion into broiler feeds resulted in improved feed conversion ratio in the last 5 days of the trial compared to the negative control (P=0.049). There was no effect of chitinase inclusion on broiler growth, feed intake, apparent ileal digestibility of crude protein or amino acids and overall caecal microbiota. There was a significant increase in *Blautia hydrogenotrophica* suggesting that chito-oligosaccharides were being utilised by bacteria leading to increased production of hydrogen and carbon dioxide for cross feeding.

In conclusion, mealworms can be manipulated through changing production conditions, feed and water sources, but inclusion of exogenous enzymes had little overall effect. Partial replacement of soybean meal with mealworms decreased feed conversion ratio but had no other negative impacts on broiler production and addition of exogenous chitinase improved feed conversion ratio in older birds.

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List of Abbreviations

MW - mealworm

FCR – feed conversion ratio

BWG – body weight gain

DM – dry matter

CP – crude protein

TF – total fat

GE – gross energy

AA – amino acid

ICPMS – inductively coupled plasma mass spectrometry

WB – wheat bran

CW – cotton wool

W – water

I – incubator

R – room

XOS – xylo-oligosaccharide

XYL – xylanase

PHY – phytase

SED – standard error of the differences of the mean

SEM – standard error of the mean

AIDC – apparent ileal digestibility coefficient

UC – unclassified

OTU – operational translation unit

1 Introduction

1.1 Overview

An increasing global population has increased demand for food, which coupled with a change in dietary preferences has resulted in more livestock products being required. The livestock production industry must increase its production to meet this new demand. However, alongside this increase there is increasing pressure to improve sustainability and become more environmentally friendly.

Environmental issues stem from many aspects of production. These include use of land and water supplies to produce feed that could be used directly for production of human food. Feed production also has a number of negative impacts on the environment. Production is frequently associated with deforestation and feed crops are often produced in countries remote from the livestock to which they are being fed, resulting in greenhouse gas emissions associated with transport. A primary example of such feed ingredients is soybean meal; although a high quality and highly digestible protein source, it has been associated with considerable negative impacts on the environment.

Alternative, more sustainable, protein sources are being investigated to either partially or fully replace these traditional ingredients. Insects represent an alternative and potentially sustainable source of protein. However, there is limited understanding of the requirements for mass production systems and the specific nutritional values of insects. There is wide variation in nutritional composition across the vast number of species and this composition

varies over the lifespan of the insect. Specific requirements for growing insects are not as well defined as with traditional livestock for mass production. There is great potential for insects as they can be reared on poor-quality feed and yield a rich protein source, with a suitable amino acid composition. Currently exogenous enzyme technologies are used to improve digestion and utilisation of nutrients in monogastric livestock (pigs and poultry); but these have not yet been used in insect feeds. Furthermore, insects have chitin in the exoskeleton and it is unclear whether this is a positive or a negative in terms of a feed component. However, enzymes could be used to help break down chitin and use in animal feeds may improve their digestibility of insects, thereby improving efficiency and reducing production costs.

The following review outlines the reasons behind the increasing demand for protein and how the livestock production industry fits into this in terms of its own environmental effects, use of technology to improve outputs and what protein sources are currently being used. Alternative protein sources are then explored, specifically outlining insects, work achieved with inclusion into animal feed and potential concerns with the mass production of insects.

1.2 Background

1.2.1 Increased demand for protein

The current increase in global population is well-documented, however alongside this global demographics are changing, with many developing countries having access to greater resources (Alexandratos and Bruinsma, 2012). As such countries are becoming more economically stable this frequently leads to changes in diet choices, with an increase in

consumption of animal products (Speedy, 2003; Thornton, 2010), moving towards a more 'Westernised diet' (Alexandratos and Bruinsma, 2012).

Worldwide meat production has almost quadrupled from 78 to 331 million tonnes between 1963 and 2015 (Michalk et al., 2019) and by 2030 meat production is predicted to exceed 455 million tonnes (Alexandratos and Bruinsma, 2012). However, there is increasing pressure to reduce consumption of animal products due to issues relating to health and environmental impact. The recent EAT-Lancet report looked at the impact of diet on the population and planet health, with the recommendations to reduce animal product intake and increase plant-based components (Willett et al., 2019). There is little doubt that many individuals in wealthier countries would benefit from reduced consumption of animal products but in developing countries increased consumption could be beneficial, as animal products are a source of high quality protein, energy and micronutrients (Speedy, 2003). In the 'Western world' socio-economic factors may play more of a role in reduced animal product intakes (Thornton, 2010), with increasing interest in vegan and vegetarian diet choices.

Current food systems have the potential to provide for human health whilst maintaining balance with the environment (Willett et al., 2019). To meet the growing demand by 2050 it has been suggested that food systems should use no additional land, reduce consumptive water usage, reduce nitrogen and phosphorus waste and zero carbon dioxide emissions (Willett et al., 2019). Thus, there is an urgent need for the global livestock industry to become more sustainable.

1.2.2 Livestock production industry

The livestock industry provides the human diet with meat, milk and eggs as food sources, supplemented with by products such as wool, leather, pharmaceuticals and medicines. The greatest increase in animal products is pig and poultry meat, followed by eggs and milk (Speedy, 2003). Worldwide trade in meat output is forecast to reach 346 million tonnes in 2021, largely being driven by Chinese production, with also notable expansions in Brazil and Vietnam (FAO, 2021). Monogastric production (pig and poultry) is predicted to grow at a faster rate compared to ruminant (meat) (Herrero and Thornton, 2013).

Global livestock production is not uniform (Rust, 2019), it is characterised by a divide between developed and developing countries (Thornton, 2010). Systems in developed countries tend to be more intensive (Rust, 2019), whereas developing countries utilise extensive grassland systems, with more small holder type production systems (Michalk et al., 2019). Extensive systems are used in developed countries, for example sheep and cattle hill farming in the UK, Lake District. This farming practice has been passed down through generations and uses land for livestock production that cannot be used for anything else. There are also a growing number of extensive type systems being utilised in developed countries, replacing intensive farming to provide a more environmentally stable style of livestock production. The extent of the intensification is driven by factors including economic development, resource availability, population dynamics and rate of urbanisation (Rust, 2019).

Currently there is an overexploitation by livestock production systems of grasslands, forage sources and land conversion (Michalk et al., 2019). Livestock play an important role in providing protein to the human diet, with ruminants converting grass into protein (Rust,

2019) but it is putting excess pressure on land and water. With an increased demand for animal products livestock systems need to become more sustainable and efficient. Previous methods used to improve production yields are use of nutrition, genetics, health and lifecycle assessments of the production system. Further intensification (higher yields per hectare) or extensification (use more hectares) (Michalk et al., 2019) will continue to overexploit the planets resources.

1.2.3 Environmental impact of livestock production

Currently, parts of food production puts unnecessary pressure on the worlds land and water resources (Willett et al., 2019). As a part of food production livestock production contributes to this significantly and increased production of animal products is expected to intensify this pressure (Henchion et al., 2017). It is important to highlight that livestock production is often negatively associated with greenhouse gas production (e.g. carbon dioxide, methane and nitrous oxide) and additionally is directly involved in land use changes, such as deforestation, that release additional carbon dioxide (Willett et al., 2019). There is still room for further improvement with the production of animal products producing more greenhouse gas emissions compared to plant products (Tilman and Clark, 2014). Eggs, poultry and pig meat have lower greenhouse gas emissions per gram of protein compared to ruminant meat (Tilman and Clark, 2014). Beef production alone contributes 41% of livestock emissions (Gerber et al., 2013). Additionally ruminants have a larger land use compared to monogastrics due to the need for grazing (Swain et al., 2018), but often monogastrics are fed a diet which could be used for human food or are grown on land that could be used for human food production.

Livestock feed requires high amounts of grassland use and land-based crop production. Current over use of these systems is contributing to reduced productivity, loss of biodiversity, increased soil erosion, reduced water yield and quality, and high greenhouse gas emissions (Michalk et al., 2019). There are improvement schemes to reduce this impact, for example leaving strips of fields and not cultivating them to encourage an increase in biodiversity and local wildlife. Agricultural systems are highly sensitive to climate, and therefore climate change is adding more uncertainty to future production (Fanzo et al., 2018).

There are differences in the environmental impacts of livestock within the different systems. Intensive systems are often highlighted as negative environmentally due to high inputs but actually, a combination of optimised nutrition, advanced animal husbandry, controlled production environment and advanced breeding techniques, can create environmental savings when compared to a traditional extensive system (Swain et al., 2018). Alternatively, if there was an increase in the number of smallholder production systems which are supplying the surrounding area utilising optimal livestock numbers, nutrition and husbandry this could reduce environmental transportation costs (Michalk et al., 2019).

It is highly important to livestock production systems that issues regarding climate change and global warming are addressed. Global warming resulting in an increased temperature will put strain on all aspects of production from impairing production outputs (growth, meat quality, milk yield, egg weight and quality), reproductive capabilities, metabolic and health status (Nardone et al., 2010). Climate smart agriculture is one potential avenue to address future problems associated with production systems (Fanzo et al., 2018).

1.2.4 Livestock Feed and use of technology

Livestock feed can be in many forms including industrial, formula, blended, or compound (FAO and IFIF, 2010). All types of feed utilise plant ingredients, which can then be mixed with synthetic amino acids, mineral, and vitamin premixes and oils. This mixing can occur in either an industrial feed mill or a simple on-farm mixer (FAO and IFIF, 2010). Processing in an industrial feed mill results in further processing into crumb, nut or pellet depending on the form needed by the species and life stage of the production animal. Livestock are fed to requirement for maintenance and production, with specific feeds for different life stages (McDonald et al., 2011).

Compound feed is supplied to all livestock species, but as of 2016, 45% of this total amount of compound feed is being supplied to global poultry market (IFF, 2016). This increased demand for compound feed is anticipated to intensify the burden on land-based crop production (Henchion et al., 2017). Monogastric feeds (poultry and pigs) represent a higher market share compared to ruminant feed, and require a large quantity of high quality protein in the diet (Sánchez-Muros *et al.,* 2014). Additionally, this protein needs to be highly digestible and of a suitable amino acid profile, so that the main essential amino acids are not limiting. Poultry feed contains two basic elements, cereal source and a protein source, these are normally wheat and soybean. When formulating a poultry diet, specifically a broiler diet, the main emphasis is aimed towards limiting amino acids (Beski *et al.,* 2015). Plants are the main supplier of protein in the diet currently, normally fortified with synthetic amino acids to reduce deficiencies in amino acids (Beski *et al.,* 2015).

Additionally, monogastric feeds utilise technologies to improve digestion. Feed enzymes have contributed greatly to meet increased consumer demand for affordable food, by

allowing for increased flexibility in which raw materials are used for monogastric feeds and additionally reducing production of animal waste which reduced negative environmental impact (Bedford and Partridge, 2010).

For example, the use of exogenous enzymes to remove negative effects of anti-nutritional factors, improve availability of nutrients bound by cell wall structures and improve nutrient utilisation in the small intestine, leading to better fermentation further down the tract (Bedford, 2000; Bedford and Partridge, 2010). Enzymes are categorised by the substrate that they act on, for example fibre, proteins, starch and phytate (Asmare, 2014). The most commonly used enzymes are phytase, to break down phytate and improve phosphorus bioavailability and xylanase to break down non-starch polysaccharides (Bedford and Partridge, 2010). Other fibre degrading enzymes include β-glucanases, β-mannanase and α-galactosidase however these are used to a lesser extent. Proteases are used to break down storage proteins in plant materials and also reduce the levels of trypsin inhibitors and lectins (Asmare, 2014). Amylase is used to breakdown starch and especially in young animals can be used to support an immature digestive system (Bedford and Partridge, 2010).

The use of enzymes is driven by reducing feed costs and thereby the value of adding enzymes is dependent on the cost of the enzyme versus the feed ingredient. As each enzyme targets a different anti-nutrient there is an opportunity within the animal feed industry to combine different types of enzymes to further improve production and reduce costs (Bedford and Partridge, 2010).

As enzymes are ultimately proteins which rely on a three dimensional structure in order to work correctly any feed processing techniques can disrupt the enzyme activity (Bedford and Partridge, 2010). For example, heat processing could disrupt or break the three dimensional

structure leading to the active site being changed and therefore will not be active within the feed. Additionally, the enzyme efficacy will be dependent on the feed ingredients nutritional value and structure, therefore the interactions between all these factors need to be understood otherwise there could be depressed animal performance or wastage (Asmare, 2014).

1.2.5 Current protein sources

Current protein ingredients used in livestock feed come from different plant sources including forages, cereals and protein concentrates. Forages are used in ruminant feeds and are variable depending on type and processing. Cereals are often the starting point for feed formulations for monogastrics, however limitations in protein and specific amino acid supply result in the need for a protein concentrate to be added. Protein concentrates include oilseed cakes and meals, leguminous seeds, synthetic amino acids and animal proteins. Currently Europe is importing 70% of its animal feed protein (PROteINSECT, 2016).

Oil seed cakes and meals are the most commonly used with 60% of EU animal feed protein imports being produced from by-products of vegetable oil production (PROteINSECT, 2016). Typical oil seed meals include soya, rape, palm kernel, and sunflower meal. Of all these soybean meal is the best source of high quality protein (with a good profile of bioavailable essential amino acids), whereas palm kernel has a relatively poor protein and amino acid content (McDonald et al., 2011). Rapemeal has a lower protein content and digestibility, while, sunflower meal is low in lysine but has twice as much methionine as soybean meal (McDonald et al., 2011).

Soybean is the most widely used plant protein source in poultry feed (Beski *et al.,* 2015), although it is generally perceived as unsustainable as a feed source. Soybean has high levels of digestible protein of both a good quality, quantity and a good amino acid profile (Sánchez-Muros *et al.,* 2014). It is a major source of lysine which is the first limiting amino acid in pig feed and second in poultry feed (de Visser et al., 2014). These qualities make it a good source of protein in animal feed. However, soybean meal negatively contributes to the environmental effect of intensive livestock production (Sánchez-Muros *et al.,* 2014). Soya beans and soybean meal are largely imported into Europe (de Visser et al., 2014) from the United States of America and South America. In South America, in particular, there are large areas of deforestation to enable the planting and growth of soya crop to keep up with global demand.

Leguminous seeds include beans, peas, and lupins. Beans have both a high protein quality and metabolisable energy content, they can be successfully grown in the UK yielding both winter and spring varieties, however the protein content varies between these varieties (McDonald et al., 2011). Peas and lupins can also be grown in the UK, compared to beans, peas have a better amino acid content with higher lysine contents and lupins have the poorest amino acid quality of all three and it is not used as widely as peas and beans (McDonald et al., 2011). At the present time, the wide availability of soybean with its superior protein quality, means these leguminous seeds are not used as widely as they could be.

Synthetic amino acids are also widely used to meet the essential amino acid requirements of monogastrics, and they are produced industrially by either chemical or microbial processes. Without the use of synthetic amino acids, high volumes of plant protein sources would have

to be used to meet requirement for essential amino acids, resulting in an oversupply of total protein and many amino acids that would be wasted and excreted as urea (McDonald et al., 2011). This is both economically wasteful and represents a potential source of pollution. Animal sources of protein include meat, bone meal, and fishmeal. Mammalian meat and bone meal cannot be given to livestock in the EU due to dangers associated with prions and bovine spongiform encephalopathy (Veldkamp et al., 2012). Fishmeal is a very high-quality protein source in terms of amino acids, with rich levels of essential amino acids and a good source of polyunsaturated fatty acids (PUFA), yet, it is used more commonly in aquaculture than traditional livestock production (Green and Pearsall, 2016).

1.2.6 Alternative protein sources

Issues surrounding the sustainability of current protein sources for animal feeds highlights the need for alternative protein sources to either partially or fully replace traditional protein sources. Examples include alternative plants, algae, bacteria and insects.

As already discussed, alternative plant protein sources include pulses and legumes are already currently used in limited amounts in livestock feed. This partial use is dependent on availability and cost but there is potential to expand their use. These plant sources have the ability to fix nitrogen (Henchion et al., 2017). Pulses contain 20-25% crude protein and 1-1.5% crude fat (Henchion et al., 2017). However these plant sources of protein can show a wide variation in protein content due to genetic and environmental factors (Henchion et al., 2017). Additionally they are often lacking in essential methionine and cysteine and contain anti-nutritional factors (Henchion et al., 2017). In contrast, white clover has been found to be highly digestibility when processed and of a suitable balanced amino acid composition for inclusion in monogastric feeds (Stødkilde et al., 2018).

Algae, including seaweed, is also being considered as an alternative protein source (Henchion et al., 2017). Algae have a composition similar to vegetables and have a high eicosapentaenoic acid and docosahexaenoic acid contents; but also contain anti-nutritional factors that could cause some problems (Henchion et al., 2017). Whole seaweed has a good protein content, however it is lacking in essential amino acids to meet requirements for monogastric production (Angell et al., 2016). There is potential for extraction of protein from seaweed to be more successful as an alternative protein source (Angell et al., 2016).

Bacterial protein could be used as an alternative protein source because it can be grown to specification, rapidly and without putting pressure on land and water resources (Overland et al., 2010). It has the potential to yield more protein per unit land area, whilst using one-tenth of the water used for soybean production (Sillman et al., 2019). Broiler chicks have been fed on a feed containing increasing bacterial protein meal with no detriments to protein or energy metabolism and carcass characteristics (Hellwing et al., 2006). However, production of these sources is still relatively a new idea so further work is needed to produce a viable product (Overland et al., 2010) of high safety and low environmental impact (Sillman et al., 2019).

Insects are the final example of an alternative protein source and the main subject of this thesis. The nutritional value of insects varies depending on the species, their diet and stage of development. In general, they are a good source of energy, high quality protein and PUFA (Kourimska and Adámková, 2016), as well as a range of readily available vitamins and minerals (Williams et al., 2016). Specific insects that are being investigated for their potential use in animal feeds, include mealworms, black solider fly larvae and housefly pupae (Makkar et al., 2014). Insects can be raised on bio-waste, so turning waste products

into a good protein source (Makkar et al., 2014). Chickens and pigs will naturally eat insects when rooting around, however this is not currently being utilised in commercial production systems. Insect farming is still in its infancy compared to traditional feed sources but it requires less resources such as land, water and feed to create a relatively dense protein source (i.e. insects), with low greenhouse gas emissions (Gahukar, 2016).

1.2.7 Insects

Insects are enhanced through physiological adaptions to be the most diverse class of the animals. As invertebrates, insect body physiology is based on a segmental plan, the cuticle forms the exoskeleton and is continuous across the whole body (Chapman, 2013), creating the major difference to traditional livestock species (vertebrates). Insect development and ultimately growth is divided into a series of molts, known as instars (Reynolds, 2013). *Tenebrio molitor* (yellow mealworm) is the species used in this PhD thesis, so therefore is going to be introduced in more detail and from here onwards referred to as mealworm. The mealworm belongs to the Coleoptera order and Tenebrionidae family. The common name for the adult is the Darkling beetle. It is holometabolous insect so undergoes an egg, larvae, pupa then beetle transformation (Park et al., 2014) (Figure 1.1).

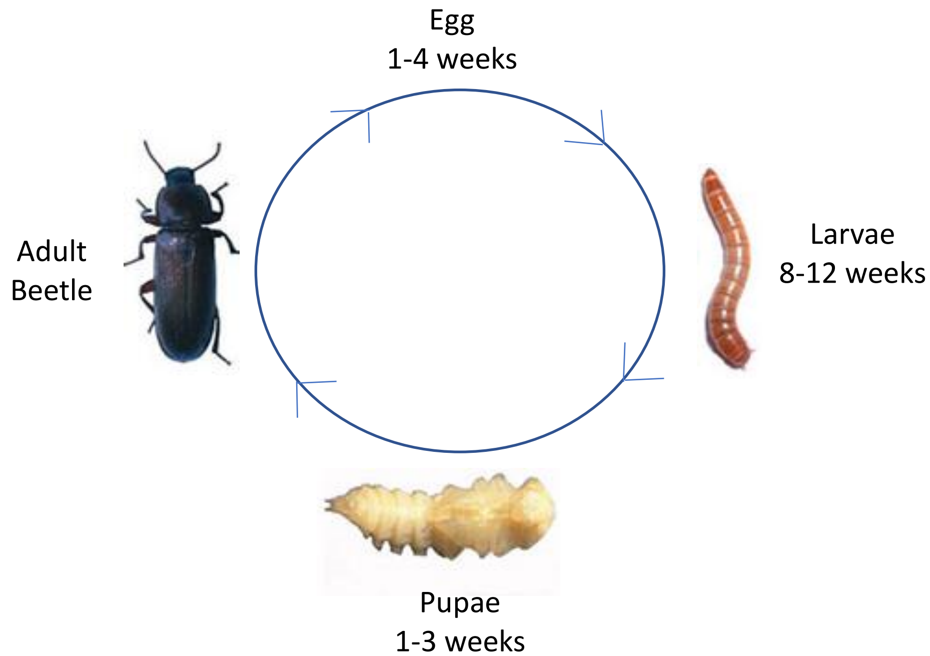


Figure 1.1 *Schematic of mealworm lifecycle*

The lifecycle of mealworms is variable in length between 280 and 630 days (Tran et al., 2019). The beetle lays eggs between 4 and 12 days after copulation. These hatch after 4 to 12 days of embryonic development and remain in the larval stage for 3 months (Park et al., 2014). During the larval stage the larvae undergo moults (8-20), getting bigger each time (Tran et al., 2019). The larvae continue to grow until the larvae turn into a pupa, remaining in this stage for 1 to 14 days then metamorphosise into the adult beetle (Park et al., 2014; Tran et al., 2019). The pupal stage is variable dependant on the temperature, and at a lower temperatures can last up to 20 days (Tran et al., 2019). Initially the beetle is white, but as the exoskeleton of the beetle develops and hardens the beetle changes colour through brown to black. All stages can be in the same ecosystem.

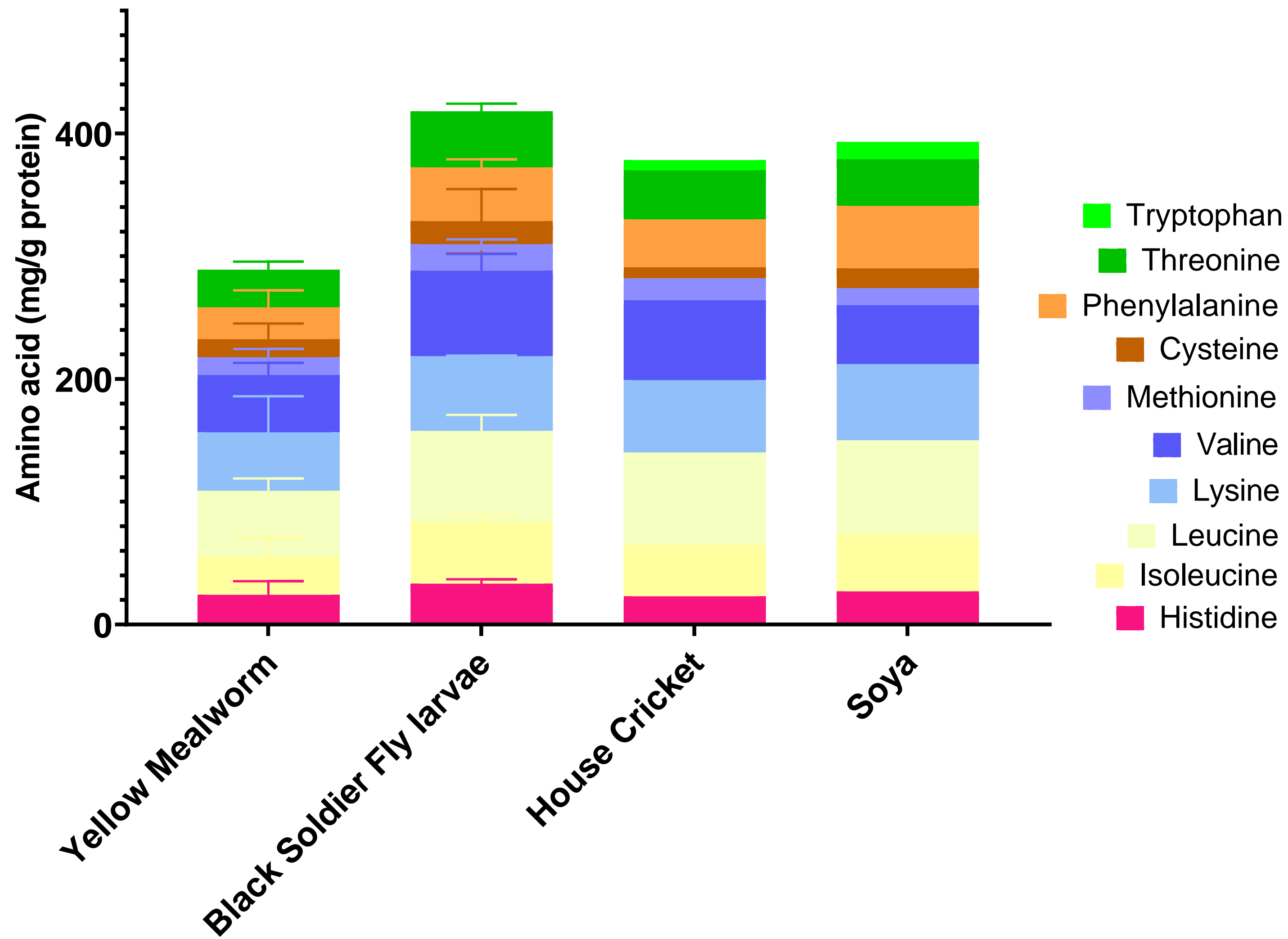
Table 1.1 reports the nutritional composition of mealworms compared to two other commonly used insects in food and feed (Black soldier fly and House cricket) and soybean meal, as a traditional source of protein in animal feed. Protein is the main component of insects followed by fat. There is more fat in mealworms compared to soybean meal, although fat is processed out of soybean meal to produce a high protein feed ingredient.

Table 1.1 *Nutritional composition (%DM) and energy content (MJ/kg) of commonly used insects in food and feed on a DM basis, parts taken from Hawkey et al 2021.*

Order	**Common Name**	**Latin Name**	**Crude Protein (g/kg DM)**	**Total Fat (g/kg DM)**	**Fibre (g/kg DM)**	**Ash (g/kg DM)**	**Energy (MJ/kg DM)**	**Sources**
Coleoptera	Mealworm	*Tenebrio molitor*	460-540	250-360	20-50	30-40	27	(De Marco et al., 2015; Jones et al., 1972; Oonincx et al., 2015; Ravzanaadii et al., 2012; Zielinska et al., 2015)
Diptera	Black soldier fly	*Hermetia illucens* (larvae meal)	340-420	250-580	70	40-200	22-24	(De Marco et al., 2015; Ewald et al., 2020; Makkar et al., 2014; Oonincx et al., 2015)
Orthoptera	House cricket	*Acheta domesticus*	590-720	100-230	50	50	ND	(Barker et al., 1998; Oonincx et al., 2015; Udomsil et al., 2019)
Hipro Soybean meal (for comparison)			55.2	1.7	4.4	7.3	20	(Heuze et al., 2020)

DM = dry matter, ND = no data

Figure 1.2 *Amino acid profile (mg/g protein) of commonly used insects in food and feed on a DM basis, parts taken from Hawkey et al 2021.*



The primary interest for the use of mealworms in livestock feed is the high protein content. The protein is of a good quality, in terms of essential amino acids, with similar levels of methionine and cysteine compared to soya (De Marco *et al*., 2015a; Heuze, Tran and Kaushik, 2020). The essential amino acid profile is displayed in Figure 1.2. There is limited data regarding the digestibility of mealworm in the gut, however using *in vitro* digestion techniques the crude protein digestibility of mealworms appears to be negatively correlated with their chitin content (Marono et al., 2016).

A negative consideration of the protein content of mealworms is that a significant proportion of the protein may not be bioavailable as it is chemically bound within the exoskeleton (Barker et al., 1998). Additionally, this may lead to an overestimation of the total protein content when based on nitrogen content, as the nitrogen bound in the exoskeleton is in the form of chitin. It has been suggested that, rather than using the traditional nitrogen to protein conversion factor of 6.25 (Jonas-levi and Martinez, 2017), a value of 5.60 may be more suitable for insects (Janssen *et al*., 2017).

The second main component of mealworms is fat, where triacylglycerol and phospholipid account for 80% and 20% of the total respectively (Kourimska and Adámková, 2016). As the fat content of whole insects (10-30%, Table 1.1) is increased compared to the 1.7% in soybean meal (Heuze et al., 2020), further processing of mealworms may be required to remove some of the additional fat (Makkar et al., 2014), similar to soya bean processing. This removed fat could then be used as a supplementary feed ingredient, similar to soya oil (Makkar et al., 2014).

Mealworms contain a range of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). The main fatty acid is the MUFA, oleic acid (C18:1) at

around 44%, followed by the PUFA, linoleic acid (n-6 C18:2) at 24% and then the SFA, palmitic acid (C16:0) SFA at 18% (Jones, Cooper and Harding, 1972; Oonincx *et al.*, 2015; Zielinska *et al*., 2015; Alves *et al*., 2016). In general, insects are relatively rich in the n-6 PUFA, linoleic acid (n-6 C18:2) with smaller amounts of n-3 alpha-linolenic acid (C18:3).

Mealworms contain sufficient levels of micronutrients and minerals to meet dietary requirements of most animals (Barker et al., 1998). In general insects can provide significant amounts of calcium, magnesium, manganese, phosphorus and selenium (Rumpold and Schlüter, 2013), with noticeably high iron and zinc levels (van Huis *et al*., 2013). Mealworms have a higher potassium and sodium content compared to black soldier fly larvae (Makkar et al., 2014; Zielinska et al., 2015) and contain high levels of vitamin B12 compared to other species (van Huis et al., 2013). Insects also contain good levels of the B vitamins, riboflavin (B2), pantothenic acid (B5) and biotin, but are lacking in vitamin A, vitamin C, niacin (B3), thiamine (B1) (Rumpold and Schlüter, 2013).

Insects are comparable to traditional livestock in their nutritional composition, but their physiology has a larger impact on the nutrient content, mainly the chitin fibre content (Williams *et al*., 2016). Chitin is a nitrogen-based carbohydrate found in the exoskeleton of most insects as a long-chain polymer of N-acetyl glucosamine and it is undecided whether chitin is positive or negative component of insects in terms of use in feed. Some evidence suggests valuable anti-cancer, antioxidant, and anti-inflammatory properties of chitin (Belluco et al., 2013). However, since chitin can bind various macromolecules it may also have anti-nutritional effects, potentially reducing digestibility (Hawkey et al., 2021).

Mealworm composition varies widely in the literature (van Huis *et al*., 2013) as content is affected by seasonality and feed provided (Kourimska and Adámková, 2016). Fat content, as total amount and fatty acid composition are the most variable components of insect composition. For example feeding increased PUFA content feeds increases the mealworm PUFA content (Fasel et al., 2017). Furthermore feeding 100% algae to black soldier fly reduced the fat content to 81 g/kg DM compared to 220 g/kg DM when fed 50% algae 50% processed wheat feed (Liland et al 2017). Housefly larvae produced on brewers grains had a lower total protein content and higher total fat content compared to larvae produced on poultry waste (Obeng et al. 2015). There is little evidence that rearing the same species on different substrates results in variation in the amino acid composition (Fitches et al., 2019).

Furthermore the developmental life stage of the insect contributes to its nutritional composition. There is a higher fat content in the larval stages of holometabolous insects compared to the adult form, and as the larvae develop towards the pupation there is an increase in fat content which acts as the main energy source for metamorphosis (Finkel, 1948). Black soldier fly larvae at 5 days old had a fat content of 97 g/kg DM compared to prepupae stage at 260 g/kg DM (Ewald et al., 2020). The nutritional changes of mealworms across the larval stages have not been reported to our current knowledge.

1.2.8 Use in animal feed

There are more published studies on the use of mealworms in monogastric feed compared to ruminant feed. There appear to be no studies on the inclusion of mealworms in ruminant diets, potentially due to their rapid digestion in the rumen and very little useful by-pass protein and associated concerns with the addition of processed animal protein to feeds.

The main studies on the use of mealworms in animal feed are in poultry. A complete replacement of soya by mealworms showed no growth effects but an improvement in feed conversion ratio (FCR) (Bovera *et al*., 2015). Whereas complete replacement of gluten meal by mealworms showed no changes in growth, feed intake and FCR (Biasato et al., 2016). Partially replacing soya with mealworms had no effects on growth, feed intake and FCR (Ramos-Elorduy et al., 2002); while inclusion of mealworms at 2% improved overall body weight gain, but there was a decrease in FCR with increasing amounts of mealworms (Ballitoc and Sun, 2013). Furthermore Sedgh-Gooya et al., (2021) reported that inclusion of mealworm larval meal up to 5% increased body weight gain for 0 to 10 days, but no effects on feed intake or FCR. Broilers which were fed mealworms 'on top' of the complete diet at 2g/kg feed has increased body weight gain and feed intake, but had no effects on FCR (Benzertiha et al., 2020). Increasing the inclusion of mealworms from 0 to 8% increased broiler body weight and bodyweight gain for 0 to 21 days but there were no effects for 0-42 days (Elahi et al., 2020). Biasato et al. (2017) reported that increasing inclusion of mealworms in male broilers improved body weight and feed intake but had a negative impact on intestinal morphology and feed efficiency.

The complete replacement of soybean meal with mealworms in broilers resulted in a lower apparent ileal digestibility coefficient of crude protein compared to the control (Bovera et al., 2016). Also it has been highlighted that mealworm fed broilers had lower albumin to globulin ratios implying that the mealworms had the potential to improve the immune response (Bovera et al., 2015). In terms of the effect on the caecal microbiome Biasato et al., 2018 and 2019a have both reported that mealworms can manipulate the Firmicutes and Bacteriodete abundances, however these studies had contrasting results.

There are fewer studies in pigs. There was an increase in body weight gain, feed intake and crude protein digestibility with partial replacement of soya with mealworms (Jin *et al*., 2016). However, it has also been reported that inclusion of mealworms into weaning pigs feed up to 10% showed no differences in body weight or feed intake (Meyer et al., 2020). Using defatted mealworms to replace fishmeal resulted in no differences in weaning pigs' growth performance or gut histology (Ko et al., 2020). Inclusion of mealworm at 9.95% in pig feeds improved apparent ileal digestibility and standard ileal digestibility of nutrients and essential amino acids (Yoo et al., 2019).

These studies show some confounding results but nearly all studies had different levels of mealworm supplementation and replaced different feed ingredients therefore direct comparisons cannot be made between the studies. For example, gluten meal has a different composition and digestibility to another feed ingredient such as soybean meal. Therefore, if mealworms are used to replace these feed ingredients, then there will be a different response by the broiler as the composition in terms of both proximate and amino acid compositions will be different, alongside the ability to digest these feed materials. Additionally, mealworms have been included in the feeds at different stages of broiler

production so therefore results are not directly comparable. For example, Sedgh-Gooya et al., (2021) fed mealworms within the starter (1-10 days) and grower (11-25 days) but not in the finisher (26-42 days) whereas Bovera et al., (2015) fed mealworms within the finisher stage (30-62 days).

It is clear that there is some value to mealworms as a potential source of protein to monogastric production animals as there are no reported negative effects on growth, however whether it is cost effective and environmentally acceptable in terms of feed intakes and feed conversion ratio remain unclear. Furthermore, studies report effects on growth and feed intake or apparent digestibility or the caecal microbiome, however there are not many studies that look at the effects of mealworm inclusion fully down the gastrointestinal tract in multiple measurement methods.

1.2.9 Mass producing insects for animal feed

The production of insects as animal feed is still at an early stage and a number of factors need to be considered, as discussed below.

As the dry matter content of mealworms is only 40% there needs to be significant scale up of production to be able to produce the amount needed to include in animal feed. This needs to be achieved in a way that is safe and cost effective. Mealworms have several positive factors for successful production including a short development cycle, high ovipositional rate and biomass productivity (van Huis *et al.*, 2013). However, considerations need to be made for the developmental cycle, since they are a holometabolous insect, meaning the timings of metamorphosis (larvae to pupae) need to be carefully controlled, as the larval stage will be the one that is harvested for mass production. Systems need to be

put in place to manage both the rearing and mass production of mealworms, with special considerations for temperature, humidity and feed and water supply.

As ectotherms, the body temperature of mealworms is reflective of their environment and metabolic heat production is insufficient to maintain homeostasis (Chapman, 2013). Insects tend to reproduce and grow bigger in tropical regions (Ortiz *et al*., 2016), which leads to the consideration that mealworms have to be kept at warm temperatures. It has been noted that the beetle form of mealworms can be produced at 23.3°C, but an increase in temperature is damaging to growth and composition (Bjørge et al., 2018).

Humidity is another factor that must be considered in mass production, which ties into water supply for the mealworms. Mealworms have lower water requirements than traditional livestock (Ortiz *et al*., 2016), but are sensitive to losing water through evaporation (Chapman, 2013). There is the potential to reduce the humidity of the system if methods of water supply are carefully controlled, thereby improving the sustainability of the system.

The nutritional requirements of mealworms are not yet fully elucidated, but most insects have similar chemical and metabolic capabilities (Chapman, 1995). Mealworms are the larval stage of the darkling beetle, which is a natural pest in flourmills, so the ideal feed source is likely to be a cereal or cereal by-product. However, this would be using a human-edible food source so flexibility in other sources of alternative or waste feeds would improve the environmental impact of mealworms. Mealworms can be raised on bio-waste (Makkar *et al*., 2014) and plant material not suitable for human consumption. However there needs to be careful consideration into the components which make up the mealworms feed due to concerns of accumulation toxins and pathogens which could be

within the feeds. There is the potential for anything harmful to accumulate within the mealworms, which is then transmitted to broilers and potentially the human food chain. For example there could be a chemical risk of mealworms from pesticides, and other chemicals from the feed source, specifically organic contaminants including DDT (dichlorodiphenyltrichloroethane) and PCB (polychlorinated biphenyl) (Hawkey et al. 2021). Furthermore heavy metals could accumulate in mealworms, for example black soldier flies grown on heavy metal supplemented feed accumulated cadmium (Diener et al. 2015).

As a new feed component, there are safety concerns concerning using mealworms in animal feeds. The biosecurity of using mealworms is unknown with both exogenous and endogenous risk factors contributing to this (Rumpold and Schlüter, 2013). There are four main categorised risks; microbial, parasitic, allergenic and chemical (Belluco *et al*., 2013). It has been suggested that there is low risk of transmission of microbial and parasitic infection (van Huis et al., 2013). The main concern is that mealworms are included in feed without removal of the gut, therefore any microbial load is included in the feed (Grau et al., 2017). There has been little reported work on the allergic responses in livestock species, there is more concern that humans directly handling the mealworms on a frequent basis could result in an allergy developing (Jensen-Jarolim et al., 2015).

Another safety concern is that currently the use of animal proteins in pig and poultry diets is prohibited in the EU (Veldkamp *et al*., 2012), due to transmissible spongiform encephalopathies, specifically bovine. However, due to a primitive nervous system, mealworms are considered incapable of expressing prions (van Raamsdonk et al., 2017). Under new legislation published by the European Union in 2015 and 2017 (EU Commission, 2017, 2015) insects are included as a 'novel' food, so it is becoming more accepted.

As with any form of production related to food or feed the environmental costs and benefits are becoming increasingly important. A life cycle assessment (LCA) for mealworm production concluded that mealworms had a lower greenhouse gas emissions compared to conventional animal protein sources, such as milk, chicken or pork due to similar requirement of energy but a lower land use (Oonincx and de Boer, 2012). However, this life cycle assessment is not useful when comparing the production of soybean meal and mealworms as protein source, but it would be expected that mealworms would have a lower land use than soybean meal. Though, Thevenot et al., (2018) assessed the environmental performance of mealworms using four parameters of feed intake, electricity consumption at the farm stage, electricity consumption at the processing stage and meal and oil yield at the processing stage. They reported that per kg of protein produced mealworm impact, in terms of energy demand, climate change, land use and eutrophication potential, was higher than soybean meal or fishmeal. However, the study did note the coefficients of variation were high and that there are several issues with the study which requires further LCA to be analysed in this area.

Utilising highly controlled conditions by the use of an incubator or a temperature-humidity controlled room may result in better growth, however does the energy required to maintain these conditions outweigh the benefits of better growth. Mealworms themselves have the positive environmental impact of being a potential replacement for soybean meal, but there is a question of whether production of mealworms is more sustainable than soybean meal.

1.3 Thesis Hypothesis and Objectives

The use of insects in food and feed is currently a ‘hot topic’ that is getting lots of media attention. With this has come a rapid increase in the number of published studies relating to the use of insects in both feed and food and the many different factors surrounding this. Even with the increase in the numbers of papers, the wide range of study designs and focuses mean they are hard to compare in any detail.

In the literature, there are many gaps in our knowledge on insect production (e.g. optimal time for harvest in terms of the best larval stage for the optimal nutritional composition, growing conditions and how controlled they need to be and nutritional requirements).

Pigs and poultry cannot digest 15-20% of feed eaten due to antinutritional factors and the use of enzymes improves this greatly (Bedford and Partridge, 2010). It is unknown if mealworms follow the same pattern in terms of feed that is not digested or utilised, however commercial enzymes could be a way to improve mealworm production further. To our knowledge there are no published papers reporting on the use of already available commercial enzymes within mealworm feed.

Mealworms have been included in many poultry studies as replacement for various feed materials with no apparent negative effects on growth, but confounding results regarding feed intake and feed conversion ratio. There is a lack of papers which explore the use of mealworms as a broiler feed on both the caecal microbiome and apparent ileal digestibility. Furthermore, there has been no work looking at utilising a chitinase enzyme to manipulate and potentially improve mealworms as a feedstuff for inclusion into broiler feeds.

The work in this thesis aims to fill some of the gaps in literature regarding mealworm production for use in the commercial animal feed industry, addressing the research question that are mealworms suitable as a partial replacement for soybean meal for use in broiler feeds and can they be manipulated to improve inclusion?

The two hypotheses to answer this question are:

- Mealworm growth and composition can be manipulated by production conditions, feed and commercially available enzymes.
- Mealworms will be suitable as a feed ingredient for sustainable broiler feeds and their value is enhanced by the inclusion of chitinase in the feed.

These two researchable hypotheses will be addressed by the following objectives:

1. To determine the effects of different environments, starvation and feed and water supply methods to mealworms (Chapter 3).
2. To investigate the effects of commercially available exogenous enzymes on mealworm growth and nutritional composition (Chapter 4).
3. To assess the partial replacement of soybean meal with mealworms in broiler diets and how this affects digestibility and the caecal microbiome (Chapter 5).
4. To evaluate chitinase both *in vitro* and *in vivo* to improve mealworms as a partial replacement of soybean meal (Chapter 6).

The four experimental chapters that follow explore (i) conditions for producing insects, (ii) the use of exogenous enzymes to manipulate nutritional composition of mealworms, (iii) the use of mealworms to partially replace soya in broiler feeds and (iv) the use of exogenous enzymes to improve the digestibility of mealworms in broilers.

1.4 Work conducted in this thesis

The work carried in this thesis was impacted by some major events. Firstly, at the end of the first year, a laboratory move resulted in 2 months of inactivity in the laboratory work. This also slowed down active insect work due to lack of facilities and a suitable growth room. A humidity-controlled incubator was bought to help with these issues, however with additional problems delivery was delayed for 6 months followed by an additional 3 months when it was not operational. The second event which impacted the work in this thesis was the COVID-19 global pandemic. The first national lockdown in March 2020 coincided with the midpoint of the second broiler trial, through additional risk assessments and health and safety procedures we were able to complete the trial at its prior agreed end date, however there was a reduced number of samples taken from the birds on the cull day. After this broiler trial finished the laboratory was shut until late July 2020, with access being gradually reintroduced during August, although there were additional issues in supply of mealworms and general laboratory consumables. The second national lockdown in November 2020 did not negatively affect the continuation of laboratory work, but the supply of consumables for molecular biology work was affected.

At the end of March 2021 the lab facilities were moved back into the newly refurbished building, while most of the lab work had been completed by this point, it did delay the remaining fat and amino acid analysis by several months. As the labs were about to reopen a flood in the new facilities delayed this work by a few weeks.

2 Materials and Methods

2.1 Materials

Tenebrio molitor (mealworm) larvae were purchased from Monkfield Nutrition Ltd, Mepal, Ely, UK for all live insect husbandry trials. These were originally acquired from Europe by the supplier and maintained on *ad libitum* feed of wheat bran and carrot.

The mealworms were purchased in the mini form, 15-18mm in length, which according to Monkfield nutrition, is less than 6 weeks old. Previous work in the Division by Ellen Tetlow reported that utilising mealworms in the mini form were more suitable for larval growth trials than regular mealworms (18-26mm, >6 weeks old) (Tetlow, 2019).

Throughout all experimental work the mealworms were kept in dark conditions. Previous work within the Division indicated that mealworms grew better in the dark compared to the light (Tetlow, 2019). On arrival, mealworms were originally maintained on wheat bran and carrot in a plastic box. After the trials in chapter 3, this changed to a feed of wheat bran and water-soaked cotton wool. Following a laboratory move, mealworms were kept in a different lab for a period of approximately five months, then kept in a temperature and humidity-controlled incubator (Trials 3, 4, 5 and 8). The specific conditions in which the mealworms were kept is defined in each chapter.

Table 2.1 displays all equipment used in mealworm growth and feeding trials including equipment used for protection, housing and handling during the trials.

Table 2.1 *Mealworm handling and feed trial equipment*

Equipment	Product Specifics	Company
Protective face mask	3M Aura Disposable Respirators 9320+ FFP2[1] (APF[2])	3M, Bracknell, RG12 8HT
Specialised fume hood	Aria CS48 Changing Station	Tecniplast, London
Incubator	SGC120IN4 Humidity and temperature controlled incubator	Weiss Technik, Loughborough LE11 3GE
Sieves	2mm slots, d: 200mm, h:50m	Retsch, Hope Valley S33 6RB
Analytical scale	4 decimal places A1205	Sartorius, Epsom KT19 9QQ
ImageJ	Computerised software for counting	NHI, University of Wisconsin
Mill	Cutting mill (1999), 0.5-3.0mm inserts	Fritsch, Hessle HU13 9PB
Platform scales	Max: 60kg, precision 20g	Kern IXS, SciQuip Wem, SY4 5NU
MW dishes	500ml plastic food takeaway dishes	Amazon UK

[1] Filtering face piece, level 2
[2] Assigned protective level

Wheat bran was obtained from Burnhills (Burnhills BRAN (Wheat Bran) – Horse Feed 20kg, code 2452. Burnhills Services Ltd. Cleckheaton) (Chapter 3) and Buy Whole Foods Online (Wheat bran. Minster, Ramsgate, North East Kent) (Chapter 4 and 5). Carrot was sourced from a local shop (Sutton Bonington Campus Costcutter) and cotton wool from Simply Organic (Organic Cotton balls, Macdonald & Taylor Healthcare Ltd, Warrington, Cheshire).

2.2 Feeding

The main feed source used in the mealworm trials was wheat bran. Wheat bran was processed before use; initially wheat bran was put through a Fritsch cutting mill to produce a set 3mm sized wheat bran. However, due to issues with measuring feed intake, the method for standardising wheat bran was changed. From the end of Chapter 3 onwards (Studies 3, 4, 5, 6, 7 and 8) the method for standardising wheat bran size was to pass wheat bran through two stacked sieves, the top sieve with holes of 2mm and the bottom sieve with holes of 0.5mm. The wheat bran retained in the middle of the two sieves was then used for feeding trials. Any wheat bran retained in the top sieve or passed through both sieves was retained for mealworm stock not being used for feeding trials. Wheat bran was weighed out for each individual trial, specific feed amounts and treatments are described in each chapter. For feeding, wheat bran was placed into the individual dishes.

Initially water was supplied to the mealworms through weighed sliced carrot. Following the second experiment in Chapter 3, the water supply was changed to utilise cotton wool soaked with water. A 10ml Gilson pipette was used to pipette a set volume of water onto the cotton wool. The wet cotton wool ball was then placed in a corner of the dish and not on the wheat bran, to avoid any mould formation.

2.3 Production Indicator Measurements

All mealworm trials were conducted at the University of Nottingham, Sutton Bonington Campus, UK.

On day 0, a set number of mealworm larvae were counted into groups and total live weight of these groups determined. These groups were then randomly allocated to different treatment groups. Larvae were then given feed and the water source; feeds were provided *ad libitum* throughout the 14-day trial period. On measurement days (days 4, 7 and 11), dishes of mealworms were removed from the rack/incubator and the cotton wool was removed. Any mealworms in the cotton wool were removed using a pair of tweezers and put back in the dish. The cotton wool was then weighed and the weight recorded.

The entire contents of the dish (mealworms, leftover feed and frass) were put onto the top of the stacked sieves. Sieves were then shaken until all feed had passed through the top sieve leaving just mealworms on the top sieve. Mealworms were then transferred to a white plastic tray for weighing and photographing. The contents retained in the bottom sieve was the remaining feed, which was weighed. The contents of the tray below the stack of sieves was the frass (faecal matter) and was also weighed.

2.3.1 Live weight

Mealworms were weighed on a group rather than individual basis. A group was defined as all the mealworms present in one replicate dish of a treatment. All live mealworms were weighed on days 0, 4, 7, 11 and 14 of each trial.

Total group body weight gain (BWG) was calculated, as in equation 2.1, on a live weight basis (living will full gut contents).

$$BWG = Final\ (day\ 14) group\ weight - Initial\ (day\ 0) group\ weight \tag{2.1}$$

Initial and final group weights and total group body weight gain were used as performance indicators.

On each measurement day, mealworms from each dish were separated from feed and frass (described further in 2.3) and placed on a tray. Tweezers were then used to remove and count any dead mealworms. A photograph was then taken of the remaining live mealworms in the tray. Analysis of this photograph was undertaken using ImageJ to perform a count of the living mealworms remaining in that dish.

Utilising the group live weight values already recorded, it was then possible to calculate an average individual live weight for the mealworms using equation 2.2.

$$Individual\ live\ weight = \frac{Group\ live\ weight}{Number\ of\ live\ individuals} \tag{2.2}$$

2.3.2 Death Proportions

Both the number of deaths and pupated individuals were used as production performance indicators. Number of deaths were calculated using the count that was performed from the live individuals' photographs, using the difference between the number of live mealworms on different measurement days gave the number of dead mealworms. Any mealworms that had pupated in these measurement days were recorded and then taken off the count of the dead mealworms.

Death percentage (%) was calculated as equation 2.3.

$$Death\,\% = \frac{No\ dead}{Starting\ No} \times 100 \qquad (2.3)$$

2.3.3 Feed Intake

Feed intake was calculated on a dish basis. Firstly, the weight of remaining feed on day 4 was subtracted from the weight supplied on day 0. This was repeated for days 7, 11 and 14. The four values for feed intake were then added together to give total feed consumption (for the dish).

2.4 Proximate Nutritional Analysis

2.4.1 Drying

All samples (feed and mealworms) had to be fully dried prior to proximate analysis. Due to restrictions on equipment availability, two drying methods were used: freeze drying (Christ) or oven drying at 60°C. The method of drying is specified for each trial in each chapter. For oven drying, samples were dried in porcelain crucibles on a baking tray, whereas for freeze drying samples were dried in plastic pots with the lids removed. Previous studies have shown no differences in the proximate composition of mealworms dried either by freeze drying or oven drying (Kröncke et al., 2019; Selaledi and Mabelebele, 2021).

Containers were weighed and then the sample was added and weighed again, giving total weight of container and sample. Samples were then either placed in the oven or freeze drier and left for 48 hours. Samples were removed from drying apparatus and re weighed, then returned to drying apparatus for a time then reweighed until a constant weight. Dry matter

(DM) percentage was calculated for the replicates of each sample and averaged. The calculation for dry matter percentage is shown in equation 2.4.

$$DM\ (\frac{g}{kg}) = \frac{(C+dS)-C}{(C+S)-C} \quad (2.4)$$

Where C = container for drying, S = sample, dS = dried sample

2.4.2 Crude Protein

Crude protein content was determined via the nitrogen content. The nitrogen content (percentage) of each sample was obtained using EA 1112 elemental analyser (Thermo Scientific). Protein contains on average 16% (w/w) nitrogen by weight of total protein, therefore a multiplier of 6.25 was used to convert nitrogen into total protein.

Standards (aspartic acid 10.52% (w/w) N%), bypass (aspartic acid to clear machine of any residual sample from previous run), samples and quality control were all prepared using the same method. 50mg was weighed into a tin capsule (10x10mm tin weighing pans, Elemental Microanalysis, Okehampton EX20 1BQ). A sealing device was used to crimp the top the of tin capsule to prevent any sample falling out. A cylindrical tool was then used to press down and create a sealed disc. All sealed tin capsules were then placed in a storage tray in a desiccator until ready for running on the analyser.

To start each run of sample, an initial bypass of aspartic acid (10.52 (w/w) N%) (D5055, Elemental Microanalysis) was used to clear the machine. Then two empty foil tins, as a negative control, were recorded as blanks in computer software and analysed. Following these, four samples of aspartic acid were weighed and analysed. The first two as nitrogen standards and the second two for quality control. Samples were then loaded into the auto-sampler, a chromatograph was produced for each sample with a nitrogen peak. After 20

samples were run, two further standards of aspartic acid were run for continued quality control.

The elemental analyser utilised a combustion reactor to combust samples at 1000°C. The products of the combustion were then carried by helium through reagents that produce CO_2, H_2O, N_2 and NO_x. Copper was then used to reduce NO_x to N_2 and samples pass through H_2O and CO_2 traps, before entering a GC column, and then N_2 was detected by a Thermal Conductivity Detector.

2.4.3 Gross Energy

Energy contents were determined by bomb calorimetry. This technique measures enthalpy change of combustion of the sample within a high pressure, constant volume reaction chamber (6300 calorimeter, Parr). The standard used was calorific grade benzoic acid (1g pellets, Parr), which has a known energy value of 26.454 MJ/kg. The equation for calculating energy equivalent is shown in equation 2.5.

$$W = \frac{Hm + e1 + e2 + e3}{T} \qquad (2.5)$$

Where:

W = Energy equivalent of the calorimeter (˚C)

H = Heat of combustion of standard benzoic acid (cal/g)

m = mass of sample (g)

T = temperature rise (˚C)

e 1 = Correction for heat of formation of nitric acid (cal)

e2 = Correction for sulphur

e3 = Correction for heating wire and combustion of cotton thread

1g of sample was weighed into a crucible and flattened down. The crucible was then placed in the bomb head with a 10cm cotton ignition thread (Parr). The exact weight of the sample

was then recorded. The sample was then ignited and the increase in temperature was proportional to the amount of energy in the sample. Energy values were recorded as MJ/kg DM.

2.4.4 Total Fat

Total fat content of samples were extracted using Gerhardt's Soxtherm system, where the lipid soluble components were separated from the water soluble components using an organic solvent.

Dried samples were ground to a fine powder and 1g was weighed onto two layers of qualitative grade filter paper (diameter: 11cm, Fisherbrand). This paper was then folded to enclose the sample and labelled. Samples were then placed within an extraction thimble and cotton wool added on top to prevent the filter paper falling out. Three boiling stones (Gerhardt) were added to each extraction flask, these flasks were then weighed and recorded. The flasks were then filled with 130-140ml petroleum ether (Fisher Chemical) in the fume hood. Metal thimble holders were then inserted into the flasks, and the thimbles placed within the holders, ensuring that the petroleum ether was surrounding the thimble. The flasks were then attached to the Soxtherm extraction apparatus and the apparatus turned on.

The extraction process involves five steps:

1. The flasks are lowered down onto the hot plates. This heats the solvent up to boiling point. The extractable material contained within the filter paper in the thimble is liberated.
2. The solvent level is lowered below the extraction thimble. Any excess solvent is collected in a recovery tank.
3. The material is extracted by the refluxed, condensed solvent and is collected in the solvent below in the extraction beaker. The condenser converted the evaporated solvent back to liquid, this then passed over the sample dissolving the fat from the material and carrying it through the filter paper and porous thimble into the flask.
4. The bulk of the solvent is distilled into the rear storage tank for later recovery. The extracted lipid remains in the flasks due its low volatility.
5. The extraction flasks are then lifted from the hot plate automatically. The flasks can then be removed from the apparatus.

When the cycle had completed, the flasks (with metal holders, thimble and filter paper containing sample) were removed from the apparatus. The metal holders and thimble containing the filter paper enclosed sample were removed from the flask. The filter paper enclosed sample was then left in the fume hood to dry.

The flasks were then placed in an oven set to 100°C for 1 hour so that any remaining petroleum ether was evaporated. After drying in the oven, flasks were placed into a desiccator to cool.

Flasks were then weighed and total crude fat was calculated as shown in equation 2.6.

$$Crude\ fat\ = \frac{(Flask + fat\ (g)) - Flask\ (g)}{Sample\ (g)} \qquad (2.6)$$

2.4.5 Acid Digestion

Weighed dried sample (200mg) was placed into a digestion tube, and 6ml of 65% nitric acid added using a stepper pipette. Stoppers were added to tubes, caps screwed hand tight and placed in order in the microwave rotor. The microwave (Anton Paar) was then turned on, the rotor placed inside and programme P1 was run to digest the samples. After the microwave digestion had finished, contents were decanted into 10ml tubes. Pure water was used to rinse the digestion tubes to ensure no sample remained.

2.4.6 Inductively coupled-plasma mass spectrometry (ICPMS)

Following microwave digestion, samples were diluted 1 in 10 with pure water as preparation for ICPMS. Multi-element analysis is undertaken using ICPMS (Thermo-Fisher iCAP-Q) with a 'Flatopole collision cell' (typically charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interference. Standard multi-element analysis includes Ag, Al, As, B, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), Tl, U, V and Zn.

As the samples were in liquid form, the samples were introduced to an argon plasma. The plasma source dries the droplets and dissociates the molecules, then forms single charged ions by oxidation. These ions then pass through a mass spectrometer which filters and sorts the ions according to their mass-charge-ratio (*m/z*). When the ions exit the mass

spectrometer they strike a detection system. The impact of the ions on the detector causes an amplification of the signal until there is a measurable pulse. Sample processing is undertaken using 'Qtegra software' (Thermo-Fisher Scientific). The software then compared these pulses to a standard to determine the concentration of each element being measured. Results are reported as gravimetric concentrations (µg L^{-1} or mg L^{-1}).

2.5 Broiler Trials

2.5.1 Feeds

Formulations

Broiler feeds were supplied by Target Feeds (Whitchurch, UK). For both broiler trials a crumb based feed was used. The soybean meal feed was used as a control feed (Dale et al., 2020). The basal feed is what was supplied by Target feeds to allow for inclusion of crushed mealworms on site.

Table 2.2 *Formulation of ingredients and proximate nutritional analysis of Control and Basal feed before addition of mealworms*

	Feeds[1]	
	Control	Basal
% of Ingredient		
Barley	9.37	12.37
Wheat	55.00	55.00
Hipro Soya	23.00	13.00
Full fat soya	5.00	5.00
L Lysine	0.20	0.20
DL Methionine	0.35	0.35
L Threonine	0.15	0.15
Soya oil	4.00	1.00
Limestone	0.95	0.95
Monocalcium phosphate	1.18	1.18
Salt	0.25	0.25
Sodium bicarbonate	0.15	0.15
Mineral Premix[2]	0.40	0.40
Titanium Dioxide	0.5	0.5
Nutrient[3]		
Oil EE (%DM)	6.39	3.66
Protein (%DM)	20.25	17.63
Fibre (%DM)	3.12	3.22
Ash (%DM)	5.40	5.40

[1]Control = soybean meal based feed, Basal = 90% feed which mealworms were added to
[2]Supplying: retinoic acid 4.8 mg/kg, cholecalciferol 75 µg/kg, α-tocopherol 75 mg/kg, thiamine 3 mg/kg, riboflavin 10 mg/kg, pyridoxine 3 mg/kg, cobalamin 15 µg/kg; nicotinic acid 60 mg/kg, pantothenic acid 14.7 mg/kg, folic acid 1.5 mg/kg, biotin 125 µg/kg, choline chloride 25 mg/kg, Fe 20 mg/kg, Cu 10 mg/kg, Mn 100 mg/kg, Co 1.0 mg/kg, Zn 82.222 mg/kg, I 1 mg/kg, Se 0.2 mg/kg and Mo 0.5 mg/kg.
[3] Analysis supplied by Target Feeds

Dried mealworms were purchased from Maltby's Stores 1904 Limited. Mealworms were formulated into broiler feed based on oil and protein content only. The basal feed was 90% of the weight of the control feed to allow for inclusion of 10% mealworms on site.

The control feed contained 23% soybean meal and 4% soya oil, which equates to 11% crude protein and 4.3% oil. In order to include 10% mealworms, 10% of the soya was removed and other components were reformulated. Therefore, the basal feed contained 13% soybean meal, 1% soya oil, 3% extra barley and 10% mealworms to give 11.50% crude protein and 4.25% oil.

Feed Mixing Protocol for all trials

Mealworms were crushed to a powder form using a handheld blender before being sealed in 2.5kg bags until mixing with other feeds.

A screw mixer was used to combine the basal feed and powdered mealworms on site. All feeds were subject to mixing, specific details for which are included in Chapters 6 and 7 for individual feeds.

2.5.2 Experimental design

Two broiler trials were carried out at the University of Nottingham Bio-Support Unit, each using 80 one day old male Ross 308 broiler chicks (obtained from P D Hook Hatcheries Limited, Bampton). Birds were housed and cared for according to the UK Animals (Scientific Procedures) Act 1986 (ASPA) Code of Practice for the care and accommodation of animals (February 2013). Throughout both trials, birds were given *ad libitum* access to feed and water. As per the Ross 308 Management Guidelines, room temperature was maintained at 32°C on arrival and reduced by 1°C per day until 21°C was reached. Lighting were

maintained at 12 hours light, 12 hours dark for the whole trial and humidity was as recommended.

For both trials there was 10 replicate pens per feed, with 4 birds in each pen (n=40 birds per feed). On arrival, the 1 day old birds were wing tagged and group housed in a single pen to start with and fed the control diet.

On day 6 birds were weighed then randomly allocated to pens (n=4 birds per pen). Pen weights and coefficient of variation were checked to ensure no significant difference between treatments at the start of the trial. Birds were then fed one of the two feeds (which were different for the 2 trials) *ad libitum* for 35 or 36 days, with the birds and any remaining feed being weighed every Monday and Thursday.

On days 35 and 36, broilers from pens 1-10 and 11-20 respectively were weighed then culled by Schedule 1 method (Animals (Scientific Procedures) Act 1986) of an overdose of Pentobarbitone (anaesthetic) through intravenous neck injection. After confirmation of death, the digestive tract of each bird was exposed and samples of gut digesta and tract (3cm pieces of each section) taken. For the first trial samples were obtained from the duodenum, ileum, jejunum, caeca and colon, as well as total liver, breast and leg muscles being dissected and weighed, then a sample taken. All samples were snap frozen in liquid nitrogen before storage at -80ºC.

For the second chicken trial, samples of gut digesta and tract were only taken from the ileum, jejunum and caeca due to COVID 19 restrictions.

2.5.3 Apparent Ileal Digestibility

2.5.3.1 *Sample Preparation*

For collection of digesta samples were taken from the terminal ileum. Measured starting from three fingers distance from the ileo-caecal junction back towards Meckels diverticulum, 3cm of terminal ileum tract cut away and the digesta content squeezed out into a labelled tube.

Ileal digesta samples from both broiler trials as well as samples of the four feeds were all freeze dried to a constant weight. Then 250g of dried ileal digesta from each bird (n=4) in the same pen was mixed to give one sample per pen, giving a total of 10 samples per treatment across both trials.

2.5.3.2 *Titanium dioxide measurement*

The titanium content of the ileal samples including titanium was measured through firstly acid digestion then ICPMS analysis (2.4.6). Feed samples were analysed by the same method at the same time.

2.5.3.3 *Amino Acid Analysis of feed stuffs and digesta*

Ileal digesta samples from both broiler trials as well as samples of the four feeds were all freeze dried to a constant weight. Then 250g of dried ileal digesta from each bird (n=4) in the same pen was mixed to give one sample per pen, giving a total of 10 samples per treatment across both trials.

Dr Jon Stubberfield, Technician Division of Food, Nutrition and Dietetics, carried out all amino acid analysis preparation.

Table 2.3 Preparation of working solutions for amino acid analysis

Working solution	Method
Formic acid/ phenol solution	Mix 735ml formic acid with 111ml deionised water and add 4.73g phenol
Oxidation solution	Dispense 10ml of 30% hydrogen peroxide into 100ml volumetric flask. Make up to volume with formic acid/phenol solution. Incubate at 20-30°C for 1 hour, solution turns yellow. Cool in fridge before adding to sample. Prepared fresh as required, approximately 2 hours prior to oxidising samples.
Hydrolysis reagent	Take 492ml of hydrochloric acid to make up to 1 litre with deionised water
4M ammonium formate in water	Weigh 252.4g of ammonium formate, transfer to a 1L volumetric flask and gauge to 1L Milli Q water
100mM ammonium formate in water	Weigh 6.31g of ammonium formate, transfer to a 1L volumetric flask and gauge to 1L with Milli Q water
20mM ammonium formate, pH=2.8	Add 200ml of 100mM ammonium formate in water to a 1L beaker, add 2ml of formic acid and approximately 700ml of water and mix thoroughly. Adjust the pH to 2.8 with formic acid, transfer again to the volumetric flask and make up to 1L with water.

Preparation of standards

Calibration solutions were prepared by dilution the original amino acid standard mix from Sigma-Aldrich: P/N AA-S-18.

Internal standard solution were prepared by dilution of the original Cell Free Amino Acid Mixture - 13C, 15N from Sigma-Aldrich: P/N 767964

Procedure

Sample containing approximately 5mg nitrogen was weighed into 20ml crimp top tubes, making sure the sample was deposited to the bottom of the tubes. Sample weight was recorded to 4 decimal places. 0.2ml of 200mM Norvaline as an ISTD was then added to tubes to make the final concentration 40uM (based on a 20 fold dilution for pure protein).

Tubes were then placed in the fridge to cool. Once removed from the fridge a dispenser was used to add 2.5ml of chilled oxidation solution to each tube, making sure the solid sample was rinsed to the bottom. Samples were return to the fridge for a minimum of 16 hours, maximum of 18 hours. After oxidation, samples were removed from the fridge and 0.42g of sodium metabisulphite was added to each. This decomposed any excess oxidation reagent. A measuring cylinder was used to add 2.5ml 12M hydrochloric acid, then 0.5ml of hydrolysis reagent (6M with 1% phenol) was added to each sample and left to cool. The tubes tops were crimped and sealed well, then put in the 110°C oven for 24 hours.

After hydrolysis, the tubes were taken out and cooled for 10 mins. The contents were quantitively transferred into 50ml falcon tubes. Ammonium formate buffer (pH 2.8, 20mM) was used to rinse the contents of the tubes into 50ml falcon tubes. Samples were then partly neutralised by adding, using a 25ml measuring cylinder, 10ml of 4M ammonium formate solution. The flask was gently swirled during the addition and not allowed rise

above 40°C. The pH of each sample was then adjusted to 2.8 using 4M 100mM ammonium formate and formic acid. Then volume was then made up to 50ml with ammonium formate buffer (pH2.8, 20mM).

Samples were then centrifuged at 3000 RPM for 10 minutes. The supernatant was passed through a 0.22µm filter into a HPLC vial. This was then diluted 20 times. 20µl of the filtered sample was taken and 180ml MPA added then 200µl ISTD (Phe13C6 @60µM) added.

Amino acid analysis was carried out by Dongfang Li, Technical Specialist for Environmental Science.

The sample was then loaded onto the Thermo Fisher Vanquish UHPLC which is attached to a Triple Quad TSQ Altis Mass spec system (Thermo Fisher).

A chromatographic method of 18 minutes was used for the analysis of the amino acids using a Thermo Vanquish uHPLC system consisting of a binary pump, a column oven, and an autosampler. The separation was performed on a Thermo Scientific™ Acclaim™ Trinity mixed mode column at 30 °C. Mobile phases consisted of ammonium formate in water at pH 2.8 for phase A and a mixture of ammonium formate in water and acetonitrile (80/20 v/v) for phase B.

Chromatographic separation was achieved by gradient elution under the conditions described in Table 2.5.

Table 2.4 *Gradient elution for Thermo Vanquish uHPLC system*

Time (min)	Flow Rate (ml/min)	%A	%B
0	0.3	100	0
5	0.3	100	0
7	0.3	0	100
11	0.3	0	100
12	0.3	100	0
12.5	0.45	100	0
14.5	0.45	100	0
15	0.3	100	0
18	0.3	100	0

Compounds were detected on a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer equipped with a Thermo Scientific™ Easy-Max NG ion source with a heated electrospray ionization probe. Sheath gas was set at 45 arbitrary units, auxiliary gas at 15 arbitrary units, and spray voltage at 3500 V for positive ionization and at 2700 V for negative ionization. Vaporizer temperature was set to 370 °C and transfer tube temperature to 70 °C, while source fragmentation was applied at 15 V. Data was acquired in Selected Reaction Monitoring (SRM) mode using a resolution of 0.7 full width at half maximum (FWHM) for both quadrupoles with a 400 ms cycle time. The SRM transitions used for this method are presented in Appendix 1.

Calculations

The concentration of amino acid was expressed as gram per kilogram sample (g/kg DM or mg/g DM). It was calculated as following (equation 2.7):

$$Amino\ acid\ \left(\frac{g}{kg}\right) = A\ \times MW\ \times \frac{F}{W} \times 10^{-3} \qquad (2.7)$$

Where:

A = concentration of hydrolysate obtained by the instrument (µM)

MW = molecular weight

W = g sample

F = ml total hydrolysate, 50ml

Cystine and cysteine were both determined as cysteic acid in hydrolysates of oxidised samples but calculated as cystine by using MW 240.3. Methionine was determined as methionine sulfone in oxidised sample but calculated as methionine by using MW using 149.21.

2.5.3.4 *Apparent Ileal Digestibility Calculations*

Apparent ileal digestibility of protein and amino acids were calculated using the following equation taken from Cowieson and Ravindran (2008) (2.8):

$$Digestiblity = \frac{(\frac{N}{Ti})_f - (\frac{N}{Ti})_i}{(\frac{N}{Ti})_f} \qquad (2.8)$$

Where:

$(N/Ti)_f$ = ratio of nutrient and titanium in the feed

$(N/Ti)_I$ = ratio of nutrient and titanium in ileal digesta

2.5.4 Caecal Microbiome

This work was carried out by Dr Elizabeth King, Division of Microbiology, Brewing and Biotechnology. The methodology has been adapted from a published paper (Kozich et al., 2013).

2.5.4.1 *Sample Preparation*

Caecal samples from individual birds from both broiler trials were analysed. The caecal content from each bird was collected by squeezing one caecum into a tube, then snap frozen in liquid nitrogen immediately.

2.5.4.2 *DNA Extraction*

The QIAMP Power Fecal kit (cat. No 51532) was used to extract DNA from caecal samples from both broiler trials according to the manufacturers instructions.

Frozen caecal samples were partially defrosted to a gelatinous viscosity, then 100mg was weighed out in a Class 2 microbiological safety cabinet into pathogen lysis tube L (cat. No 19092). 650µl of pre-warmed Buffer PW1 was added to each sample. After the addition of Buffer PW1 all steps were carried out at room temperature. Each sample was homogenized using the MP homogeniser set at 6.5m/s for 45 seconds, waiting 5 minutes then repeating the 45 seconds. Samples were then centrifuged for 1 minute. 400µl of supernatant was pipetted into a new tube and 150µl of Buffer C3 was added and mixed thoroughly though pipetting. This was incubated at 4°C for 1 hour then centrifuged for 1 minute.

For each sample, 20µl of Proteinase K was added to a new S-Block well and 300µl of supernatant was added to these wells and mixed by pipetting until frothy. This was then incubated for 10 minutes at room temperature.

The QIcube HT instrument was used to extract the DNA.

2.5.4.3 *Initial setup*

The Illumina Experiment Manager was used to create a sample plate map of samples and indexed primers layout for each 96 well plate. Two index primer pairs were as follows: A701 – A712 with B501 – B508 and B701 – B712 with A501 – A508. The index and sequencing primers were reconstituted to 100µM (See appendix 2 for primer design). 100µl of 10µM aliquots of indexed primers were prepared. The sequencing primers (Red 1, Index and Read 2) were not diluted. 5µl aliquots of each indexed primers were arrayed into two 96 well plates using the plate map design. The primer plates were then stored at -20°C until needed for the PCR step. The extracted gDNA (including kit controls) were arrayed in a 96 well format. Two wells were left out for a negative water control and another for the positive mock community control.

2.5.4.4 *PCR*

All PCR steps were carried out under sterile conditions in a microbiological safety cabinet. All consumables needed for the PCR were decontaminated using UV irradiation for 30 minutes.

A multichannel pipette was used to transfer 2µl of each paired set of index primers from the primer plate to the corresponding well on the 96-well PCR plate. 17µl of Accuprime Pfx Supermix was added to each well of the PCR plate and the 1µl of template DNA, negative control (molecular biology grade water), positive control (ZymoBIOMICS microbial community 10ng) and kit controls were added to the 96 well PCR plate. The plate was then sealed using adhesive seal roller and applicator on the PCR block. A second seal, (Biorad Microseal A, non-adhesive) was used to maintain the seal on the plate to avoid loss by

evaporation. The plate was then briefly vortexed on the IKA Vortexer and spun down using the Axygen Axyspin. The PCR plate was then put in the Thermal Cycler (Biorad T-100) and underwent 2 minutes at 95°C, then 30 cycles of 95°C for 20 seconds, 55°C for 15 seconds, 72°C for 2 minutes. Then at 72°C for 10 minutes and finally 4°C for 20 minutes.

2.5.4.5 *Gel Electrophoresis*

A 1% agarose gel was prepared using the 96-well, 12 ladder wells casting and running tray. 2µl of sample and 4µl of 1.5x diluted loading dye was loaded onto the 1% agarose gel. This was run at 95V for 40 minutes alongside a 100bp ladder. The gel was then photographed under UV, using the Gel Doc XR+. The gel was checked for a band for every well (excluding the kit and negative controls) to ensure the PCR had worked.

2.5.4.6 *Clean up, Normalisation and Pooling*

The normalisation was carried out using the SequalPrep Normalisation Plate (96) Kit.

15µl of PCR product was transferred from the 96 well PCR plate to the corresponding well on the normalisation plate. Then 15 µl of Binding Buffer was added to the normalisation plate, mixed by pipetting, sealed briefly vortexed using the IKA vortexer and then spun using the Axygen Axyspin. This plate was then incubated at room temperature for 60 minutes. The liquid from the wells was then aspirated and stored in at microplate at -20°C. 50µl of wash buffer was added and pipetted up and down twice then aspirated. The plate was then inverted and tapped on blue paper towel to ensure there was no residual wash buffer remaining in any well. 20µl of Elution buffer was added to the plate and mixed through pipetting. This was then sealed, vortexed and spun briefly as described above. After

incubation at room temperature for 5 minutes a pool was created from the entire contents of the plate by taking 5µl of each well and combining in a 1.5ml Eppendorf tube. The remaining sample in the normalisation plate was then frozen for use later.

Library QC and Quantification

Agilent 2200 TapeStation System Trace was used using the D1000 ScreenTape.

The sample buffer was allowed to equilibrate at room temperature for 30 minutes and then vortexed before use. The ladder was prepared by mixing 2µl of D1000 sample buffer with 2µl D1000 ladder. The diluted samples were then prepared by mixing 2µl of D1000 sample buffer with 2µl of DNA library diluted samples. This was then spun down, then vortexed using IKA vortexer and adaptor at 2000 rpm for 1 minute. Then spun down again to position the sample at the bottom of the tube. Samples were then loaded into the 2200 TapeStation instrument. The average size of the library of base pair was then recorded.

Qubit Assay

7 0.5ml Qubit assay tubes (Cat. No Q32856) were set up and labelled only on the lids for 2 standards and 3 replicates from each trial. The Qubit working solution was then prepared by diluting the Qubit dsDNA HS reagent 1:200 dilution in Qubit dsDNA HS Buffer (1µl reagent in 199µl buffer). 190µl of Qubit working solution was added to each of the standard tubes and then 10µl of Qubit standard was added, then briefly vortexed. Then 198µl of Qubit working solution was added to each of the sample tubes (6 in total, 3 for each trial). Then 2µl of each sample was added to individual assay tubes. All tubes were incubated at room temperature for 2 minutes. Samples and standards were then read on the Qubit 3.0 Fluorometer.

Standards were read first to give a standard curve. The output sample concentration from the Fluorometer was given in ng/µl, this was then converted to nM using equation 2.8:

$$\frac{(concentration\ in \frac{ng}{\mu l})}{(\frac{660g}{mol} \times average\ library\ size\ in\ bp)} \times\ 10^6 = concentration\ in\ nM \quad (2.8)$$

2.5.4.7 *MiSeq*

The 500 cycle MiSeq Reagent Kit v2 cartridge was removed from the -20°C freezer and then put in a 4°C incubation to defrost overnight. 0.2N sodium hydroxide (NaOH) was prepared fresh. Then to a 1.5ml Eppendorf tube, 10µl of library was added to 10µl of 0.2N NaOH. This was then incubated at room temperature for 5 minutes and after this 980µl of ice-cold HT1 was added immediately.

In a separate tube 2µl of PhiX (Library control), 3µl of PCR grade water and 5µl of 0.2N NaOH were added together and mixed by pipetting. This was then incubated at room temperature for 5 minutes and after this 900µl of ice-cold HT1 was added immediately. The concentration of the PhiX was then 20pm.

The two tubes where then diluted to 4.75pm. For the library tube 281.35µl of library was added to 718.65µl of HT1. For the PhiX tube 237.5µl of PhiX (20pm) was added to 762.5µl. The loading solution was then created, 150µl of the PhiX (4.75pm) was then added to 850µl of library (4.75pm).

The cartridge was inverted before loading and wells were checked to ensure there was no condensation.

Using Biosphere extra-long pipette tips 10µl of well 12 in the cartridge was removed and placed in a sterile PCR tube. 3µl of 100µM Read 1 sequencing primer was then added to the tube, this was mixed by pipetting then placed back into well 12 in the cartridge. This was then repeated for well 13 and the index primer and for well 14 with the read 2 sequencing primer. To well 17 600ul of loading solution was then added. The cartridge was then placed in the fridge until loading in the MiSeq.

The flow cell for the MiSeq was cleaned thoroughly before use with 100% ethanol, Milli-Q water and blotting with lint free wipes (Kimwipes) to ensure there were no blemishes, particles or fibres on the glass. The flow cell, reagent cartridge and PR2 bottle was placed in the MiSeq and set to run. During the run, the run was monitored using the Illumina Sequence Analysis Viewer, with the view that the ideal parameters for a 90% 16S run are cluster density 700-800k/mm2, >85% clusters passing filter, 15% aligned, no spikes in corrected intensity plot, all indices following index reads and a final >Q30 score of >70%

2.5.4.8 *MiSeq Data Analysis*

The Schloss lab in Mothur (online software) was used to quality filter 16S rRNA sequences and cluster into operational translation units (OTUs). Analysis of molecular variance (AMOVA) was calculated within Mothur (Kozich et al., 2013) to give an indication of any significant changes within the groups. OTU's were exported into excel format allowing for graphs of relative abundance of phyla and species to be drawn.

Linear discriminant analysis effect size (LEfSE) plots were then used to analyse changes between feed treatments for specific families. Significant OTU from the LefSe report were further analysed by taking the FASTA sequence and running through NCBI BLAST to give an indication of the specific species. The BLAST results were analysed to ensure that there was a high (>95% identity) and that the family was the same as that was being reported by the LefSe report.

3 Commercialisation of Insect Production

3.1 Introduction

Over the last 50 years there has been significant developments in the variety of ingredients, equipment and technologies which are used for animal feed (Coffey et al., 2016). Alongside this traditional livestock animals growth patterns are well understood and have been optimised utilising genetics and breeding to optimise product output (Thornton, 2010). The combination of both improvements in feeding methods for animals and understanding of growth parameters have improved efficiency greatly. As a novel feed ingredient, insect production is in its infancy with limited understanding of growth patterns and production requirements.

Utilisation of mealworms as an alternative protein source in compound animal feed would require a significant mass production system. The production of mealworms has been estimated to be 4.8 times more expensive than that of traditional chicken feed (van Huis et al., 2013), owing to factors including high amounts of manual labour (Rumpold and Schlüter, 2013), use of expensive feeds that may not meet the nutritional requirements of the insects and sub-optimum growing conditions.

Insects are often described has having a high production efficiency, although potentially this is when they are grown in optimal conditions, and frequently on feed which could be used directly for human or animal feed (Oonincx et al., 2015). In traditional livestock species, feed conversion ratio (FCR) is used to describe the efficiency of a feed source and a production

system. Production of traditional livestock feed has been developed over decades resulting in high production efficiencies and a low feed conversion ratio (FCR).

Temperature, humidity, nutrition and water supply are known factors to affect insect growth rates (Mirth and Riddiford, 2007), however their relationship to subsequent nutritional composition is not well described. The specific nutritional requirements of mealworms have not yet been fully identified. Producers usually use an *ad libitum* style feeding system that feeds to excess. Many commercial suppliers feed insects chick crumb, which is expensive and inefficient since it can be directly used in poultry production. Another feed which is used is wheat bran, a good source of dietary fibre, vitamins and minerals; however, while this is often seen as a by-product from the food industry, and, as such is relatively inexpensive, it can be consumed by humans (Chalamacharla et al., 2018; Onipe et al., 2015). There have been studies where insects have been grown on alternative bio waste (Makkar et al., 2014) or waste food stream (Dossey et al., 2016) that cannot be used for either human or animal consumption. Providing this maintains growth and nutritional composition, this would be financially beneficial, improve the environmental aspects, and remove the highlighted concerns that insects are consuming food which could be used for either humans or animals directly.

Another consideration is the source of water that is used, with the most popular method being the use of vegetables, such as carrot (Aguilar-Miranda et al., 2002). Carrot has a high water content so makes an excellent source for insects, but also contains high levels of dietary fibre and carotenoids such as beta carotene (Sharma et al., 2012). The vegetables could be an additional source of nutrients, thereby improving growth or altering nutritional composition. However, using vegetables as a source of water on a mass -level adds cost,

complexity and risks contamination should mould start growing. Other methods include sprinkling the feed with water or utilising cotton wool soaked in water (Morales-Ramos et al., 2011; Weaver and McFarlane, 1990), however these methods have not previously been compared directly to vegetables as a water source in terms of growth rates and efficiency.

As mealworms are holometabolous insects, they hatch from an egg into a larvae (Chapman, 2013). The larval form is what is then harvested as the potential animal feed ingredient. However, if left to grow the larval stage will undergo molting (shedding of the exoskeleton) into larger larval forms and ultimately will transform into a pupa. Which after a period of time, will then metamorphosise into the adult beetle form (Reynolds, 2013). The factors which initiate molting and metamorphosis are relatively unknown, the general view is that insect responds to reaching a critical weight but this is variable depending on the starting insect size (Chapman, 2013). With all three stages, the final weight of the insect varies depending on the conditions in which it was reared (Nijhout, 2003). Factors which are known to affect such development include nutrition, temperature and density of population (Reynolds, 2013). However, there is limited data reporting how growth rate relates to the nutritional composition of the insect.

Alongside picking the right time for harvesting mealworms to be used in feeds, the processes associated with harvesting need to be established. One important question is whether to clear the gastrointestinal tract of mealworms before they are killed, dried, and used in animal feeds. Most traditional feed ingredients are plant based, therefore there is no gastrointestinal tract which is passed onto the consumer. A major safety concern with utilising insects as feed is that they are used as a feed ingredient without removing the gut, therefore potentially passing on their microbial content to the livestock consumer (Grau et

al., 2017; Veldkamp et al., 2012). Currently in Europe, the use of animal proteins in pig and poultry feeds is prohibited (Veldkamp et al., 2012) and this stems from concerns related to prions, bovine spongiform encephalitis and other transmissible spongiform encephalopathies. Insects are considered incapable of expressing prions (van Raamsdonk et al., 2017) but if they are produced on feeds containing waste materials, possibly including animal manure, there could be a transmissible vector route (Hawkey et al., 2021).

This chapter aims to investigate some of these factors relating to mass producing mealworms and provide more information into optimal growing and harvesting conditions.

Aims

1. To investigate an alternative system (to vegetables) for supplying water and compare the effects on growth, feed intake and nutritional composition of mealworms.
2. To compare the use of wheat bran and chick crumb as feed sources and their impacts on growth rates and nutritional composition of mealworms.
3. To determine whether temperature and humidity impact upon growth rates and nutritional composition of mealworms.
4. To define the optimal harvesting age and method for mealworms to be used in animal feed

Hypothesis

It is hypothesised that:

- Manipulating the source or the content of water of the mealworms feed will affect their growth characteristics.
- Mealworms fed chick crumb will have improved growth and nutritional composition in comparison to those fed wheat bran.
- More controlled environment (temperature and humidity) will improve mealworm growth characteristics.
- Mealworm nutrient composition will change with both larval growth age and starvation.

3.2 Materials and methods

Multiple studies were carried out as part of this chapter, all were carried out using the same experimental methods but with differing treatment groups and conditions. Detailed methods are presented in chapter 2.

3.2.1 Animal Husbandry

Mini mealworms were obtained from Monkfield Nutrition Ltd (Ely, UK) and maintained on an *ad libitum* diet of wheat bran and carrot until day 0 for studies 1 and 2, whereas cotton wool soaked in water was used as a water source for study 3 and 4. For study 5 mealworms from our own breeding colony were used and maintained on *ad libitum* wheat bran and water-soaked cotton wool.

3.2.2 Feed Preparation

Study 1: Feed Rehydration trial

Wheat bran was milled to 3mm using a Fritsch cutting mill and stored at 2-5°C until use. At day -3 (3 days before the start of the study) wheat bran was placed in an oven at 100˚C for 36 hours and dried to a constant weight. Wheat bran was then rehydrated with controlled volumes of water, to give moisture percentages of 15, 30 and 50% on a v/wt basis (Table 3.1). Feeds were rehydrated on the morning of measurement days. This resulted in five treatment groups: control (wheat bran + carrot), WB 0% (no water) then WB 15%, WB 30%, and WB 50% (wheat bran hydrated to 15, 30 and 50% respectively).

Table 3.1 *Study 1: Formulation of mealworm feeds*

	Treatments[1]				
	Control	0%	15%	30%	50%
Wheat Bran (g)	5	5	5	5	5
Water (ml)	-	-	0.75	1.5	2.5
Carrot (g)	10	-	-	-	-

[1]Percentages indicate the percentage of water added to the wheat bran. 0.75ml is 15% of 5g

Study 2: Water-soaked cotton wool trial

Wheat bran was milled to 3mm using a Fritsch cutting mill and then stored in a dry place. Treatment groups were aimed to replicate 10g of carrot, which equates to 8.8g water, 82.85μg beta carotene and 0.359g sucrose. Both the beta carotene and sucrose were serially diluted with cellulose and then mixed with the wheat bran, with just cellulose added to the WB for all other treatments. Cellulose was therefore included in all treatment groups.

To supply water in the treatments without carrot, cotton wool was used as an external carrier for the water. A separate treatment group was included which had cotton wool but with no water, the cotton wool was weighed before and after inclusion into mealworm dishes to obtain whether the mealworms were consuming it. There were therefore 5 treatment groups: Control (WB + carrot), WB (wheat bran), CW (wheat bran + dry cotton wool), W (wheat bran + cotton wool + water), BC+S (wheat bran with beta carotene & sucrose + cotton wool + water) (Table 3.2).

Table 3.2 *Study 2: Formulation of mealworm feeds and water sources*

	Feed Treatments[1]				
Feed	Control	WB	CW	W	BC+S
Wheat bran (%)	99.885	99.885	99.885	99.885	93.900
Cellulose (%)[2]	0.115	0.115	0.115	0.115	0.115
Beta Carotene (%)	-	-	-	-	0.001
Sucrose (%)	-	-	-	-	5.983
Water Source					
Carrot (g)	10.00	-	-	-	-
Cotton Wool (g)	-	-	1.00	1.0	1.0
Water (ml)	-	-		8.80	8.80

[1]Control (wheat bran + carrot), WB (wheat bran), CW (wheat bran + cotton wool), W (wheat bran + cotton wool/water), BC+S (wheat bran, beta carotene, sucrose + cotton wool/water)
[2]Cellulose was used to serially dilute beta carotene and sucrose, therefore was included in all treatments

Study 3: Chick crumb and environment feeding trial

Wheat bran was sieved on a stack of sieves, the top sieve was 2mm and the bottom 0.5mm to retain a particle size of 0.5-2mm. This method of sieving wheat bran made it easier to obtain a value for feed intake as when a mixture of feed and frass was sieved, only the frass passed through the bottom smaller sieve. Chick crumb was obtained from a broiler trial

carried out at the University of Nottingham (Dale et al., 2020). There were 4 treatment groups, since wheat bran or chick crumb were fed either in the temperature and humidity-controlled incubator or in the room (under standard laboratory conditions) in a 2x2 factorial design, to determine the effects of the feed, the environment and their interaction on growing mealworms (Table 3.3).

Table 3.3 *Study 3: Experimental design where mealworms were fed either wheat bran or chick crumb in either an incubator or in a box within a room*

Location	Wheat bran feed	Chick crumb feed
Incubator	WB-I	CC-I
Room	WB-R	CC-R

WB-I (wheat bran, incubator), CC-I (chick crumb, incubator), WB-R (wheat bran, room) and CC-R (chick crumb, room).

Study 4 (Larval development) and Study 5 (Gut clearance)

In both studies mealworms were fed *ad libitum* wheat bran and water-soaked cotton wool.

3.2.3 Experimental Design

Study 1, 2 and 3

At day 0, groups of 200 larvae were counted into dishes and the total live weight determined. Dishes were randomly allocated to treatment groups. Larvae were then given their appropriate feed and water source; with the feeds being provided *ad libitum* throughout the 14-day trial period. On days 4, 7 and 11, stacked sieves were used to separate larvae, feed, and frass. Any dead larvae were removed, along with any skin that had been shed, and the remaining larvae were photographed. Total live weight, remaining

feed and frass weights were recorded, then larvae were returned to the dish with the appropriate fresh feed and water source. On day 14, the same process was followed, but separated live larvae were then killed through cold exposure (-20˚C). Samples (frozen remaining larvae from each dish) were stored at -20°C prior to further analyses.

Study 4

Mini-mealworms obtained from Monkfield nutrition were left to grow and pupate on *ad libitium* wheat bran and water soaked cotton wool. Once mealworms had started pupating, pupae were removed from the main mealworm container and placed in a new container. This process was continued until there was 300 pupae. Pupae were then randomly allocated to 4 separate containers (n=75) and allowed to transform to beetles on a wheat bran and water-soaked cotton wool. Once all beetles had emerged from pupae, beetles were left for 2 weeks. After these two weeks beetles were removed from containers and eggs allowed to hatch. After a further 2 weeks mealworms emerged and this marked day 0 of experiment. Weekly stacked sieves were used to separate larvae, feed, and frass. Any dead larvae were removed, along with any skin that had been shed. Then larvae were returned to the dish with the appropriate fresh feed and water source. Starting at week 6 samples were taken for proximate analysis, 2g live mass of mealworms were killed by cold exposure at -20˚C.

Study 5

At day 0, groups of 200 larvae were counted into 9 dishes and the total live weight determined. Larvae were then given *ad libitum* wheat bran and water-soaked cotton wool for 9 days. For this trial, all 9 replicates of mealworms were treated the same for the first 8 days. On day 9 stacked sieves were used to separate larvae, feed, and frass. Any dead larvae were removed, along with any skin that had been shed, and the remaining larvae were

photographed. On the 9th day 3 replicates were randomly chosen, total live weight recorded and then live larvae were then killed through cold exposure (-20˚C). For another 3 replicates, feed was removed for 3 hours and then total live weight recorded and then live larvae were then killed through cold exposure (-20˚C). The final 3 replicates, feed was removed for 24 hours, total live weight recorded and then live larvae were then killed through cold exposure (-20˚C). All samples (frozen remaining larvae from each dish) were stored at -20°C prior to further analyses.

3.2.4 Experimental Conditions

Studies 1 and 2

Black box files were used to keep the mealworms in dishes in the dark, but the temperature and humidity of the room were not controlled. The temperature fluctuated between 10-20˚C, humidity was not recorded.

Study 3

A temperature and humidity-controlled incubator set at 25˚C and 60% humidity was used for the incubator treatment groups based on the published literature. The room temperature fluctuated between 15-22˚C and 38-43% humidity and those larvae in these treatment groups were kept in a storage box in the lab.

Study 4 and 5

A temperature and humidity-controlled incubator set at 25˚C and 60% humidity was used for the incubator treatment groups based on the published literature.

3.2.5 Proximate nutritional analysis

Proximate nutritional analysis was carried out on remaining live larvae for each dish after drying to constant weight. Study 2 mealworm samples were oven dried at 80˚C whereas study 3, 4 and 5 mealworms were freeze dried. In both studies, samples were crushed to a fine powder using a pestle and mortar. Proximate nutritional analysis included crude protein analysis by nitrogen analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy content by bomb calorimetry (6300 Calorimeter, Parr) and total fat content via Soxhlet extraction (Soxtherm, Gerhardt) (see Chapter 2 for detailed methods).

3.2.6 Statistical analysis

All graphs were drawn using Graph Pad Prism 8. Genstat (20th Edition) was used to perform statistical analysis. For studies 1 and 2, effect of treatments on performance indicators (initial group weight, final group weight, group body weight gain and feed intake) were determined by one-way ANOVA, whereas effects on average individual mealworm weight were analysed by repeated measures 2-way ANOVA (time x treatment). Changes in cotton wool weight in study 2 were determined by pairwise T-test. Similarly, effects of treatment on proximate nutritional composition (dry matter, crude protein, total fat and total energy contents) were analysed by one-way ANOVA. For study 3 a two-way ANOVA (environment x feed) was used to determine effects on both performance indicators and proximate nutritional composition. Average individual mealworm weight was analysed by repeated measures 3-way ANOVA (time x feed x environment). For study 4 and 5 a one-way ANOVA was used to determine the effects of age or starvation on proximate nutritional composition

(dry matter, crude protein, total fat and total energy). Significance was set at P<0.05, with trends set at P<0.1 but >0.05. Where significant differences were observed, post hoc Bonferroni Tests were carried out to identify where the differences occurred.

3.3 Results

3.3.1 Study 1: Feed rehydration trial

There was no significant difference in the initial group weights (Table 3.4). Removing carrot and supplying water through rehydration of the wheat bran significantly reduced final group weights, body weight gain and feed intakes (Table 3.4), while numbers of deaths tended to be higher in the WB 50% group (P=0.087) (Table 3.4). Proximate nutritional analysis was not carried out on Study 1.

Table 3.4 *Study 1: The effect of increasing the water content of wheat bran on performance indicators over a 14-day period.*

Performance	Feed Treatments[1]						
	Control	WB 0%	WB 15%	WB 30%	WB 50%	SED[2]	P value
Initial Group Weight (g)	9.74	10.35	9.60	9.75	9.32	0.428	0.229
Final Group Weight (g)	18.39^{a}	10.36^{b}	10.28^{b}	11.01^{b}	10.76^{b}	0.521	<0.001
Deaths (%)	5.66	7.18	8.75	7.05	10.66	1.755	0.087
Body Weight Gain (g)	8.65^{a}	00.1^{b}	0.68^{bc}	1.26^{cd}	1.44^{d}	0.217	<0.001
Feed Intake (g)	15.50^{a}	11.56^{b}	13.42^{c}	13.91^{c}	12.25^{b}	0.294	<0.001

[1] Control (wheat bran + carrot), WB 0% (dried wheat bran), WB 15% (15% hydrated wheat bran), WB 30% (30% hydrated wheat bran), WB 50% (50% hydrated wheat bran)
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=5 for all treatment groups.
[a-d] Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

3.3.2 Study 2: Water-soaked cotton wool trial

There were no significant differences in the initial group weights (Table 3.5). Utilising cotton wool to hold the water (W) had no effect on the final group weight or body weight gain compared to the Control of just carrot (Table 3.5). Similarly, there was no effect of adding beta-carotene and sucrose (BC+S) on the final group weight or body weight gain compared to Control or W groups. However, completely removing the water source in the wheat bran (WB) and cotton wool (CW) groups significantly reduced final group weight and body weight gain (Table 3.5). This was associated with a significant decrease in total feed intake in the WB and CW groups compared to Control, whereas there was a significant increase in feed intake in the groups consuming water in the cotton wool (W and BC+S) (Table 3.5). There were no significant differences in the numbers of deaths (Table 3.5).

Table 3.5 *Study 2: The effect of replacing carrot with water-soaked cotton wool in wheat bran-based feeds on performance indicators over 14-day period*

	Feed Treatments[1]						
Performance	Control	WB	CW	W	BC+S	SED[2]	P value
Initial Group Weight (g)	13.90	13.80	14.09	13.96	14.32	0.373	0.680
Final Group Weight (g)	17.27^{a}	10.50^{b}	10.53^{b}	16.90^{a}	17.02^{a}	0.723	<0.001
Deaths (%)	27.30	30.40	33.50	31.10	31.80	3.220	0.428
Body Weight Gain (g)	3.37^{a}	-3.29^{b}	-3.56^{b}	2.94^{a}	2.70^{a}	0.485	<0.001
Feed Intake (g)	17.87^{a}	13.73^{b}	12.62^{b}	19.55^{c}	19.09^{ac}	0.451	<0.001

[1]Control (wheat bran + carrot), WB (wheat bran), CW (wheat bran + cotton wool), W (wheat bran + cotton wool/water), BC+S (wheat bran, beta carotene, sucrose + cotton wool/water)
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=5 for all treatments.
$^{a-c}$ Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

Mealworms did not consume the dry cotton wool in the CW group as the initial and final cotton wool weights were not significantly different for each of the measurement days (P=0.522) (Figure 3.1).

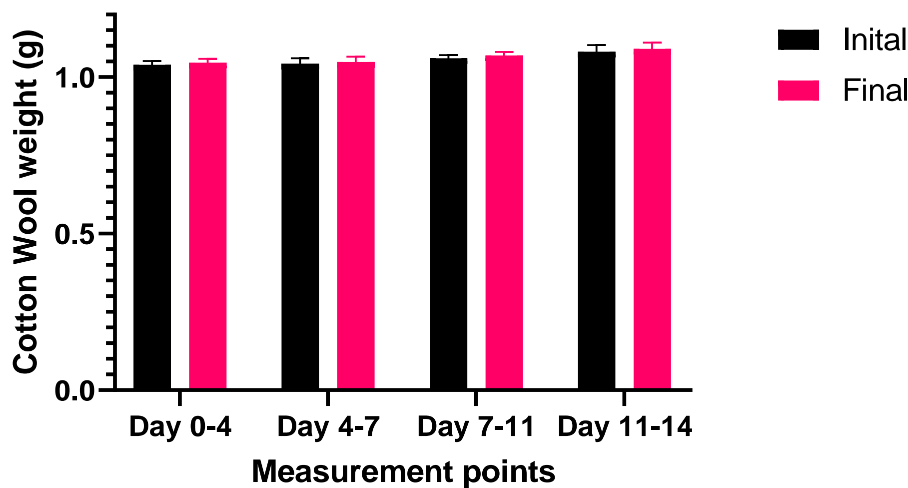


Figure 3.1 *Study 2: Initial and final cotton wool weights for each measurement day*

Initial = initial cotton wool weight. Final = final cotton wool weight after being in dish with mealworms. Data is presented as means±SEM, n=5 for each point

There was a significant interaction between time and treatment for average individual mealworm weight (P<0.001). Completely removing the water source in the WB and CW groups resulted in a dramatic reduction in growth over the 14 days (Figure 3.1) with the average individual mealworm weights of the WB and CW groups hardly increasing at all, which contrasts with the three groups supplied a water source (Control, W and BC+S) which all grew well.

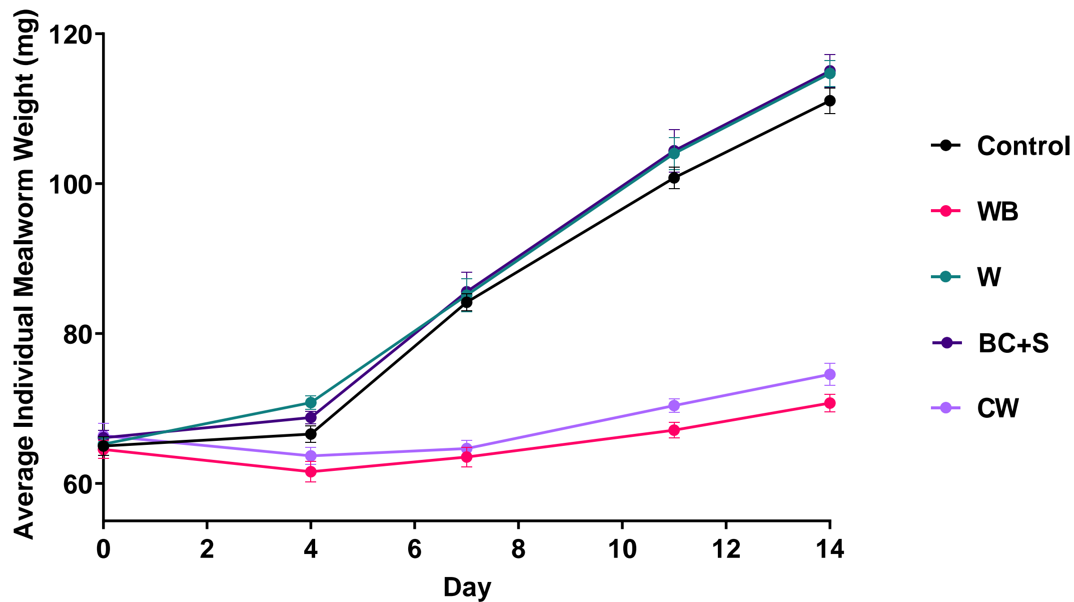


***Figure 3.2** Study 2: The effect of replacing carrot with water-soaked cotton wool in wheat bran based feeds on average individual mealworm weight over 14 day period.*

Control (wheat bran + carrot), WB (wheat bran), CW (cotton wool), BC+S (Beta-carotene + sucrose), W (wheat bran + cotton wool/water). Data is presented as means±SEM, n=5 for all treatment groups

Water supply method significantly affected proximate nutritional composition of the mealworm larvae (Table 3.6). Completely removing the water source (WB and CW groups) decreased mealworm dry matter content compared to the groups supplied a water source ($P<0.001$) and increased the crude protein content compared to Control and BC+S groups ($P<0.001$). Supplying water through cotton wool (W and BC+S) decreased the total fat content ($P<0.001$) and gross energy ($P=0.038$); however gross energy was not significantly different using a post hoc Bonferroni test (Table 3.6).

Table 3.6 *Study 2: The effect of replacing carrot with water-soaked cotton wool in wheat bran based feeds on proximate nutritional composition of mealworms after a 14-day period*

	Feed Treatments[1]						
Composition	Control	WB	CW	W	BC+S	SED[2]	P value
Dry Matter Yield (g)	5.60^{a}	4.17^{b}	4.23^{b}	5.35^{a}	5.36^{a}	0.256	<0.001
Crude Protein (g/kg DM)	545.3^{a}	573.8^{c}	577.9^{c}	562.7^{bc}	555.4^{ab}	0.506	<0.001
Total Fat (g/kg DM)	279.6^{a}	274.3^{a}	267.5^{a}	240.3^{b}	242.5^{b}	0.771	<0.001
Gross Energy (MJ/kgDM)	26.25^{a}	25.91^{a}	26.40^{a}	25.53^{a}	25.52^{a}	0.316	0.038

[1]Control (wheat bran + carrot), WB (wheat bran), CW (wheat bran + cotton wool), W (wheat bran + cotton wool/water), BC+S (wheat bran, beta carotene, sucrose + cotton wool/water)
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=5 for all treatment groups.
$^{a-b}$ Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

3.3.3 Study 3: Chick crumb and environment feeding trial

From here on all mealworm trials utilised water soaked cotton wool as the water source. There were no significant differences in initial group weights (Table 3.7). There was a trend for feed to influence final group weights (P=0.065) and body weight gain (P=0.085), with both being slightly lower in the WB-fed mealworms. Death percentage was significantly affected by environment (P<0.001), with a higher death rate of the mealworms housed in the incubator compared to the room (Table 3.7). There was a significant interaction between feed and environment for feed intake (P<0.001). This was due to higher intakes when mealworms were housed in the incubator, with no difference between the 2 feeds (CC-I and WB-I), whereas intakes were reduced when mealworms were housed in the room (CC-R and WB-R) with higher intakes of chick crumb (CC-R) compared to wheat bran (WB-R, Table 3.7).

Table 3.7 *Study 3: The effects of feeding chick crumb or wheat bran to mealworms in two environments on performance indicators over 14-day period*

	Feed Treatments[1]					P Value		
Performance	WB-I	WB-R	CC-I	CC-R	SED[2]	Feed	Environment	Interaction
Initial Group Weight (g)	12.56	12.42	12.65	12.66	0.439	0.602	0.840	0.801
Final Group Weight (g)	18.27	19.19	19.53	20.19	0.785	0.065	0.181	0.817
Deaths (%)	36.82	24.58	37.23	27.05	2.377	0.409	<0.001	0.551
Body Weight Gain (g)	5.71	6.78	6.89	7.53	0.719	0.082	0.119	0.685
Feed Intake (g)	39.10^{a}	31.29^{b}	38.82^{a}	34.76^{c}	0.622	0.006	<0.001	<0.001

[1]WB-I (fed wheat bran in incubator), WB-R (fed wheat bran in room), CC-I (fed chick crumb in incubator), CC-R (fed chick crumb in room)
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=4
$^{a-c}$ Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

There was a significant 3-way interaction between feed, environment, and time for average individual mealworm weight (P<0.037, Figure 3.2). From day 7 onwards CC-I mealworms were heaviest and WB-R mealworms were lightest, with WB-I and CC-R being in the middle. From day 11 onwards the mealworms fed chick crumb in the incubator had higher average individual mealworm weights compared to those fed wheat bran or housed in the room (Figure 3.2).

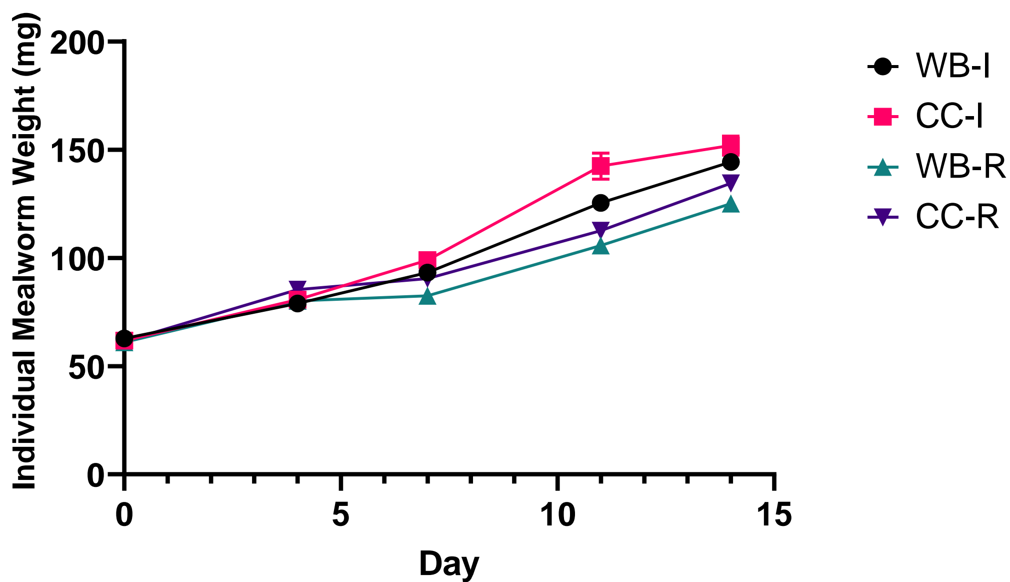


Figure 3.3 *Study 3: The effect of feeding chick crumb and wheat bran in 2 environments on average individual mealworm weight over 14-day period.*

WB-I (wheat bran, incubator), CC-I (chick crumb, incubator), WB-R (wheat bran, room) and CC-R (chick crumb, room). Data is presented as mean±SEM, n=4 for all treatment groups

There were no significant differences in proximate nutritional composition of mealworms fed chick crumb or wheat bran in either of the two environments (Table 3.8). There was a trend for an effect of feed on dry matter (P=0.062) and total fat (P=0.070) contents, with chick crumb increasing the dry matter, but reducing the fat content slightly compared to WB. In addition, there was a trend for an effect of environment on the total fat content (P=0.070), being slightly lower in mealworms housed in the Room compared to the Incubator.

Table 3.8 *Study 3: The effect of feeding either wheat bran or chick crumb in different environments on proximate nutritional composition of mealworms after a 14-day period.*

	Feed Treatments[1]					**P Value**		
	WB-I	WB-R	CC-I	CC-R	SED[2]	Feed	Environment	Interaction
Dry Matter Yield (g)	7.10	6.93	7.68	7.69	0.457	0.062	0.804	0.786
Crude Protein (g/kg DM)	499.7	494.3	479.3	483.9	1.618	0.204	0.972	0.671
Total Fat (g/kg DM)	261.0	240.4	240.3	223.2	1.346	0.070	0.070	0.856
Gross Energy (MJ/kg DM)	24.46	24.14	24.21	23.78	0.367	0.256	0.174	0.838

[1]WB-I (fed wheat bran in incubator), WB-R (fed wheat bran in room), CC-I (fed chick crumb in incubator), CC-R (fed chick crumb in room)
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at $P<0.05$. $n=4$ for all treatment groups.

3.3.4 Study 4: Larval development trial

There was a significant effect of time on dry matter, crude protein and total fat content of mealworms across an 8-week growth period (Table 3.9). Mealworm dry matter significantly increased with increasing age ($P<0.001$). The crude protein (g/kg DM) and total crude protein (g/gMW fresh weight) were also significantly affected by age, with the former decreasing between weeks 6 and 11 and the latter increasing between weeks 6 and 12 (Table 3.9). Both the total fat (g/kg DM) and total fat (g/gMW fresh weight) significantly changed with age ($P<0.001$), with both increasing between weeks 6 and 12 (Table 3.9).

Table 3.9 *Study 4: Larval development trial proximate nutritional composition of mealworms over an 8-week growth period*

	Week									
	6	7	8	9	10	11	12	13	SED[1]	P Value
Dry Matter Yield (g)	0.24[a]	0.30[ab]	0.35[abc]	0.40[abcd]	0.43[bcd]	0.46[cd]	0.51[d]	0.46[bcd]	0.043	<0.001
Crude Protein (g/kgDM[2])	604.6[a]	584.1[ab]	568.4[ab]	556.3[ab]	529.5[ab]	507.7[b]	574.5[ab]	520.7[ab]	2.428	0.013
Total Crude Protein (g/gMW FW[3])	0.15[a]	0.17[ab]	0.20[ab]	0.22[ab]	0.23[ab]	0.24[ab]	0.30[b]	0.24[ab]	0.031	0.006
Total Fat (%DM)	115.5[a]	969.0[a]	124.1[a]	172.5[b]	181.5[b]	202.6[bc]	260.9[d]	243.9[cd]	1.062	<0.001
Total Fat (g/gMW FW)	0.028[a]	0.029[a]	0.043[a]	0.069[b]	0.078[bc]	0.094[cd]	0.132[e]	0.111[de]	0.005	<0.001

[1]SED – standard error of the differences of the means, [2] DM – dry matter, [3]MW – mealworm (fresh weight)
Data is presented as means, significance set at P<0.05. n=3 for all treatment groups, apart from week 13 n=2.
[a-e] Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

3.3.5 Study 5: Gut clearance trial

There was a significant effect of starvation on dry matter (P<0.001), crude protein (P=0.037) and total fat (P<0.001). There was a significant decrease in dry matter and total fat content with 24 hours starvation compared to no starvation and 3 hours starvation (Table 3.10). There was a significant increase in crude protein content in the 24-hour starvation period group compared to the control but not the 3-hour starvation group (Table 3.10). There were no significant differences in gross energy between any of the groups, however there was a trend for reduced gross energy content in the 24 hour starved mealworms (P<0.1) (Table 3.10).

Table 3.10 *Study 5: Proximate composition of mealworms after varying periods of starvation*

	No starvation	**3 hours starvation**	**24 hours starvation**	**SED**	**P Value**
Dry Matter Yield (g)	9.49^{a}	8.57^{a}	6.44^{b}	0.320	<0.001
Crude Protein (g/kg DM)	496.0^{a}	518.1^{ab}	544.1^{b}	1.390	0.037
Total Fat (g/kg DM)	243.5^{a}	259.6^{a}	198.2^{b}	0.569	<0.001
Gross Energy (MJ/kg DM)	24.48	24.74	23.91	0.290	0.068

[1]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=4 for all treatment groups.
[a-b] Means within a row with different superscripts are significantly different (P<0.05, Bonferroni)

3.4 Discussion

Study 1 and 2: Complete removal of water source and rehydration of wheat bran has negative effects on mealworm growth and feed intake but there were no differences between supplying water through carrot or water-soaked cotton wool

Both studies 1 and 2 revealed that removing carrot and feeding WB alone significantly reduced mealworm growth. Complete removal of the water source (study 2) resulted in the lowest group final weight and lowest feed intakes compared to those mealworms that were supplied a water source. Hence, wheat bran does not supply enough water for growth of mealworms and the hypothesis that manipulating the water content affects the mealworm growth can be accepted. Wheat bran has previously been suggested to provide all the nutrients required for growth and development of mealworms (Ortiz et al., 2016), but at 87% dry matter, it has a low moisture content (Heuze et al., 2015). Low moisture feeds have been implicated with halting development of mealworms if there is no other water supply (N. Ribeiro et al., 2018). Indeed, a low water supply can result in the disruption of haemolymph pressure and therefore disruption of metabolic function (Gäde, 2004). In study 2 there was a significant decrease in feed intake in the water deprived groups, potentially causing the reduction in weight compared to those supplied a water source. Water deprived mealworms have been shown to ingest less food and therefore have a decreased body weight (N. Ribeiro et al., 2018). The larvae of *Tribolium* (Coleoptera) can meet most of their water requirement through food oxidation (Chapman, 2013). Mealworms belong to the same family, suggesting that they could possibly obtain sufficient water through the oxidation of food source for survival and the balance to maintain metabolic functions, but

maybe not growth. Interestingly, mealworms will eat (Yang et al., 2015), therefore dry cotton wool was included as an individual treatment group in study 2, just to check if they consumed the cotton wool. There were no changes in the weights of cotton wool, confirming that the mealworms are not eating it and not gaining any nutritional value from the cotton wool. However, there is the potential that if there was no other feed source apart from cotton wool, then mealworms could start to eat the cotton wool.

Supplying water by rehydrating wheat bran negatively affected mealworm growth and feed intake compared to the control, which utilised carrot as the water source. Hence the hypothesis can be accepted that manipulating the water source affects both mealworm growth and feed intake. Mealworms that were supplied water within the wheat bran reduced feed consumption. This suggests that a wet feed is not suitable for mealworms, potentially due to an altered viscosity with increasing rehydration, correlating to the lowest feed intakes in the 50% group. This indicates that mealworms require an external source for water which is not part of their feed or that the carrot in the control group could be providing further nutritional value in addition to being a water source. van Broekhoven *et al.* (2015) suggested that carrot is utilised by mealworms to compensate for a poor-quality feed, since carrots are a good source of beta carotene and sucrose, alongside other sugars (Sharma et al., 2012), which could be an additional energy source for growth.

Study 2 further investigated alternative methods for supplying water and whether carrot was an additional nutrient source for growth as well as an external source of water. It again confirmed reduced growth rates in the absence of water. However, there were no growth differences using carrot, water-soaked cotton wool or water-soaked cotton wool containing beta-carotene and sucrose, suggesting water-soaked cotton wool could be used instead of

carrot and that the specific nutrients in carrot were not impacting on growth. This agrees with other studies where mealworms produced on dry substrates had higher growth rates when there was a suitable source of water (Liu et al., 2020; Urs and Hopkins, 1973).

Supplying water through cotton wool resulted in increased protein, decreased fat and no differences in dry matter compared to the control (WB + carrot). Furthermore, completely removing the water source resulted in a lower dry matter and higher crude protein content compared to those mealworms that had a water supply. Therefore, manipulating the water source does affect the proximate composition of mealworms. It is important to state that the proximate composition of all mealworms in study 2 was similar to previously published data (Barker et al., 1998). However, a previous study using a carrots, red cabbage and oranges as a water source compared to wheat bran with no water source resulted in no differences in mealworm proximate composition (Liu et al., 2020). Similarly, in study 2 the inclusion of carrot, as a water source, resulted in no change in total fat content, however there was a decrease in crude protein content compared to no water source. Another study which utilised cotton wool pads to hold water reported an increase in total fat (Hopkins and Urs, 1973), whereas there was a reduction in total fat content when water-soaked cotton wool was used as a water source compared to no water source in study 2. Even though there were significant differences in total fat content in study 2, there were no significant differences in the gross energy, when it would be expected that a higher fat content would be associated with higher energy. This suggests that there may be differences in other energy sources such as carbohydrates.

Study 3: Feeding chick crumb does not improve growth or composition of mealworms compared to wheat bran.

The study comparing chick crumb and wheat bran as feed sources found that there were no differences in the growth of the mealworms. This contrasts with (Bordiean et al., 2020) who reported an increase in larval weight when feeding wheat bran compared to chick crumb. However younger mealworms were used in that study compared to study 3, which may account for the differential responses to the feeds. Feeding wheat bran reduced feed intakes compared to chick crumb, which may be due to wheat bran having an increased crude fibre content (10.4% DM) compared to chick crumb (3.07% DM) (Dale et al., 2020; Heuze et al., 2015), and is often perceived as a bulking ingredient (McDonald et al., 2011). An increased fibre content could have led to an increased satiety in the mealworms, subsequently decreasing feed intake. However, a decreased feed intake might be expected to reduce mealworm growth or impact on proximate composition, but there were no effects. The lack of changes observed in proximate composition of mealworms was unexpected, but there was a trend for the feed to affect dry matter and total fat contents, therefore a longer feeding trial may result in significant differences. The chick crumb would be expected to have a higher metabolisable energy compared to wheat bran and therefore could result in increased fat deposition within the mealworms, but this was not seen in study 3. There was no change in the crude protein content of the mealworms, suggesting that the additional synthetic amino acids provided in the chick crumb were either not absorbed or not utilised by the mealworms.

Study 1,2,3: Temperature and humidity impacts on the growth but not the proximate composition of mealworms.

The environment in which the three studies were carried out varied considerably due to the facilities available at the time. Study 3 was a direct comparison between a controlled and uncontrolled environment, but there were no differences in final group mealworm weight or proximate composition between the two environments, although individual mealworm weight was higher in the incubator compared to the room. Therefore, temperature and humidity do appear to impact on mealworm growth.

The constant temperature in the incubator could have increased physical activity of the mealworms and resulted in increased intakes and consequently growth. Feeding mealworms in the room resulted in bigger individual mealworm weights but higher death rates so overall there was no differences in group weight. Mealworms have been reported to have increased larval mass when produced at a humidity of 84% compared to 43% (Johnsen et al., 2021). However, in study 3 there were no differences in weights of mealworms housed in the incubator where the humidity was set at 60% compared to those kept in the room at 40% humidity. Surprisingly, growing mealworms in the incubator actually resulted in a higher proportion of deaths than in the room, which may be due to the conditions in the incubator not being ideal for the mealworms. The temperature and humidity chosen for the incubator were those reported in the literature (N. Ribeiro et al., 2018). However, if the mealworms were previously kept in different environmental conditions by the commercial supplier prior to delivery, then they may not be able to adapt to these different conditions.

As both studies 1 and 2 were carried out under normal room conditions and not in a temperature-controlled incubator, temperature fluctuations could have influenced the growth of the mealworms. Stress conditions for mealworms have been reported as below 10°C and above 35°C (Ribeiro et al., 2018). Recorded temperatures during studies 1 and 2 did not exceed these, but it should be noted that night temperatures were not recorded so could have dropped below 10°C. It was recorded that temperatures fluctuated between 10°C and 20°C during these studies, and it has been reported that larvae maintained at a temperature lower than 23°C will gradually lose weight (Punzo, 1975) while temperatures below 17°C can inhibit development (Koo et al., 2013). Additionally, colder conditions could decrease physical activity levels leading to reduced feed intake and potentially contribute to reduced growth. In agreement with this, Study 3 was carried out in either a temperature-controlled incubator or a warm room and resulted in higher average individual mealworm weights compared to study 2, carried out in a colder room. This was after a two-week growth period and even though study 2 started with higher weights, further suggesting that temperature plays a critical role in the growth of mealworms. However it has been highlighted that relative humidity plays a major role in combination with temperature (Ribeiro et al., 2018) and humidity was only controlled in study 3.

Study 4 and 5: Larval age and period of starvation impact on proximate nutritional composition of mealworms

The crude protein of mealworms on a dry matter basis decreased with increasing larval age while total protein content per unit weight of mealworms fresh weight increased due to increasing dry matter. There was an increase in total fat content fresh weight as the mealworms increased in age. Starving mealworms for 24 hours increased crude protein and decreased total fat content of mealworms. Therefore, the hypothesis can be accepted that mealworm nutrient composition changes with both larval age and starvation.

As the total fat content of mealworms increased with age, it suggests that fat was being stored as the mealworm reached pupation age. The larval body form needs to have an adequate nutritional composition in a storage format for the pupal form to survive through to metamorphosis into the adult form (Nijhout, 2003). The increase in total protein content per unit weight of mealworm due to increasing dry matter was potentially due to growth and metabolic changes before metamorphosis and suggests that harvesting mealworms closer to pupation is most appropriate for use as a feed ingredient.

Starving mealworms resulted in an increase in crude protein content, suggesting that mealworms were utilising fat stores for energy whilst no food source was present. In insects, the fat body is the main tissue involved in starvation induced autophagy, where stored triglycerides are metabolised to support energy (Chapman, 2013). Furthermore, Wynants et al., (2017) noted that there was no difference in microbial numbers between larvae that were fed and starved. If starvation does not remove the microbial load from the guts (Wynants et al., 2017), this still could be an issue with inclusion in animal feed. Ensuring

that mealworms are fed a controlled feed source would help to control the gut microbial load, for example using a cereal source compared to manure.

3.5 Conclusions

Water can be supplied to mealworms via water-soaked cotton wool as a viable alternative to vegetables, such as carrot, with no effects on performance or proximate nutritional composition. However, mealworms do need a water source, as complete removal of a water source results in a significant decrease in both feed consumption and growth. Adding water to the wheat bran is not a solution, as this also results in decreased feed consumption and growth, while also appearing to increase the percentage deaths.

Utilising a temperature and humidity-controlled incubator resulted in higher individual average weights of mealworms with no impact on composition, but further work is needed to investigate the increased death rates compared to the room (laboratory), as this suggests that the conditions are not yet ideal.

Importantly, there were no benefits of feeding mealworms on chick crumb compared to wheat bran over a 14-day trial period, as there were no differences in growth performance or proximate nutritional composition. Feeding chick crumb would be much more expensive than wheat bran, and the inclusion of a vegetable, such as carrot, as a water source also increases the cost of production.

Mealworm composition changes with age, with increasing fat and decreasing of protein content the closer to pupation, suggesting that mealworms closer to pupation should be used as a feed ingredient. Additionally, starving mealworms for 24 hours resulted in

increased crude protein and decreased total fat, however this does not address the potential microbial load which could be passed on if used in animal feed.

Overall, this series of experiments suggests that mealworms can be produced as a protein source for animal feed by feeding relatively low cost ingredients (wheat bran opposed to chick crumb) and water can be supplied (used soaked cotton wool) without the need for added vegetable matter. Further work is required to define the optimal environmental conditions and how best to avoid potential hazards associated with microbial contamination.

4 Use of commercial enzymes to manipulate the nutritional value and growth of mealworms

4.1 Introduction

Insects are attracting growing attention, as a potential supplementary feed ingredient for animal feeds, specifically because they can be grown on poor quality feed. Development of traditional livestock production systems has seen the increased use of enzymes to help with digestion of poor quality feeds. To our knowledge, no work has been carried out to see if commercially available enzymes can improve mealworm production.

Wheat bran is a by-product from milling wheat into flour and it is routinely used in traditional livestock feed, as well as in human diets (Onipe et al., 2015). It is very palatable (Fuller, 2004), but is only included at low levels in animal feed as it is perceived as a high fibre bulking ingredient (McDonald et al., 2011). As the adult form of mealworms, the darkling beetle, is a known flour pest (Park et al., 2014), wheat bran has been suggested to represent a suitable feed source for mealworms. Wheat bran consists of the outer pericarp layer of wheat seed (Chalamacharla et al., 2018). It contains 46% w/w DM non-starch polysaccharides, including arabinoxylan, cellulose and beta glucan (Carré and Brillouet, 1986), with a crude protein content of 14-19% (on a dry matter basis) and a good mineral content (Heuze et al., 2015), making it a well-balanced feed source. Minerals found in wheat

bran include iron, zinc, manganese, magnesium and phosphorus (Onipe et al., 2015). It also contains bioactive compounds such as phenolic acids and phytosterols (Onipe et al., 2015). The main concern with wheat bran as a feed ingredient is the presence of anti-nutritional factors (ANFs) (Chalamacharla et al., 2018), including phytate (626-795mg/100g wheat bran) and tannins (0.03-0.07 mg/100g wheat bran) (Hassan et al., 2008), which influence the availability of many nutrients.

Phytate is the salt and stable form of phytic acid, which is bound to metal cations (Humer et al., 2015). Phytic acid is synthesised from a myo-inositol ring bound to six phosphate ester bonds (Dersjant-Li et al., 2015). The twelve replaceable negative active sites on the phytic acid molecule mean it is able to bind to minerals, proteins and amino acids to form complexes (Dersjant-Li et al., 2015). Phytate is most common in plant derived foods such as cereals or legumes (Kumar et al., 2010) and approximately 80% of the phosphorus in wheat bran is stored as phytate (Onipe et al., 2015).

Hence, phytate can have negative impacts on feed and protein digestibility (Gupta et al., 2013), both of which are decreased due to the insoluble complexes formed. Protein digestibility is diminished as these insoluble complexes alter the protein structure thereby reducing solubility, enzyme activity and proteolytic digestibility. Furthermore phytate can supress amylase activity which then depresses carbohydrate digestion (Deshpande and Cheryan, 1984).

Non-starch polysaccharides can hold significant amounts of water (Bedford and Partridge, 2010), creating viscous solutions which reduce passage time through the gut and thereby reduce nutrient digestion, growth and performance (Annison and Choct, 1991).

Exogenous enzymes can be used to reduce the effects of some ANF's, including phytate. Commercial feed enzymes have been used since the 1980's to alter the nutritional profile of feed ingredients in monogastrics (Bedford and Partridge, 2010). Feed enzymes act in a number of different ways: (i) to improve the availability of protein, oil and polysaccharides which are otherwise unavailable due to cell wall structures; (ii) to break down anti-nutritional factors, such as phytate; (iii) to supplement young animal's endogenous enzymes; and (iv) to increase utilisation of nutrients in the small intestine (McDonald et al., 2011). All these modes of action help to increase the efficiency of feed digestion, improve consistency and help maintain gut health (Bedford and Partridge, 2010; Cowieson et al., 2006). Common feed enzymes include various carbohydrases (e.g. xylanase/ B glucanase and amylase), proteases and phytases (Bedford and Partridge, 2010). The action of these enzymes can be affected by moisture content, temperature, pH, enzyme concentration and substrate concentration (Ravindran, 2013).

Phytases hydrolyse the removal of phosphate from phytic acid in a step wise manner (Selle and Ravindran, 2007), starting with the fully phosphorylated phytic acid (IP6) that is hydrolysed to penta- (IP5), tetra- (IP4) and so on down to the mono-ester of inositol (Dersjant-Li et al., 2015). Complete hydrolysis will result in the release of myo-inositol and phosphate, as well as any amino acids, minerals or other nutrients which were previously bound to the phytic acid, thereby reducing the anti-nutritional effect of phytate, improving digestibility and enhancing nutrient utilisation (Kryukov et al., 2021). Plants actually have intrinsic levels of phytase, but it is inactive when fed as dry matter. Wheat bran is reported to have a high phytase activity (915-1561 FTU/kg) as well as a high phytate content (2.1-7.3 g/100g) (Guo et al., 2015).

Xylanases digest complex non-starch polysaccharides (NSP) found in the cell walls of plants (Bedford and Partridge, 2010). They are thought to degrade the NSPs in 2 stages, firstly the xylanase breaks down cell walls, releasing fermentable xylo-oligosaccharides (XOS) in the ileum and then the bacteria in the caeca ferment the released XOS producing volatile fatty acids and increasing the growth of beneficial bacteria (Bedford, 2000). This alters the microbial profile in the gut, potentially leading to improved animal performance. Hence, the XOS released by the action of xylanase are thought to act as prebiotics in the caeca (Courtin et al., 2008).

Multiple studies feeding XOS to monogastrics have shown a reduction in feed intake (Zhenping et al., 2013). XOS has been suggested to have prebiotic effects on the microflora, aiding in the breakdown of fibre improving digestion (T. Ribeiro et al., 2018). The prebiotic effect on the microflora has included an increase of butyrate-producing bacteria in the caecum and lactobacilli in the colon in broilers (De Maesschalck et al., 2015). In broilers, supplementation of a high level of XOS had a positive effect on the concentration of NSP in the ileum but there was limited effect on performance (Craig et al., 2020), but the combination of both xylanase and XOS together may have added benefits.

This chapter aims to determine whether inclusion of commercially available enzymes in mealworm feeds can alter mealworm performance and nutritional composition.

Aims

1. To determine impact on growth of addition of exogenous enzymes and xylo-oligosaccharides to mealworm feed.
2. To investigate changes in proximate nutritional composition of mealworms when fed exogenous enzymes and xylo-oligosaccharides.
3. To determine the effect of exogenous phytase on the phytic acid content of wheat bran.

Hypothesis

The addition of commercially available phytase, xylanase and XOS will alter mealworm growth characteristics and nutritional composition.

4.2 Materials and Methods

Multiple mealworm trials were carried out as part of this chapter, all were carried out under the same experimental methods but with differing treatment groups and conditions. Detailed methods are presented in chapter 2.

4.2.1 Animal Husbandry

Mini mealworms were purchased from Monkfield Nutrition (Ely, UK) and maintained on an *ad libitum* feed of wheat bran and water-soaked cotton wool until day 0 for all studies in this chapter.

4.2.2 Exogenous Enzymes

All exogenous enzymes used in this chapter were supplied by AB Vista (Woodstock, Marlborough). The exogenous phytase used was an enhanced *E.Coli* phytase (commercial name Quantum Blue). Two different forms of phytase were used, solid (granules) or liquid and had stock concentrations of 5000 FTU/g and 5000 FTU/L respectively. The activity of phytase is expressed as FTU, which is the amount of phytase that liberates 15µmol per L of sodium phytate at 37°C (Kerr et al., 2010).

The exogenous xylanase used was a thermostable beta 1-4, endo-xylanase (commercial name Econase XT). It was in a solid powder form and had a stock concentration of 100g/tonne.

The xylo-oligosaccharide used was XOS 35A (Batch A20170611 – supplied from AB Vista). This fermentable xylo-oligosaccharide was combined with Econase XT to form the commercial product, Signis, with stock concentrations of 100g/tonne Econase XT and 50g/tonne XOS.

4.2.3 Phytic Acid Assay

The phytic acid content of wheat bran, with and without treatment of water or increasing doses of exogenous phytase, was determined to establish if treatment with exogenous phytase reduced the wheat bran phytic acid content compared to water treatment alone.

Sample preparation

Wheat bran was soaked in water or water + phytase for 6 hours. Liquid phytase enzyme (stock concentration 500 FTU/ml) was used for easy incorporation into water for soaking in

wheat bran. After soaking wheat bran was then dried to a constant weight in an oven at 60°C.

The six treatment groups are as shown in Table 4.1.

Table 4.1 *Study 7: composition of dose response trial feeds*

Group[1]	Treatment
WB	Wheat bran
WB+W	Wheat bran soaked in water
WB+100	Wheat bran soaked in water + 100 FTU/L Exogenous Phytase
WB+500	Wheat bran soaked in water + 500 FTU/L Exogenous Phytase
WB+2000	Wheat bran soaked in water + 2000 FTU/L Exogenous Phytase
WB+5000	Wheat bran soaked in water + 5000 FTU/L Exogenous Phytase

[1]WB = wheat bran, W = water. All feeds were soaked in the treatment above dried to constant weight, then fed to the mealworms.

Phytic Acid Assay

Megazyme Phytic acid / total phosphorus assay (Megazyme, Co. Wicklow, Ireland) was used to measure phosphorus released by phytase and alkaline phosphatase. Total phosphate released was measured using a modified colorimetric method.

1g of soaked wheat bran sample was mixed with 20mL of 0.66M hydrochloric acid, then left mixing (using a magnetic flea) in a foil covered beaker overnight. Following acid digestion overnight, 1ml of extract was pipetted into a 1.5ml tube and centrifuged at 13000 rpm for 10 minutes. After this 0.5mL of the supernatant was neutralised with 0.5ml of 0.75M sodium hydroxide. The sample extract was then ready for assay.

For the enzyme dephosphorylation reaction assay two reactions were run simultaneously, with and without enzyme (Table 4.2). The free phosphorus pathway measured the phosphorus in the sample that was not bound to phytate. The total phosphorus pathway used phytase or alkaline phosphatase to release all the phosphorus (both bound to phytate plus free phosphorus) to give a measure of total phosphorus. The two pathways are demonstrated in Figure 4.1.

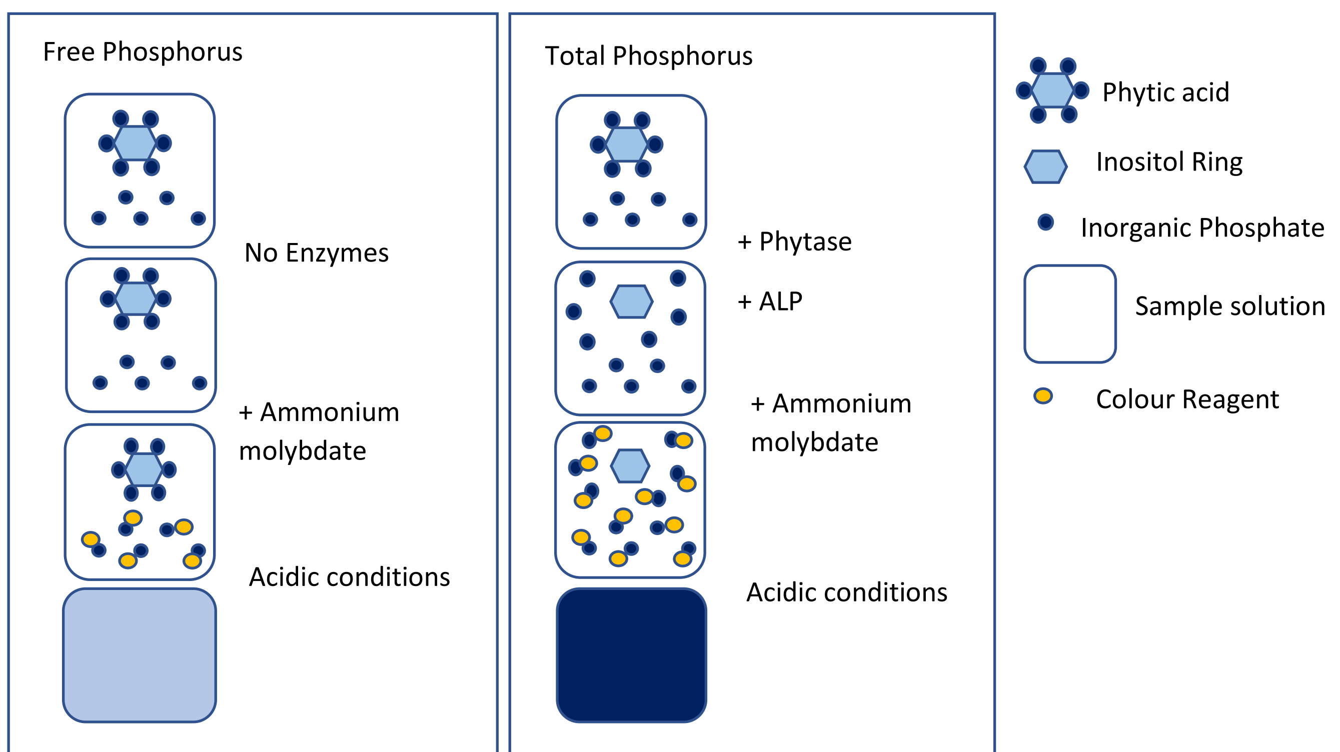


Figure 4.1 *Diagram demonstrating the reactions in the free and total phosphorus pathways.*
ALP = alkaline phosphatase

For the two-pathway methodology, the following solutions were prepared and used as follows: Solution 1 was a buffer (pH 5.5) with sodium azide (0.02% w/v); Suspension 2 was a phytase suspension, the bottle was swirled each time before use; Solution 3 was a buffer (pH 10.4) with magnesium chloride, zinc sulphate and sodium azide (0.02% w/v); Suspension

4 was alkaline phosphatase suspension, and the bottle was swirled each time before use. In both solutions 1 and 3 sodium azide was acting as a preservative.

Table 4.2 *Two-way pathway methodology for enzymatic phytic acid assay*

Pipette into 1.5ml microfuge tubes	Free Phosphorus	Total Phosphorus
Distilled water Solution 1 (Buffer) Sample Extract Suspension 2 (Phytase)	0.62ml 0.20 ml 0.05 ml -	0.60 ml 0.20 ml 0.05 ml 0.02 ml
Mix by vortex and incubate in a water bath set at 40°C for 10 mins. After 10 minutes, start the next reaction by addition of:		
Distilled water Solution 3 (Buffer) Suspension 4 (ALP)	0.02 ml 0.20 ml -	- 0.20 ml 0.02 ml
Mix by vortex and incubate in a water bath set at 40°C for 15 min. After 15 min, stop the reaction by addition of:		
Trichloroacetic acid (50% w/v)	0.30 ml	0.30 ml
Centrifuge the terminated reaction at 13,000 rpm for 10 min.		

ALP = alkaline phosphatase

Colorimetric determination

The enzymatic reaction produced molybdenum blue, which was proportional to the amount of phosphorus in the reaction. The molybdenum blue was measured through colorimetric determination using a wavelength of 655nm in a 1cm light path.

The colorimetric determination involves a colour reagent made from two solutions. Solution A was ascorbic acid (10% w/v) and sulphuric acid (1M): 10g of ascorbic acid was added to 90ml of distilled water, 5.35ml concentrated sulphuric acid was then added to dissolve the ascorbic acid. The total volume was then made up to 100ml with distilled water. Solution B was ammonium molybdate (5% w/v): 1.25g of ammonium molybdate was added to 20ml of distilled water and dissolved. The total volume was then made up to 25ml with distilled water. During the incubation steps of the enzyme dephosphorylation reaction, the colour

reagent was made by mixing 1 part of solution B with 5 parts of solution A, with enough colour reagent being made to analyse all samples plus blanks and standards.

Following the final centrifugation step of the enzyme dephosphorylation reaction, 1ml of the supernatant was pipetted into a 1.5ml microfuge tube and 0.5ml of colour reagent was added. The same was carried out for blanks and standards. This was then mixed via vortex and incubated at 40°C for 1 hour. Following this incubation, the mixture was vortexed again and 1ml transferred into a semi-micro cuvette and absorbance was read at 655nm within 3 hours on a spectrophotometer.

Phytic acid content calculation

Phosphorus was quantified from a calibration curve generated using standards of known concentrations of phosphorus ranging from 0µg to 7.5µg (Table 4.3).

Table 4.3 *Preparations of phosphorus solutions to be used as standards in phytic acid assay*

	Standard[1]				
	STD0 (0µg)	STD1 (0.5µg)	STD2 (2.5µg)	STD3 (5µg)	STD4 (7.5µg)
Distilled water (ml)	5.00	4.95	4.75	4.50	4.25
P.Std.Sol 5 (ml)[2]	-	0.05	0.25	0.50	0.75

[1]STD = standard
[2]P.Std.Sol 5 = Phosphorus standard solution 5 (50µg/ml) and sodium azide (0.02% w/v)

The absorbance of each phosphorus standard was measured. The absorbance of the 0 standard (0µg) was subtracted from the absorbance of all the other standards to obtain the

change in the absorbance of phosphorus ($\Delta A_{phosphorus}$). M was then calculated for each standard (STD1-4) as in equation 4.1.

$$M = \frac{P\ (\mu g)}{\Delta A_{phosphorus}} \quad (4.1)$$

These values for M were then averaged for all standards giving a mean M value, as in equation 4.2.

$$mean\ M = \frac{(M_{STD1}+M_{STD2}+M_{STD3}+M_{STD4})}{4} \quad (4.2)$$

Following this calculation the absorbance for the 'Free phosphorus' was subtracted from the 'Total phosphorus' obtaining a $\Delta A_{phosphorus}$ value. The concentration of phosphorus (c) was then calculated using equation 4.3.

$$c = \frac{mean\ M*20*55.6}{10\ 000*1.0*1.0} \times \Delta A_{phosphorus} \qquad (4.3)$$

Where:

mean M = mean value of phosphorus standards (ug/$\Delta A_{phosphorus)}$

20 = original sample extract volume (ml)

55.6 = dilution factor

ΔA = absorbance change of sample

10000 = conversion from µg/g to g/100g

1.0 = weight of original sample material (g)

1.0 = sample volume (used in the colourmetric determination)

The value for phosphorus was then converted into phytic acid (Equation 4.4).

$$Phytic\ acid\ [\frac{g}{100g}] = \frac{c}{0.282} \qquad (4.4)$$

4.2.4 Feed preparation

Study 6: Initial Phytase Trial

Five treatments were prepared (Table 4.4) with a base of wheat bran and water-soaked cotton wool (Control). Phytase was included in either the wheat bran (in the granule form) or the water (in the liquid form) at either high (5000 FTU per L or Kg) or low (500 FTU per L or Kg) concentrations, resulting in 4 Phytase treatments: High Phytase in Feed (HPF), Low Phytase in Feed (LPF), High Phytase in water (HPW) or Low Phytase in Water (LPW).

Table 4.4 *Study 6: Composition of initial phytase trial feeds with exogenous phytase concentrations used*

Feeds[1]	Wheat Bran	Water	Phytase Concentration	Phytase Form
Control				
HPF	✓		5000 FTU/kg	Granules
LPF	✓		500 FTU/kg	Granules
HPW		✓	5000 FTU/L	Liquid
LPW		✓	500 FTU/L	Liquid

✓ Indicates which feed component the exogenous phytase was added to.
[1]Control (wheat bran plus cotton wool with water), HPF (high dose phytase in feed (5000FTU/kg)), LPF (low dose phytase in feed (500 FTU/kg)), HPW (high dose phytase in water (5000 FTU/L)) and LPW (low dose phytase in water (500FTU/L))

Study 7: Phytase dose response trial

Feeds had already been prepared for the phytic acid assay. From the results of the phytic acid assay the feeds chosen to be used were WB, WB + water, WB + 500FTU/L and WB + 5000FTU/L (Table 4.1). The first concentration of 500 FTU/L is commonly used in livestock practise, whereas the second concentration of 5000 FTU/L is classed as super dosing. This is 10 times higher than would be used in any commercial setting and used to look at extreme effects of enzymatic inclusion.

Study 8: Combination enzyme trial

The enzymes used in this study were phytase (Quantum Blue) and xylanase (Econase XT), along with xylo-oligosaccharides (XOS 35A), which were all added either individually or in combinations, resulting in eight treatment groups (Table 4.5). Xylanase and XOS were both in solid form so were diluted with cellulose prior to mixing with the wheat bran. Wheat bran was then soaked with an equal measure of water, 1ml per g. Phytase (liquid form) was added to water, which was then used to soak an equal measure of wheat bran. All feed treatments were left to soak for 6 hours and then oven dried at 50°C to a constant weight. All feeds were fed to mealworms as the dried form, with water supplied through cotton wool.

Table 4.5 *Study 8: Feed treatments used in the combination enzyme mealworm trial*

Treatment[1]	Description
Control	Wheat bran
XYL	Wheat bran mixed with 500g/tonne xylanase
XOS	Wheat bran mixed with 250g/tonne xylo-oligosaccharides
PHY	Wheat bran mixed with 5000FTU/L phytase
XYL+XOS	Wheat bran mixed with 500g/tonne XYL and 250g/tonne XOS
XYL+PHY	Wheat bran mixed with 500g/tonne XYL and 5000FTU/L phytase
XOS+PHY	Wheat bran mixed with 250g/tonne XOS and 5000 FTU/L phytase
XYL+XOS+PHY	Wheat bran mixed with 500g/tonne XYL, 250g/tonne XOS and 5000 FTU/L phytase

[1] XYL (xylanase), XOS (xylo-oligosaccharides), PHY (phytase)

4.2.5 Experimental Design

For both studies 6 and 7 (phytase trials), 200 mealworms were counted into dishes and the total live weight determined, whereas 300 mealworms per dish were used for study 8 (combination enzyme trial). Dishes were randomly allocated to treatment groups. Larvae were then given their appropriate feed and water source, both of which were provided *ad libitum* throughout the 14-day trial period. On days 4, 7 and 11, stacked sieves were used to separate larvae, feed and frass. Any dead larvae were removed, along with any skin that had been shed, and the remaining larvae were returned to the dish with the appropriate fresh feed and water source. On day 14, the same process was followed, but separated live larvae

were then killed through cold exposure (-20°C). Samples (frozen remaining larvae from each dish) were stored at -20°C prior to further analyses.

Study 6

Black box files were used to keep the mealworms in dishes in the dark, but the temperature and humidity of the room were not controlled. The temperature fluctuated between 10-20˚C, humidity was not recorded.

Study 7

A cardboard box in a temperature-controlled room was used to keep the mealworms in dishes in the dark. The temperature was set at 30°C, humidity was not recorded or controlled.

Study 8

Dishes were kept in a dark temperature and humidity-controlled incubator set at 25˚C and 60% relative humidity.

4.2.6 Proximate nutritional analysis

Proximate nutritional analysis was carried out on remaining live larvae for each dish after drying to a constant weight. In study 6 and 7, mealworm samples were oven dried at 80°C, whereas in study 8, mealworms were freeze dried. Samples were crushed to a fine powder using a pestle and mortar. Proximate nutritional analysis included crude protein analysis by nitrogen analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy content by bomb calorimetry (6300 Calorimeter, Parr), total fat content via Soxhlet extraction (Soxtherm, Gerhardt) and mineral analysis (Acid digestion and ICPMS) (see Chapter 2 for detailed methods).

4.2.7 Statistical analysis

All graphs were drawn using Graph Pad Prism 8. Genstat (20th Edition) was used to perform statistical analysis. For studies 6 and 7, effects of treatments on performance indicators (initial group weight, final group weight, group body weight gain and feed intake) were determined by one-way ANOVA. Similarly, effects of treatment on proximate nutritional composition (dry matter, crude protein, total fat, and total energy contents) and mineral content were analysed by one-way ANOVA. Phytic acid content of wheat bran was analysed by one-way ANOVA. For study 8, effects of treatment on performance indicators (initial group weight, final group weight, group body weight gain and feed intake) were analysed by three-way ANOVA (phytase x xylanase x XOS) and individual mealworm weight were analysed by repeated measures 2-way ANOVA (time x treatment). Significance value was set at $P<0.05$, trends set at <0.1. Where significant differences were observed post hoc Bonferroni Tests were carried out to identify where the differences were.

4.3 Results

4.3.1 Study 6: Initial Phytase Trial

There were no effects of adding exogenous phytase into either the feed or water on any performance indicators of final group weight, body weight gain or feed intake (Table 4.6).

Table 4.6 *Study 6: Initial phytase trial performance indicator results after treatment with differing doses of exogenous phytase for 14-day period*

	Feed Treatments[1]						
Performance	Control	HPF	HPW	LPF	LPW	SED[2]	P value
Initial Group Weight (g)	17.71	18.08	17.83	18.21	18.20	0.296	0.369
Final Group Weight (g)	19.42	19.73	19.43	19.22	19.39	0.764	0.976
Deaths (%)	38.70	38.59	35.64	40.61	38.97	2.742	0.510
Group Body Weight Gain (g)	1.71	1.65	1.59	1.01	1.19	0.893	0.910
Feed Intake (g)	36.19	35.99	35.99	35.13	36.49	1.002	0.725

[1]Control (wheat bran plus cotton wool with water), HPF (high dose phytase in feed (5000FTU/kg)), LPF (low dose phytase in feed (500 FTU/kg)), HPW (high dose phytase in water (5000 FTU/L)) and LPW (low dose phytase in water (500FTU/L))
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at $P<0.05$.

There were also no significant effects of phytase treatment on dry matter weight, crude protein, total fat or energy content of mealworms (Table 4.7).

***Table 4.7** Study 6: Proximate nutritional analysis of mealworms following treatment with differing exogenous phytase concentrations for 14-day trial period*

	Feed Treatments[1]						
	Control	HPF	HPW	LPF	LPW	SED[2]	P value
Dry Matter Yield (g)	6.18	6.40	6.44	6.13	6.47	0.284	0.656
Crude Protein (g/kg DM)	562.3	552.3	563.4	562.7	549.7	0.812	0.316
Total Fat (g/kg DM)	291.6	305.0	298.0	301.9	297.2	1.155	0.817
Gross Energy (MJ/kg DM)	26.39	26.26	26.66	26.59	26.12	0.261	0.251

[1]Control (wheat bran plus cotton wool with water), HPF (high dose phytase in feed (5000FTU/kg)), LPF (low dose phytase in feed (500 FTU/kg)), HPW (high dose phytase in water (5000 FTU/L)) and LPW (low dose phytase in water (500FTU/L))
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=4 for all treatment groups

4.3.2 Study 7: Phytase dose response trial

There were significant decreases in phytic acid contents in wheat bran following treatment with water or water plus phytase (P<0.001) (Figure 4.2), but there was no further effect of adding phytase enzyme compared to water alone (Figure 4.2), suggesting that the addition of water activated the endogenous phytase in the wheat bran.

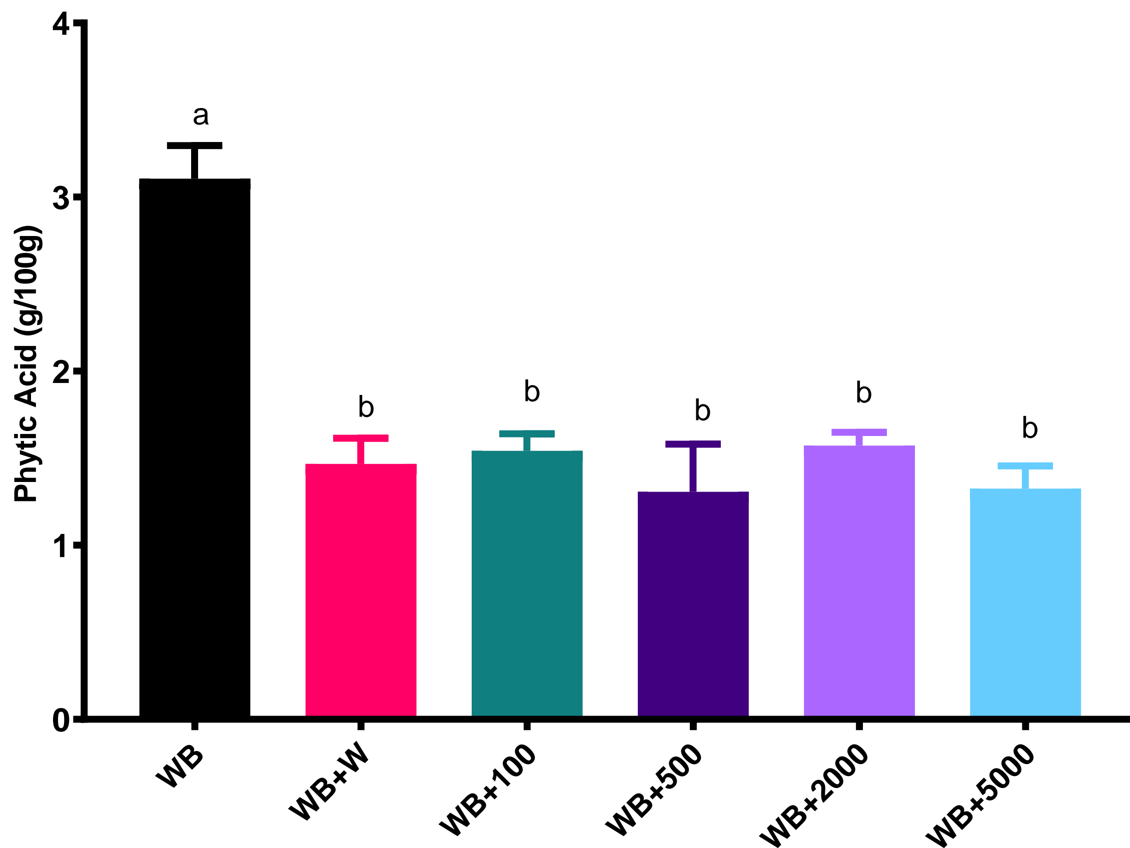


***Figure 4.2** Study 7: Phytic acid content of wheat bran (control) and wheat bran treated with water or increasing doses of exogenous phytase.*

WB (wheat bran), WB+W (wheat bran + water), WB+100 (wheat bran + 100FTU/L exogenous phytase), WB+500 (wheat bran + 500FTU/L exogenous phytase), WB+2000 (wheat bran + 2000FTU/L exogenous phytase), WB+5000 (wheat bran + 5000FTU/L exogenous phytase). Bars are means±SEM, n=5 for all treatment groups. Different lower-case letters are significantly different (P<0.05, Bonferroni)

There were no significant differences in initial group weight of mealworms (Table 4.8), but there was a significant effect of treatment on final group weight (P=0.020) and body weight gain (P=0.014), with soaking wheat bran in water (WB+W) and treatment with 5000 FTU/L both significantly decreasing final group weight and group body weight gain compared to the control (Table 4.8). These differences were not due to differences in feed intake as there were no significant differences (P=0.199) and soaking with water or water and phytase had no effect on the percentage of dead mealworms (P=0.884).

Table 4.8 *Study 7: Dose response phytase mealworm trial performance indicator results after treatment with differing doses of exogenous phytase for 14-day trial period*

	Feed Treatments[1]					
Performance	Control	WB+W	WB+500	WB+5000	SED[2]	P Value
Initial Group Weight (g)	9.99	9.85	9.88	10.04	0.283	0.898
Final Group Weight (g)	16.50[a]	14.45[b]	14.74[ab]	14.35[b]	0.648	0.020
Deaths (%)	30.93	31.36	32.38	32.67	2.506	0.884
Group Body Weight Gain (g)	6.50[a]	4.60[b]	4.86[ab]	4.32[b]	0.596	0.014
Feed Intake (g)	31.46	30.17	31.05	30.50	0.603	0.199

[1]Control (wheat bran), WB+W (wheat bran + water), WB+500 (wheat bran + 500 FTU/L), WB+500 (wheat bran + 5000 FTU/L).
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at P<0.05. n=4 for all treatment groups
[a-b] Means within a row with different superscripts are significantly different (P<0.05, Bonferroni).

Feeding the control, un-soaked wheat bran, resulted in the highest dry matter and crude protein contents of mealworms compared to the treated groups (P=0.002 for both), but there were no differences between the treated groups (Table 4.9). Total fat composition of mealworms was also significantly affected by treatment (P=0.044), feeding WB +500 resulted in significantly more fat than the control but not to the other two treatments (Table 4.9). There was not enough sample for gross energy analysis.

Table 4.9 Study 7: Dose response phytase mealworm trial proximate nutritional analysis results of mealworms after treatment with differing does of exogenous phytase for 14-day trial period

	Feed Treatments[1]					
Composition	Control	WB+W	WB+500	WB+5000	SED[2]	P value
Dry Matter Yield (g)	5.58^{a}	4.70^{b}	4.78^{b}	4.60^{b}	0.215	0.002
Crude Protein (g/kg DM)	532.0^{a}	471.1^{b}	480.8^{b}	477.7^{b}	1.307	0.002
Total Fat (g/kg DM)	282.4^{a}	301.1^{ab}	320.7^{b}	311.4^{ab}	1.209	0.044

[1]Control (wheat bran), WB+W (wheat bran + water), WB+500 (wheat bran + 500 FTU/L), WB+500 (wheat bran + 5000 FTU/L).
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at $P<0.05$. n=4 for all treatment groups
$^{a\text{-}b}$ Means within a row with different superscripts are significantly different ($P<0.05$, Bonferroni).

There were significant differences in phosphorus ($P<0.001$), selenium ($P=0.003$), magnesium ($P<0.001$), iron ($P<0.001$), calcium ($P<0.001$), sodium ($P<0.001$), manganese ($P<0.001$) and zinc ($P<0.001$) contents of mealworms when fed un-soaked wheat bran (Control) or WB that had been soaked with either water or differing doses of exogenous phytase (Figure 4.3).

The phosphorus content of mealworms was significantly higher in WB+W and WB+5000 compared to the control and WB+500 groups (Figure 4.3, A). Selenium was the only mineral to decrease in mealworms fed pre-soaked WB (water or phytase) compared to the control (Figure 4.3, B). Pre-soaking WB with water increased the magnesium, iron, manganese, calcium and sodium content of mealworms compared to the control but did not increase the zinc content (Figure 4.3). Treatment with WB+500 had no further effect compared to the control for manganese and magnesium (Figure 4.3, C + E). The addition of 5000 FTU/L, but not 500 FTU/L, significantly increased magnesium, manganese, calcium, sodium, and zinc contents of mealworms compared to the control (Figure 4.3). Both the sodium and zinc concentrations of mealworms increased significantly with increasing concentration of phytase added to the wheat bran feed (Figure 4.3, G and H).

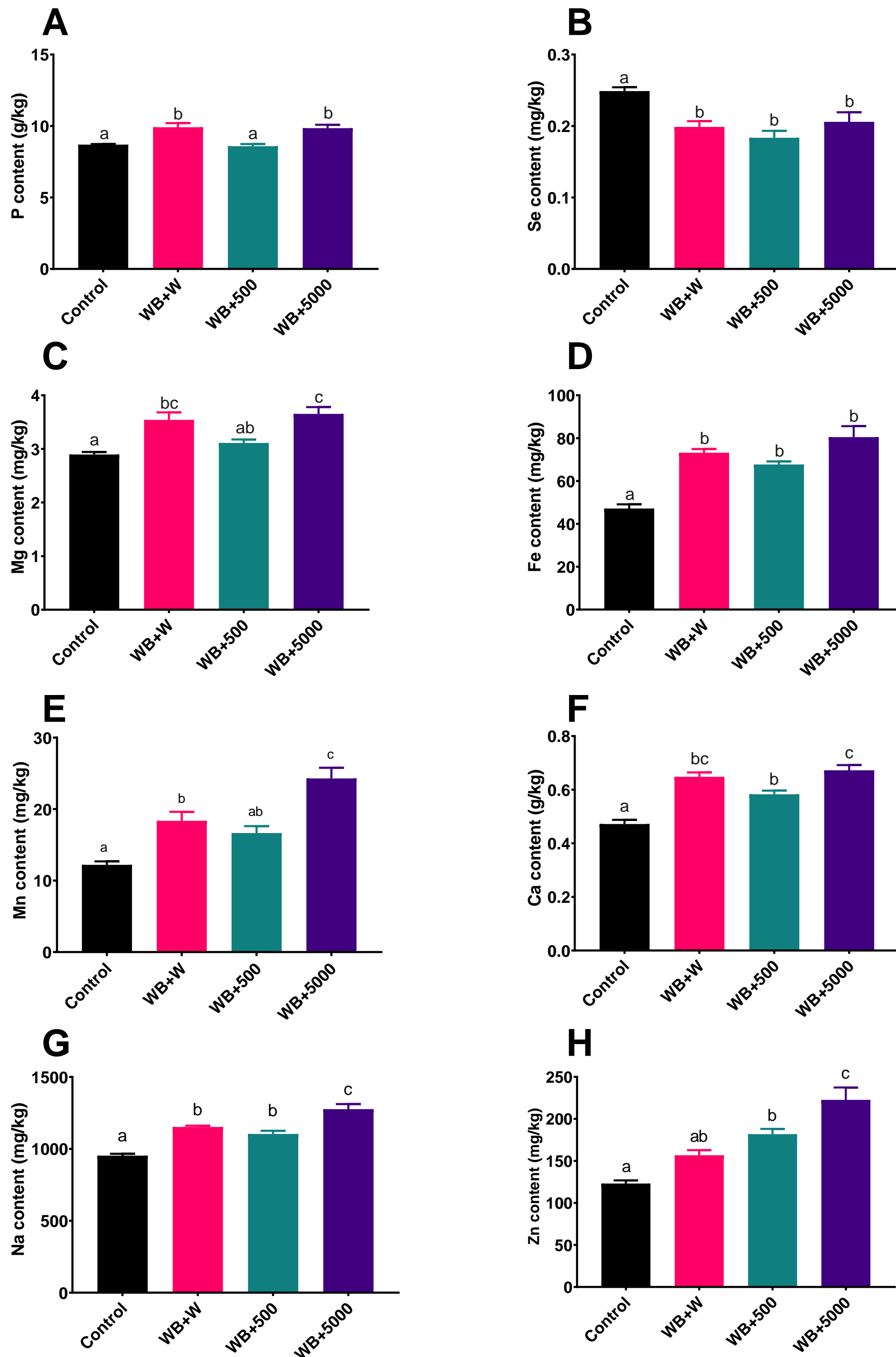


Figure 4.3 *Study 7: Mineral content of mealworms on a dry matter basis treated with increase doses of exogenous phytase: Phosphorus, P (A), Selenium, Se (B), Magnesium, Mg (C), Iron, Fe (D), Manganese, Mn (E), Calcium, Ca (F), Sodium, Na (G) and Zinc, Zn (H).*

Control (wheat bran), WB+W (wheat bran soaked with water), WB+500 (wheat bran soaked with 500FTU/L) and WB+5000 (wheat bran soaked with 5000 FTU/L). Bars are means±SEM, n=4 for all treatment groups. Different lower-case letters are significantly different (P<0.05, Bonferroni).

4.3.3 Study 8: Combination enzymes trial

The addition of xylanase significantly reduced final mealworm group weight (P=0.033) (Table 4.10 /Table 4.11). There was a 3-way interaction between phytase, XOS and xylanase on total feed intake (P=0.030), due to a significantly higher feed intake in the group which had no enzyme treatment compared to the xylanase only group, with the other groups being intermediate between those two extremes. There were no significant effects of treatment on death percentage of mealworms (Table 4.10 / Table 4.11).

Table 4.10 *Study 8: Combination enzyme trial performance indicator results after treatment with differing combinations of exogenous xylanase, xylo-oligosaccharides and phytase for 14-day trial period*

Performance	Feed Treatments[1]								SED[2]
	Control	XYL	XOS	PHY	XYL+XOS	XYL+PHY	XOS+PHY	XYL+XOS+PHY	
Initial Group Weight (g)	20.28	21.10	22.36	21.11	21.63	22.18	21.19	21.16	0.772
Final Group Weight (g)	21.04	18.56	23.05	20.08	20.14	21.34	21.13	19.24	1.326
Feed Intake (g)	30.39[a]	23.94[b]	30.10[ab]	24.91[ab]	25.72 [ab]	27.63 [ab]	27.78 [ab]	24.42 [ab]	1.769
Deaths (%)	49.73	52.70	46.45	51.46	49.56	48.09	48.07	49.80	2.937

[1]Control (wheat bran + cotton wool/water), XYL (xylanase), XOS (xylo-oligosaccharides), PHY (high dose phytase), XYL+XOS (xylanase and XOS), XYL+PHY (xylanase and high dose phytase), XOS+PHY (XOS and high dose phytase), XYL+XOS+PHY (xylanase, XOS and high dose phytase).
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at $P<0.05$ and n = 4 for each treatment.
[a-b] Means within a row with different superscripts are significantly different with 3-way interaction ($P<0.05$, Bonferroni).

Table 4.11 *Three-way ANOVA P values for performance indicator results reported in Table 4.10*

	P Values						
Performance	PHY	XOS	XYL	PHY. XOS	PHY. XYL	XOS. XYL	PHY. XOS .XYL
Initial Group Weight	0.869	0.294	0.476	0.091	0.543	0.097	0.097
Final Group Weight	0.707	0.349	**0.033**	0.092	0.086	0.189	0.315
Feed Intake	0.139	0.749	**0.003**	0.611	**0.008**	0.270	**0.030**
Deaths	0.864	0.180	0.457	0.428	0.200	0.381	0.408

XYL (xylanase), XOS (xylo-oligosaccharides), PHY (high dose phytase), XYL.XOS (xylanase and XOS interaction), XYL.PHY (xylanase and high dose phytase interaction), XOS.PHY (XOS and high dose phytase interaction), XYL.XOS.PHY (xylanase, XOS and high dose phytase interaction).
Data is presented as P values from 3 way ANOVA, P values highlighted are significant, significance set at $P<0.05$ and $n = 4$ for each treatment.

There was a significant effect of time on average individual mealworm weight (P<0.001), but no effect of treatment (P=0.693) nor a time x treatment interaction (P=0.672) (Figure 4.4).

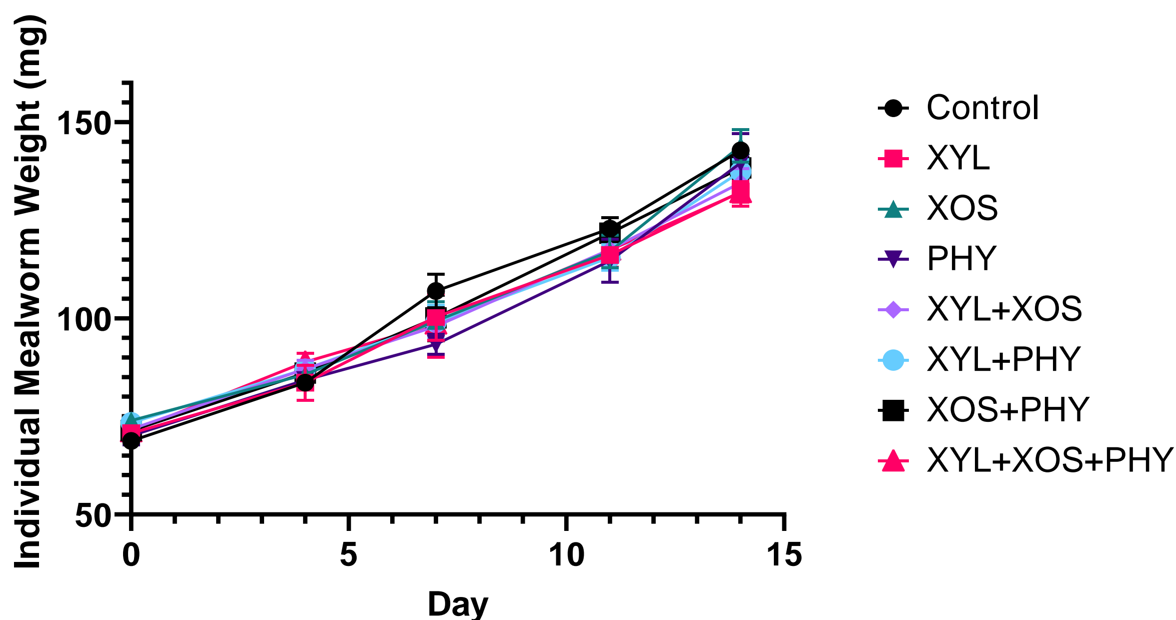


Figure 4.4 *Study 8: The effect of feeding mealworms wheat bran based feeds which have been treated with differing combinations of exogenous xylanase, xylo-oligosaccharides and phytase on average individual mealworm weight for 14-day trial period.*

Control (wheat bran + cotton wool/water), XYL (xylanase), XOS (xylo-oligosaccharides), PHY (high dose phytase), XYL+XOS (xylanase and XOS), XYL+PHY (xylanase and high dose phytase), XOS+PHY (XOS and high dose phytase), XYL+XOS+PHY (xylanase, XOS and high dose phytase).
Data is presented as means±SEM, n=4

Inclusion of xylanase into wheat bran significantly decreased dry matter content of mealworms (P=0.004). There was a trend for an interaction between xylanase and phytase to affect dry matter content of mealworms (P=0.056), with inclusion of either appearing to reduce the DM, but no further reduction when both were added together (Table 4.12/ Table 4.13). There was a significant interaction between xylanase and phytase on the crude protein (P=0.002) and total fat (P=0.007) content of mealworms, with xylanase reducing the CP and increasing TF content but only in the absence of phytase. While treatment with XOS significantly reduced the CP content of mealworms (P=0.017). There were no significant effects of inclusion of any of the enzymes on mealworm energy content, although there was a trend for xylanase to increase the energy content (P=0.079) (Table 4.11/ Table 4.13).

Table 4.12 *Study 8: Proximate nutritional analysis of mealworms following treatment with differing exogenous enzymes for 14-day trial period*

	Feed Treatments[1]								
Composition	Control	XYL	XOS	PHY	XYL+XOS	XYL+PHY	XOS+PHY	XYL+XOS+PHY	SED[2]
Dry matter Yield (g)	7.90	6.77	8.32	7.03	7.19	7.40	7.60	6.73	0.436
Crude Protein (g/kg DM)	508.4	469.6	480.1	483.4	458.8	480.3	458.2	483.3	1.195
Total Fat (g/kg DM)	250.2	299.5	266.5	275.6	293.2	286.1	296.2	276.1	1.461
Gross Energy (MJ/kg DM)	25.23	25.22	24.71	24.70	25.36	25.35	25.06	25.12	0.369

[1]Control (wheat bran + cotton wool/water), XYL (xylanase), XOS (xylo-oligosaccharides), PHY (high dose phytase), XYL+XOS (xylanase and XOS), XYL+PHY (xylanase and high dose phytase), XOS+PHY (XOS and high dose phytase), XYL+XOS+PHY (xylanase, XOS and high dose phytase).
[2]SED – standard error of the differences of the means
Data is presented as means, significance set at $P<0.05$ and $n = 4$ for each treatment

Table 4.13 *Three-way ANOVA P values for proximate nutritional analysis of mealworms reported in Table 4.12*

	P value						
Composition	PHY	XOS	XYL	PHY. XOS	PHY. XYL	XOS. XYL	PHY. XOS .XYL
Dry matter Yield	0.118	0.401	**0.004**	0.294	0.056	0.169	0.168
Crude Protein	0.629	**0.017**	0.123	0.485	**0.002**	0.068	0.661
Total Fat	0.406	0.490	**0.033**	0.983	**0.007**	0.082	0.786
Gross Energy	0.716	0.723	0.079	0.492	0.920	0.916	0.103

XYL (xylanase), XOS (xylo-oligosaccharides), PHY (high dose phytase), XYL.XOS (xylanase and XOS interaction), XYL.PHY (xylanase and high dose phytase interaction), XOS.PHY (XOS and high dose phytase interaction), XYL.XOS.PHY (xylanase, XOS and high dose phytase interaction).
Data is presented as P values from 3 way ANOVA, P values highlighted are significant, significance set at P<0.05 and n = 4 for each treatmen

4.4 Discussion

The overall aim of the studies described in this chapter was to investigate whether the addition of exogenous enzymes to mealworm feeds impacted significantly on the growth or composition of larvae. Two enzymes, phytase and xylanase, were investigated, together with the impact of adding xylo-oligosaccharides. All these feed additives have been shown to positively impact the performance of monogastric livestock (Bedford and Partridge, 2010; Craig et al., 2019) and were expected to have similar effects in mealworms.

Impact of adding exogenous phytase to feed or water (Study 6)

In study 6 the impact of adding exogenous phytase to either the feed or the water was investigated. There were no significant differences in any of the performance indicators measured in this initial trial and there were no significant differences in the proximate nutritional composition either, with similar proximate composition to that recorded in Chapter 3. This suggests that the exogenous phytase, whether supplied in the feed or water, had no effects on the mealworms in terms of growth or nutritional value. The potential effects on growth were identified from the effects of phytase in commercial practise, where super dosing has been shown to increase average daily gain in pigs (Zeng et al., 2014) while phytase improves feed efficiency in commercial broiler chickens (Pirgozliev et al., 2008).

The lack of effects of exogenous phytase may relate to the fact that the enzyme has been designed to work in the monogastric digestive system (Humer et al., 2015). A monogastric digestive system has distinct regions of differing digestion with an acidic proventriculus followed by small intestine made up of duodenum, jejunum and ileum, followed by the caeca and colon. Each of these regions has a differing pH and therefore effects on feed

digestion. In contrast, the mealworm digestive system is much simpler, being split into foregut (stomodeum), midgut (mesentron) and hind gut (proctoderm) (Chapman, 2013). The midgut is the primary site of enzymatic digestion and absorption and the pH ranges from 5.2 to 8.2 (Vinokurov et al., 2006).

In chickens, phytate is most soluble at pH 2-4 (Selle et al., 2000), meaning that the phytase is designed to work between the crop and duodenum covering this area of solubility, with pH ranging from 2-6 (AB Vista, 2021). The mealworm digestive system has not been studied in as much detail as the chicken, but the pH ranges from 5 to 8 throughout the gut (Terra et al., 1985). This higher pH than the broiler chicken gut presents two potential issues with using phytase: (i) the enzyme is not active and (ii) the phytate is still insoluble, so the enzyme cannot act on it. Hence, no active enzyme-substrate interactions are taking place. Additionally, proteases in the mealworm gut could be acting to alter the tertiary structure of the protein, thereby changing the active site so the phytase will not work.

Impact of pre-treating wheat bran with phytase on growth and composition of mealworms (Study 7)

An alternative strategy could be pre-treat the wheat bran with phytase prior to feeding to mealworms. Initial experiments were undertaken to determine whether the treatment reduced the phytic acid content of wheat bran using a phytic acid assay.

The phytic acid assay was used to assess the effects of exogenous phytase on the phytic acid contents following *in vitro* incubations of wheat bran with either water or water and exogenous phytase. Soaking the wheat bran in either water or water plus phytase enzyme significantly reduced the phytic acid content of the wheat bran. The action of soaking the wheat bran in water presumably activated the endogenous phytase contained within the

aleurone layer of the wheat bran. Guo *et al.* (2015) found similar results following incubation of wheat bran with distilled water, as the water significantly increased the phytase activity thereby decreasing the phytic acid content. There was no further effect of adding exogenous phytase on the phytic acid content in wheat bran. However, the water used was at pH 7.5, but the enzyme is designed to work at a more acidic pH, meaning that it simply was not active. Thus, these finding suggest that while soaking with water alone reduced phytic acid content of feed, there is no additional benefit of including the exogenous enzyme.

The soaked wheat bran, with or without added phytase, was then fed to mealworms in its dried form. All of the soaked feeds (with or without enzyme, which had been dried for feeding) tended to reduce growth of the larvae (though this failed to reach significance in the 500 FTU/L treated group). No differences in the feed consumption on a fresh weight basis were seen between the groups. The negative effect on growth was unexpected, as the exogenous phytase is designed to improve availability of nutrients bound within, or to, the phytate, thereby increasing growth (of pigs and poultry), whilst maintaining the same level of feed consumption (Pirgozliev et al., 2008). In the mealworms, soaking the feed may potentially be resulting in the release of an unidentified factor (s) which has a negative effect on growth (but not feed consumption).

There was decreased crude protein and increased total fat content of mealworms when fed soaked feeds (with or without exogenous enzymes) compared to the untreated wheat bran. As there were no differences between the pre-treatment of just soaking with water and soaking with water and enzyme, the exogenous phytase was not having any further effect on mealworm composition compared to the water alone. This appears to confirm the

findings of the previous experiment where phytase was directly added to the feed or water. Pre-soaking reduces phytate content which may be responsible for the negative effects on composition but this seems unlikely due to the positive effects in other species.

Therefore, the negative effect on composition could have been caused by phytase action in the mealworm gut. It would be expected that the exogenous phytase would work in the gut to further reduce the negative effects of phytate by breaking down insoluble complexes (Selle et al. 2000), thereby resulting in increased protein available for digestion. Potentially the exogenous phytase could be interacting with amino acids in the wheat bran, both positive and negative interactions between phytase and amino acids have been reported in broilers (Selle et al., 2000). Hence there could be negative effects of exogenous phytase on amino acid uptake in the mealworm gut which then reduces the overall crude protein content. Overall, there is no evidence that adding exogenous phytase has any beneficial effects nor, indeed, that breaking down phytate by release endogenous phytase is beneficial.

In this trial we also investigated the impact of pre-soaking wheat bran and addition of exogenous phytase on mineral content of the mealworms. The effects of the treatments varied depending on the mineral considered. While it is unclear why there was different responses between the minerals, it is well established that phytase can improve their bioavailability. Activation of the endogenous phytase by the soaking in water can release minerals that were bound to the phytate through dephosphorylation of the phytate. This removal of phosphate groups from the inositol group is in a step wise manner, thereby reducing the ability and strength to bind minerals (Hallberg et al., 1990).

Selenium was the only mineral that decreased by soaking the wheatbran (with no further effect of added phytase). In chicks it has been reported that there is a positive relationship between phytate and selenium, with increased phytate consumption associated with increased selenium content of tissues (Shan and Davis, 1994). However, the mechanism for this relationship is unknown.

Phosphorus, magnesium, calcium and iron were all increased by soaking but with no effect of added phytase. Variability in the original mealworm mineral contents before treatment could be a reason for this. When phytase is supplied to pigs or poultry in their feed, there can be incomplete hydrolysis of phytate so a mixture of inositol-phosphate esters is formed (Dersjant-Li et al., 2015). The same could be happening here either in the soaking of the wheat bran or in the mealworm gut. These intermediate inositol-phosphate esters could be at a level where the minerals are not being bound into the phytate salt (Kumar et al., 2010), but due to the higher pH of the mealworm gut there are ternary protein-phytate complexes being formed which then reduces digestibility (Selle et al., 2000).

Manganese, sodium and zinc all tended to increase with pre-soaking but were further increased by inclusion of phytase (particularly at the highest dose). This suggests this additional phytase may be further dephosphorylating the phytate beyond the first two phosphorus molecules and releasing additional minerals. The greatest differences were in zinc levels, which might be expected, as phytate is known to chelate zinc making it unavailable (Kumar et al., 2010).

Impact of xylanase, phytase and xylo-oligosaccharide and their interactions (Study 8)

Study 8 investigated the use of xylanase, XOS and phytase individually and in different combinations. As previously explained, in monogastric livestock species xylanase can break down NSPs to produce XOS which may be fermented by gut microbes releasing volatile fatty acids as an energy source (Bedford, 2000). Similarly, direct feeding of XOS may also have the same effect (Courtin et al., 2008). Despite, the lack of effect of added phytase in Study 6 and 7 it was decided to include this in Study 8 as well. This was because this was the first study in which we were able to carefully control environmental conditions using an incubator. Furthermore, it provided an opportunity to investigate possible interaction between the different treatments.

Xylanase was the only enzyme to affect mealworm growth with a reduction in final mealworm group weight compared to no xylanase treatment, but no effects on feed intake. This was unexpected as xylanase supplementation in pig feeds results in higher average daily gains (Dong et al., 2018). The role of a xylanase is to cleave a NSP into smaller fragments (Zhang et al., 2014), thereby reducing the NSP's water holding capacity and increasing nutrient uptake. In broilers, supplementation of xylanase enzymes improves nutrient uptake from wheat-based feeds (T. Ribeiro et al., 2018). This improved nutrient uptake can then lead to improved growth performance, the opposite of what was seen in the mealworms, suggesting that the nutrients (or other substrates) which are released by the xylanase may be having a negative effect on mealworm growth. One of the main NSP's in wheat bran is arabinoxylan, which releases XOS, xylose and arabinose when digested by xylanase. It has previously been reported that mealworms failed to grow on pentose sugars such as arabinose, xylose, ribose and fructose (Fraenkel, 1955). Hence, xylanase action could be releasing xylose and/or arabinose, which have a negative effect on mealworm growth.

As the feed consumption was not different between the different treatment groups, the sugars being released by the xylanase are either not being absorbed in the mealworm gut or not being utilised for energy metabolism. There were increased death rates in study 8, with 20% higher deaths compared to study 7. This higher death rate was apparent across all treatments and the control group so is unlikely to be a consequence of the treatments and more likely to be due to the batch of mealworms.

Since different combinations of enzymes affected the mealworm composition, the hypothesis that mealworm composition can be manipulated with enzyme inclusion in feeds can be accepted. Inclusion of xylanase resulted in decreased DM content of mealworms, but this could be as a result of the decreased final mealworm group weight compared to the other treatments. There was an interaction between xylanase and phytase for crude protein and total fat content of mealworms with xylanase appearing to reduce the crude protein and increase the total fat content but only in the absence of phytase. As with the results for the growth of the mealworms, xylanase could be releasing pentose sugars or xylo-oligosaccharides that are not absorbed or utilised by the mealworm, therefore this sugar could be excreted and not used. In vertebrates the majority of additional xylose in the feed ends up being excreted in the urine, suggesting that it is not metabolised (Huntley and Patience, 2018). With previous reports that mealworms have reduced growth when fed on xylose (Fraenkel, 1955), if the xylose or xylo-oligosaccharides cannot be utilised as an energy source for growth, could it potentially be used by gut bacteria resulting in increased stored fat. In other insect species feeding xylose resulted manipulated nutritional composition, for example black solider fly fed xylose supplemented feeds resulted in increased fat content of the larvae (Li et al., 2015).

Furthermore, XOS inclusion resulted in reduced crude protein content of mealworms but there was no effect on total fat content. Inclusion of XOS in broiler feeds resulted in a shift in the gut microbiome content (Ribeiro et al., 2018), therefore XOS supplied in mealworm feed could be being digested by the gut microbiome to release a secondary metabolite which is not beneficial to the mealworms. Alternatively, the XOS could be acting as a prebiotic (Ribeiro et al., 2018) and changing the mealworm gut microbiome resulting in a reduced crude protein content.

4.5 Conclusion

Exogenous phytase added directly to the feed or water with no further treatment had no effect on mealworm production or proximate composition. Treating wheat bran-based feeds with water reduced the phytic acid content with little further effect of adding exogenous phytase. However, pre-soaking the wheat bran-based feeds had a negative effect on mealworm growth and crude protein content again with little impact of adding exogenous phytase. There were increases in the mealworm content of some, but not all, minerals associated with super dosing phytase. Inclusion of xylanase into mealworm feeds had a negative effect on growth and crude protein content but had no effect on feed intake. Xylo-oligosaccharide inclusion also reduced crude protein content of mealworms but had no effect on growth compared to the other enzymes and combinations.

The results observed are clearly different to those seen in monogastrics livestock. In pigs and poultry, both phytase and xylanase improve growth whereas in the mealworms there was a negative effect on growth. XOS decreases feed intake in poultry, but there was no effect of this in mealworms. Pre-treatment of mealworm feeds with phytase did have a positive effect of increasing the mealworm content of some minerals. Overall, the studies

described in this chapter have failed to demonstrate the positive impacts that inclusion of these enzymes clearly have in monogastric livestock species. This is likely to be due to significant differences in the gastrointestinal system and/or microbiome of the larvae compared to monogastric livestock.

5 Inclusion of mealworms into broiler feeds

5.1 Introduction

Traditional livestock feeds utilise a combination of plant sources and synthetic amino acids to provide a suitable protein supply and meet requirements for both maintenance and production qualities of the species being fed. However, with increasing pressure on the livestock industry to improve the sustainability of meat production, new alternative sources of feed proteins, with lower land and water requirements are needed.

Soya is used as a protein supplement to increase intakes of limiting amino acids. It is a good source of highly palatable (Selaledi et al., 2020) and high-quality protein (Khan et al., 2018). Soya beans have a high anti nutritional factor content and therefore cannot be included in animal feed as the raw bean (Dei, 2011). It has to be solvent extracted to obtain soya oil which is used for human consumption, leaving a residual meal, which is of a higher protein and energy content than the raw bean, to be used in animal feed (McDonald et al., 2011). Soybean meal is the most commonly used form of soya bean for animal feed (Dei, 2011). It is highly digestible (Dei, 2011) and contains all essential amino acids, although levels of methionine and cysteine are suboptimal (McDonald et al., 2011). Methionine is the first limiting essential amino acid, resulting in synthetic amino acids needing to be added to poultry feeds (Dei, 2011).

As seen in table 5.1, mealworms have a similar composition to soya in terms of crude protein. The major difference is the fat content, since the majority of the oil is removed

during the processing of soybean meal. The nitrogen-correct apparent metabolisable energy (AMEn) for mealworms has been reported to be between 3965 (De Marco et al., 2015) and 5004 kcal/kg DM (Nascimento Filho et al., 2021). With careful formulation, mealworms are suitable to replace some of the soybean meal content, particularly when soya oil is used as an adhesion agent for dried feeds and increases the energy content of the feeds.

Table 5.1 *Comparison of proximal nutritional composition of mealworm larvae and Hipro soybean meal, parts taken from* Hawkey et al., 2021

	Crude Protein (g/kg DM)	Methionine (mg/g protein)	Lysine (mg/g protein)	Leucine (mg/g protein)	Cysteine (mg/g protein)	Crude Fat (g/kg DM)	Gross Energy (MJ/kg DM)
Mealworm	460-540	14.44	47.61	52.96	14.68	250-360	27
Hipro Soybean Meal	552	14.00	62.00	77.00	16.00	17	20

It has been highlighted that mealworms could impact on economic value, animal welfare and the environment (Selaledi et al., 2020). Broiler production is highly focussed on performance, fast growth, breast meat yield, feed conversion ratios, skeletal quality and heart and lung function (Borda-Molina et al., 2018). Partial replacement of traditional protein feed ingredients needs to equal the current broiler performance of fast growth and low FCR's or improve them to be able to be considered for mass broiler production. However, the mealworms also need to be safe and financially viable for inclusion.

Chickens reared in a free-range system will naturally root around for insects and are therefore likely to have some ability to digest them. There is little information regarding the variability in the nutritional composition of mealworms between producers that could

impact on their nutritional value. Mealworm physiology, particularly the exoskeleton, impacts on the nutrient content and consequently the digestibility (Williams *et al*., 2016). Evaluating digestibility of animal feed components allows for determination of the proportion of nutrients being utilised by the broiler and determining whether mealworms are a useful supply of accessible nutrients. Digestibility coefficients based on collection and analysis of digesta from the terminal ileum are generally considered to give a more accurate measure of the nutrients being absorbed (McDonald et al., 2011). Furthermore, the use of apparent amino acid digestibility can give further insight into how protein is being utilised, by measuring the digestibility of amino acids of both dietary and endogenous origins (Bryden and Li, 2010).

The microbiota present in the gastrointestinal tract play a role in gut physiology, nutrition, immune responses and maintenance of gut health. Gut microbes are involved in the degradation of complex substrates which often require specific enzymes (Sergeant et al., 2014). Use of high levels of animal protein was previously avoided in broiler feeds, due to associations with necrotic enteritis and increases in the abundance of *Clostridium perfringens* in the gut (Drew et al., 2004). It is unclear whether insect protein would have similar effects as animal protein, as their composition may vary substantially from vertebrates.

Previous studies have investigated the impact of mealworm inclusion on performance, apparent digestibility or the caecal microbiome, but there have been no studies which looked at all three of these in the same study. This chapter therefore explores the use of mealworms as a partial replacement for soybean meal in broiler feeds and impacts on broiler performance, apparent digestibility, and the caecal microbiome.

Aims

To determine the effect of partial replacement of soybean meal with mealworms in broiler diets on:

1. Growth, feed efficiency and body composition
2. Crude protein and amino acid apparent ileal digestibility coefficients
3. Caecal microbiome

Hypothesis

Partial replacement of soybean meal with mealworms will not alter growth characteristics of broilers and therefore will be a suitable alternative protein.

5.2 Materials and Methods

5.2.1 MW Broiler Trial

5.2.1.1 *Feeds*

Two mash feeds were used for the MW broiler trial. A wheat-based broiler feed formulated for all stages of chick development (Dale et al., 2020), was used as the control (SBM) feed. The experimental feed (MW) had 10% of the soya removed and replaced with crushed mealworms. To balance the oil content of the feeds, the MW feed had 3% less soya oil and then this 3% was increased on the barley. Titanium dioxide was included in both feeds at 5g/kg as an indigestible marker to determine apparent ileal digestibility.

Table 5.2 Percentage makeup of the composition of broiler feeds.

	Control	**Mealworm**
Control (%)	100	0
Basal (%)	0	90
Mealworms (%)	0	10

Further details of specific composition of control and basal can be found in Table 2.2.

Both feed types were mixed on site prior to chicks arriving. Each 25kg bag of SBM feed was opened and 1 scoop of feed taken out of the bag and mixed in a separate bag with 125g of titanium dioxide. Another 4 scoops were added gradually with continual manual mixing of the bag. The remainder of the 25kg was added to the screw mixer and the mixer was turned on. Whilst the screw mixer was mixing, the mix of titanium dioxide and feed was added. The same procedure was carried out for another 25kg bag of SBM feed resulting in 50kg in the screw mixer. This was mixed for 5 minutes after all components had been added, then feed was bagged in two 25kg bags and sealed before use.

The experimental MW feed was supplied as 22.5kg of the basal feed in a 25kg bag, to allow the addition of 2.5Kg of mealworms. The same procedure for titanium dioxide was followed as the control feed. After the remaining amount was put in the mixer, 2.5kg of mealworms were added per 22.5kg of basal feed. Again, after all components had been added, the feed was mixed for 5 minutes then bagged in two 25kg bags and sealed.

Feeds were then stored in a cool dry room until needed to feed chicks.

5.2.1.2 *Experimental Design*

Eighty 1-day old broiler chicks were acclimatised on control (SBM) feed for 6 days, then allocated to one of 20 pens with 4 birds per pen. There were 10 replicate pens per feed (Control and MW) each containing 4 birds. Birds were fed the appropriate feed and water *ad libitum* for 30 days. Birds were weighed and feed intakes recorded twice weekly.

5.2.1.3 *Sample collection*

On day 35, pens 1-10 were weighed then culled by Schedule 1 method (Animals (Scientific Procedures) Act 1986) of an overdose of pentobarbitone (anaesthetic) through intravenous neck injection), with pens 11-20 being similarly weighed and culled on day 36. After confirmation of death, the digestive tract was exposed and samples of digesta taken from the duodenum, ileum, jejunum, caeca and colon of each bird. Alongside these digesta samples, 3cm lengths of tract were also sampled. From each bird whole liver, breast and leg (quadriceps) muscles were dissected from the left side and weighed before a smaller sample was taken. All digesta and tissue samples were snap frozen in liquid nitrogen before storage at -80ºC.

5.2.1.3.1 Gut Digesta Sample Analysis

Apparent Ileal Digestibility

Ileal digesta samples collected from each bird were freeze dried until a constant weight and crushed to a fine powder using a pestle and mortar; the 4 samples from each pen were then pooled. Pooled pen samples (n=10 per feed) were analysed for crude protein (by nitrogen analyser, FlashEA 1112 Series, Thermo Fisher Scientific), mineral (via acid digestion and ICPMS, Thermo- Fisher iCAP-Q) and amino acid (LCMS-MS, Thermo Fisher) contents.

Similarly, feed samples (n=4 for each feed) were freeze dried until a constant weight, samples were crushed to a fine powder using a pestle and mortar. Proximate nutritional analysis included crude protein (by nitrogen analyser, FlashEA 1112 Series, Thermo Fisher Scientific), gross energy (by bomb calorimetry, 6300 Calorimeter, Parr), mineral (via acid digestion and ICPMS, Thermo- Fisher iCAP-Q) and amino acid (LCMS-MS, Thermo Fisher) contents.

Apparent ileal digestibility coefficients (AIDC) were calculated on a pen basis according to the equations described in Chapter 2.

Caecal Microbiome Analysis

See chapter 2 for detailed methods on caecal microbiome analyses. Briefly, DNA was isolated from caecal digesta samples collected from each bird and used individually for microbiome analysis. Initially the V4 regions of the bacterial 16S rRNA genes were amplified by PCR; an Illumina MiSeq platform was used to sequence amplicons using 2x 250-bp cycles. The Schloss lab in Mothur (online software) was used to quality filter 16S rRNA sequences and cluster into operational translation units (OTUs). Linear discriminant analysis effect size (LEfSE) plots were then used to analyse changes between feed treatments for specific families.

5.2.2 Statistical Analysis

All graphs were produced using Graph Pad Prism 8. Genstat (20th Edition) was used for statistical analyses. For the broiler trial the effect of feed on performance indicators (initial live weight, final live weight, body weight gain, feed intake and FCR) were determined by one-way ANOVA, whereas effects on live weight over time were analysed by repeated measures 2-way ANOVA (time x treatment). Similarly, effects of treatment on apparent ileal digestibility coefficients (AIDC) (crude protein and amino acids) were analysed by one-way ANOVA.

For the caecal microbiome, analysis of molecular variance (AMOVA) was calculated within Mothur (Kozich et al., 2013). Linear discriminant analysis effect size (LefSe) was also implemented within Mothur. Significant OTU from the LefSe report were highlighted and the FASTA sequences were analysed using NCBI BLAST to give an indication of the specific species.

Significance was set at $P<0.05$, with trends set at $P<0.1$ but >0.05.

5.3 Results

5.3.1 Feed Analysis

Due to a single sample of the broiler feeds statistical analysis was not possible, the means represented in Table 5.2 are based on technical replicates and give an indication into the composition differences between the two feeds.

Table 5.2 *Nutritional analysis of control and mealworm feeds used in the broiler trial*

	Feeds[1]	
	Control	MW
Dry Matter (g/kg)	911.9	904.3
Crude Protein (g/kg DM[2])	222.1	233.7
Gross Energy (MJ/kg DM)	17.51	18.26
Calcium (g/kg DM)	18.53	12.91
Phosphorus (g/kg DM)	10.59	8.95
Titanium (mg/kg DM)	115.52	81.99
Essential AA (mg/g DM)		
Histidine	4.62	5.55
Isoleucine	7.85	8.54
Leucine	12.94	13.35
Lysine	12.07	12.00
Methionine	10.88	9.11
Phenylalanine	9.60	9.96
Threonine	8.47	8.76
Valine	9.08	10.98

[1]Control (soybean based broiler feed), MW (10% soybean replaced with mealworms feed)
[2] DM – dry matter
Data is presented as means. n=4 for each feed (across different feed bags)

5.3.2 Performance Indicators

As expected, there was no difference in initial bird live weights (Table 5.3). Feeding mealworms (MW) as a partial replacement for soybean meal had no effect on final bird live weight or body weight gain (Table 5.3, $P>0.4$ for both), but total pen feed intake ($P=0.010$) and FCR ($P=0.002$) were both significantly increased in the MW fed group compared to the control group (Table 5.3).

Table 5.3 *Mealworm broiler trial performance indicator results after feeding two different feeds for 30-day trial period*

	Feeds[1]			
	Control	MW	SED[2]	P Value
Initial Bird Live weight (kg)	0.109	0.1085	0.002	0.818
Final Bird Live weight (kg)	2.071	2.023	0.062	0.450
BWG (kg)[3]	1.962	1.915	0.062	0.456
Total Pen Feed Intake (kg)	11.560	12.690	0.396	0.010
FCR (pen)[4]	1.476	1.661	0.051	0.002

[1]Control (soybean based broiler feed), MW (10% soybean replaced with mealworms feed)
[2] SED – standard error of the differences of the means
[3]BWG (Body weight gain)
[4]FCR (Feed conversion ratio).
Data is presented as means, significance set at $P<0.05$.
For Initial bird weight, final bird weight and BWG n= 40 for each treatment, for Total pen feed intake and FCR n=10 for each treatment

Bird live weight significantly increased with time ($P<0.001$), but there was no effect of feed ($P=0.774$) (Figure 5.1).

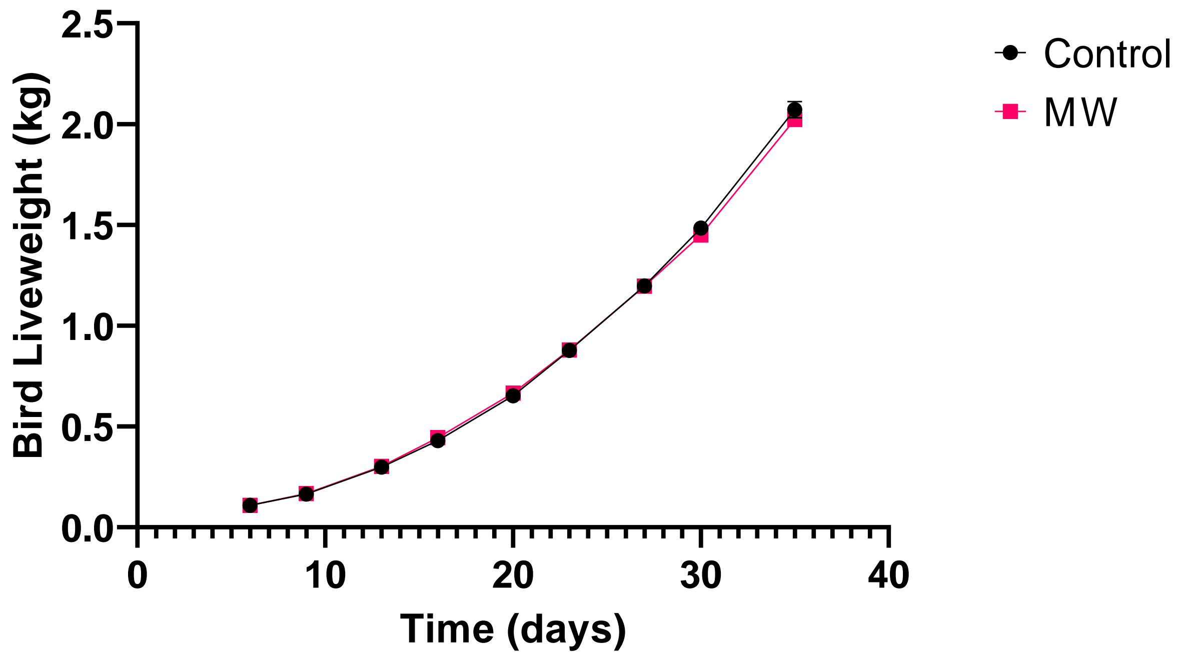


Figure 5.1 *The effect of feeding broilers either the control (soybean meal) or mealworm feed on average bird live weight for 30-day trial period when weighed twice weekly*

Control = soybean meal feed, MW = mealworm feed. Data is presented as mean±SEM, n=40 for both treatments

5.3.3 Body Composition

Inclusion of mealworms as a partial replacement for soybean meal had no significant effects on liver, breast or leg muscle weights after 30 days of feeding (Table 5.4). This was also true when the tissue weights were expressed as percentage of live weight (Table 5.4).

Table 5.4 *Average body composition as liver, breast and leg muscle of broilers after 30 days on feeds*

		Feeds[1]			
		Control	MW	SED[2]	P value
Liver	(g)	43.83	44.62	1.931	0.688
	(%LW[3])	2.13	2.21	0.097	0.395
Breast Muscle	(g)	112.37	113.26	7.430	0.906
	(%LW)	5.39	5.56	0.237	0.481
Quad Leg Muscle	(g)	147.63	139.38	4.940	0.112
	(%LW)	7.14	6.89	0.152	0.119

[1]Control (soybean meal based broiler feed), MW (10% soybean replaced with mealworms feed)
[2] SED – standard error of the differences of the means
[3]LW - live weight
Data is presented as means, significance set at $P<0.05$. n= 40 for each treatment

5.3.4 Apparent Ileal Digestibility

There were no significant differences in the average ileal titanium content between control and mealworm fed birds (P=0.947) (Figure 5.2). However, there was a large variation in the titanium contents from the different pens across both feed groups (Figure 5.2). For the control birds, the standard error of the mean was 149, which is 21% of the mean. For the MW birds, the standard error of the mean was 110, which is 15% of the mean.

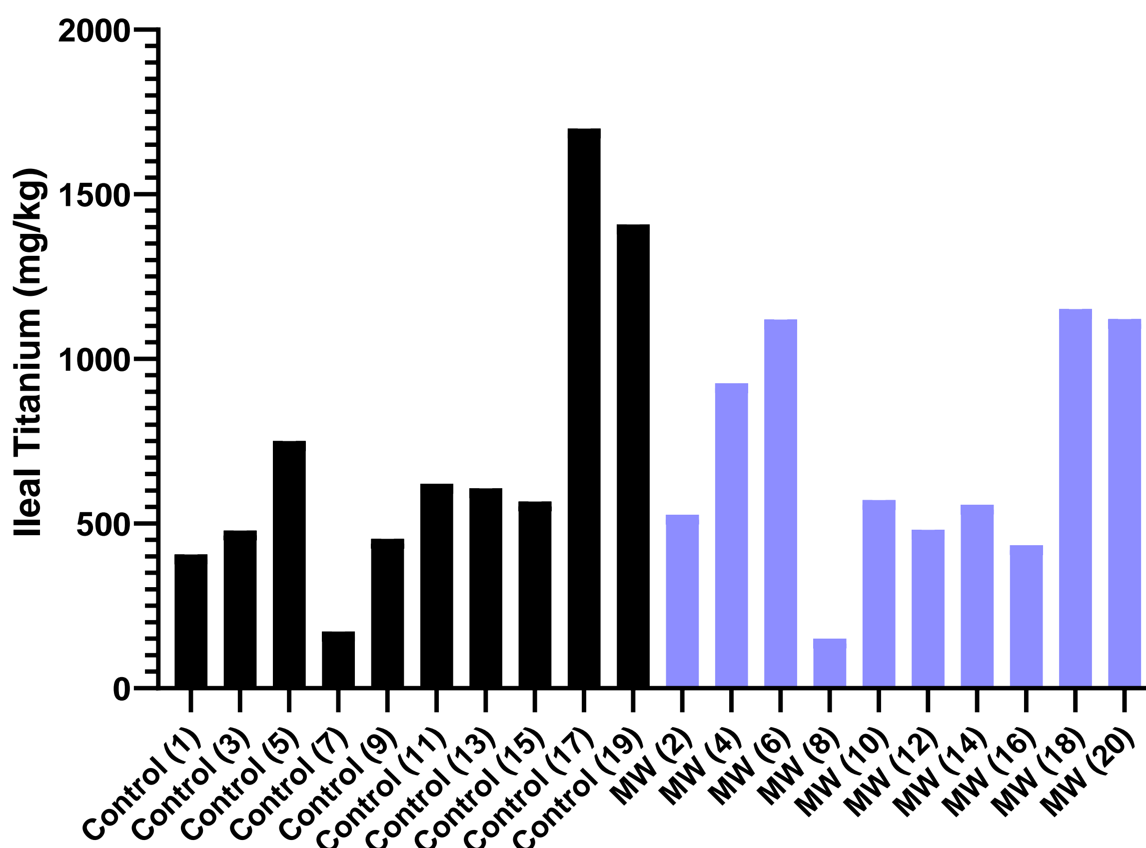


Figure 5.2 *Average pen ileal titanium content displayed on a pen basis after 30 days on feeds*

Control = soybean meal feed, MW = mealworm feed. Data is presented as individual values, 250g of freeze-dried ileal digesta from each of the 4 birds was combined together to represent 1 pen.

There was a significant difference in the ileal crude protein contents, with the MW fed birds having a higher protein content in the ileum compared to the control birds ($P<0.001$) (Table 1.4). However, there were no significant differences in the apparent ileal crude protein digestibility coefficients ($P=0.142$) (Table 5.5) between the two feed groups.

Table 5.3 *Ileal Crude protein content and apparent ileal crude protein digestibility coefficient after 30 days on feeds*

	Feeds[1]			
	Control	MW	SED[2]	P Value
Ileal CP (g/kg)	121.61	144.41	5.58	<0.001
$AIDC_{cp}$[3]	0.89	0.92	0.017	0.142

[1]Control (soybean based broiler feed), MW (10% soybean replaced with mealworms feed)
[2] SED – standard error of the differences of the means
[3]$AIDC_{cp}$ – Apparent ileal digestibility coefficient for crude protein
Data is presented as means of pen, significance set at $P<0.05$. n= 10 for each treatment

The inclusion of mealworms as a partial replacement for soybean meal significantly increased the alanine, glycine, histidine, isoleucine, leucine, lysine, methionine, tyrosine and valine contents in the ileum (Figure 5.3), but there was no effect on the arginine, aspartate, cysteic acid, glutamate, phenylalanine, proline, serine and threonine contents (Figure 5.3).

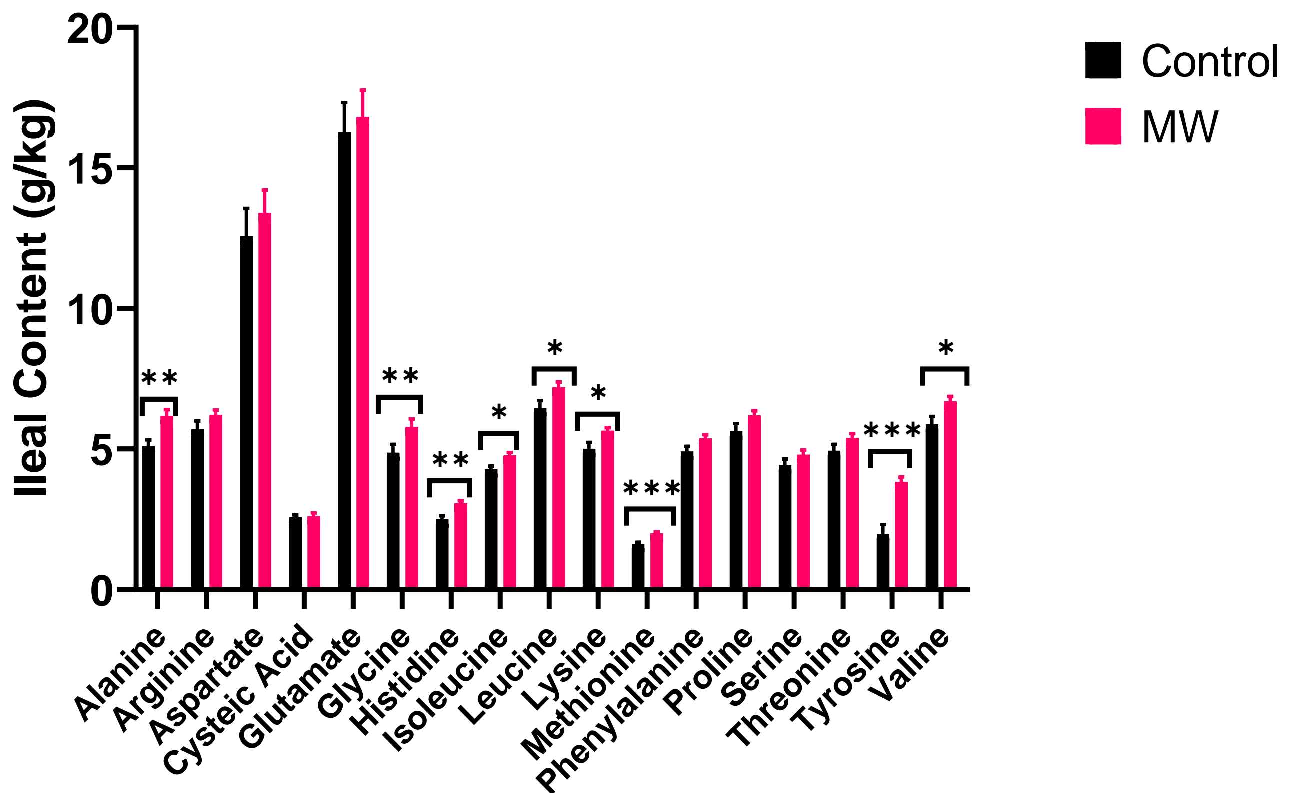


Figure 5.3 *Ileal amino acid content of broilers after 30 days on feeds*

Control = soybean meal feed, MW = mealworm feed. Data is presented as means±SEM, n=10 per feed treatment. Data analysed through 1 way ANOVA, * P<0.05, **P<0.01, *** P<0.001

Importantly, there was no effect of inclusion of mealworms on the apparent ileal amino acid digestibility coefficients compared to the control (soybean meal) feed (Figure 5.4), although there was a tendency for mealworm inclusion to increase the apparent ileal digestibility of alanine (P=0.09), cysteic acid (P=0.07) and isoleucine (P=0.09)(Figure 5.4).

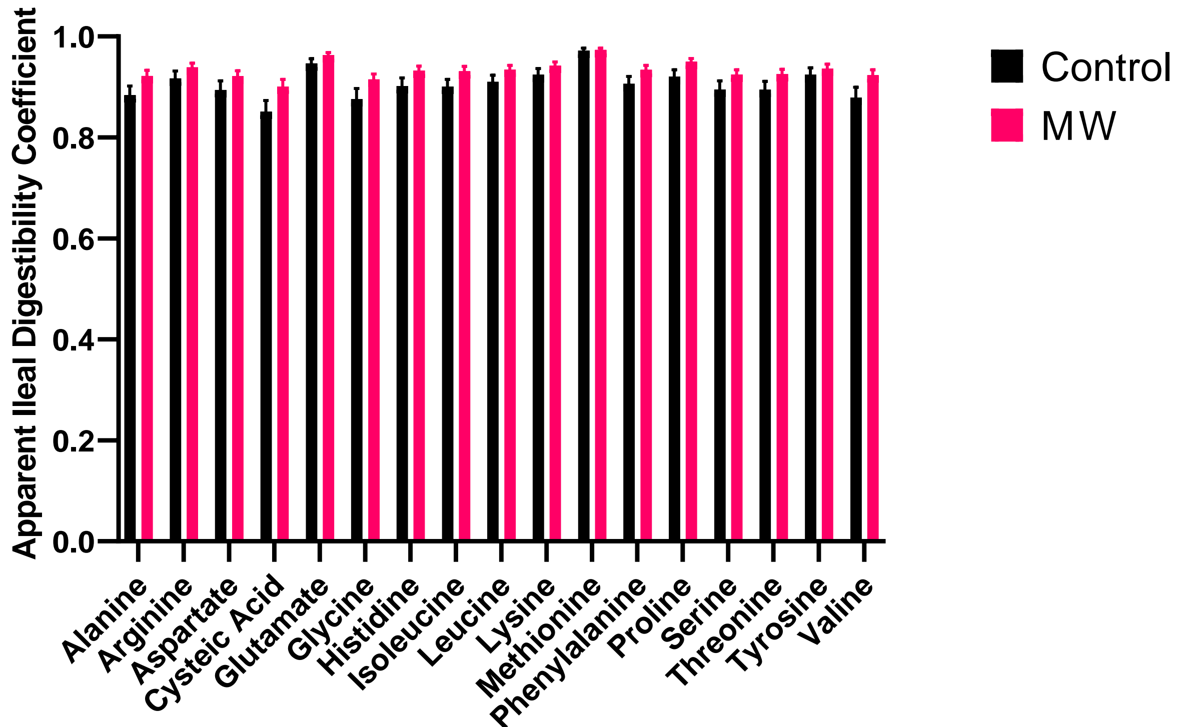


***Figure 5.4** Apparent ileal amino acid digestibility coefficients (AIDC) of broilers after 30 days on feeds*

Control = soybean meal feed, MW = mealworm feed. Data is presented as means±SEM, n=10 per feed treatment.

5.3.5 Caecal Microbiome

There was a significant effect of the partial replacement of soybean mealworm with mealworms on the caecal microbiota of broilers ($P<0.001$) (Figure 5.5). Firmicutes were the dominant phylum accounting for over 95% in the caecal contents of both feed groups (Figure 6.2) and there was an increase in the relative abundance of Firmicutes in the mealworm compared to the control (soybean meal) feed. The second most abundant phylum was proteobacteria, followed by actinobacteria, unclassified bacteria (i.e. could not be identified) and then others (Figure 6.2). The Bacteroidetes phylum did not appear at all in the control group but did in the MW group ($4.8x10^{-5}$ %) (Figure 6.2). Further analysis into the

specific operational taxonomic unit (OTU) contributing to the Bacteroidetes revealed that the effect was due to just 1 bird out of the 40 in the MW group (Pen 8 Bird 86, OTU=2321).

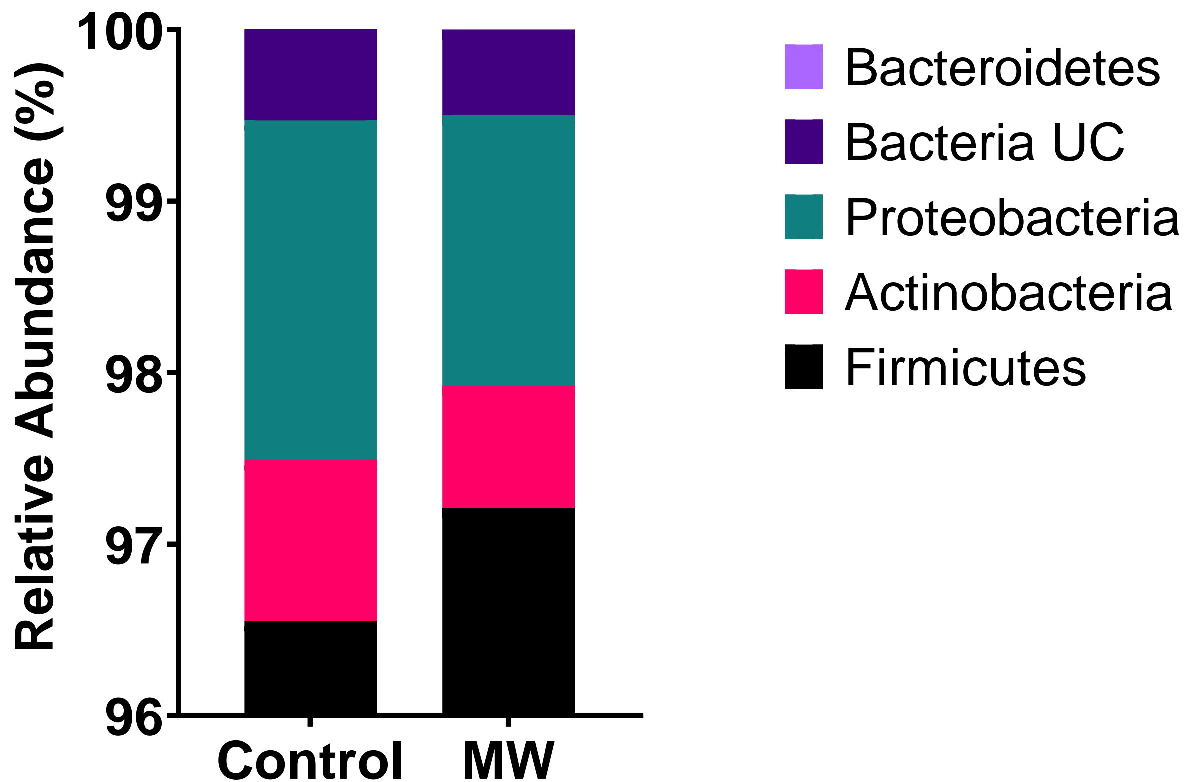


Figure 5.5 *Phyla relative abundance of caecal content of broilers after 30 days on feeds*

Control (soybean based broiler feed), MW (10% soybean replaced with mealworms feed). Bacteroidetes = 4.8×10^{-5} % relative abundance. Bacteria UC = Bacteria unclassified Data is presented as relative abundance means. n=40 for both feeds.

The top 10 OTU's with the greatest relative abundances are shown in Figure 5.6A for all birds sampled. Figure 5.6B shows the OTU's that were discriminative between the control and the MW fed birds. Interestingly, Romboustia and Clostridium_IV only feature in the MW group, whereas Lactobacillus and Anaerostipes only feature in the control group (Figure 5.6B). In Figure 5.6A there is an increase in the Lachnospiraceae unclassified and a decrease in Ruminococcaceae unclassified in the MW fed birds compared to the control, but

from figure 5.6B there are log changes in Lachnospiraceae_unclassified and Ruminococcaceae_unclassified with both the mealworm and control feeds.

Table 5.6 displays further analysis utilising the base sequences of the OTU sequences that were significantly different in the LefSe report, which were then run through NCBI Blast to identify the potential species of bacteria. Lacnospiracae_unclassified and Ruminococcaceae were reported as changing in both the SBM and MW feeds (Figure 5.3B), so Table 5.6 reports the specific OTU's and the potential species causing the increase in either the MW or SBM fed birds. Further analysis indicated that the significant increase in Lachnospiraceae UC (P<0.001) (OTU0002) in the MW fed birds may be due to *Sellimonas instestinalis,* while the significant increase in the Ruminococcaccae UC (P=0.004) (OTU0009) may be due to *Actualibacter muris* strain KB18. Additionally, there was a high percentage identity for OTU0014 to also be *Actualibacter muris* strain KB18. The significant decrease in Lachnospiraceae UC (P<0.001) (OTU0007) in the MW fed birds may be due to *Ruminococcus torques* strain VPI B2-51 and the decrease in Ruminococcaceae UC (P=0.038) (OTU0048) may be due to *Agathobaculus desmolans* strain ATCC. In MW fed birds the significant increase in romboutsia (P=0.006) (OTU0005) was identified to potentially be *Romboutsia timonesis* strain DR1 while the decrease in lactobacillus (P<0.001) (OTU0017) appears to be *Lactobacillus reuteri* DSM 20016 and anaerostipes (P=0.001) (OTU0008) appears to be *Anaerostipes butyraticus* strain 35-7.

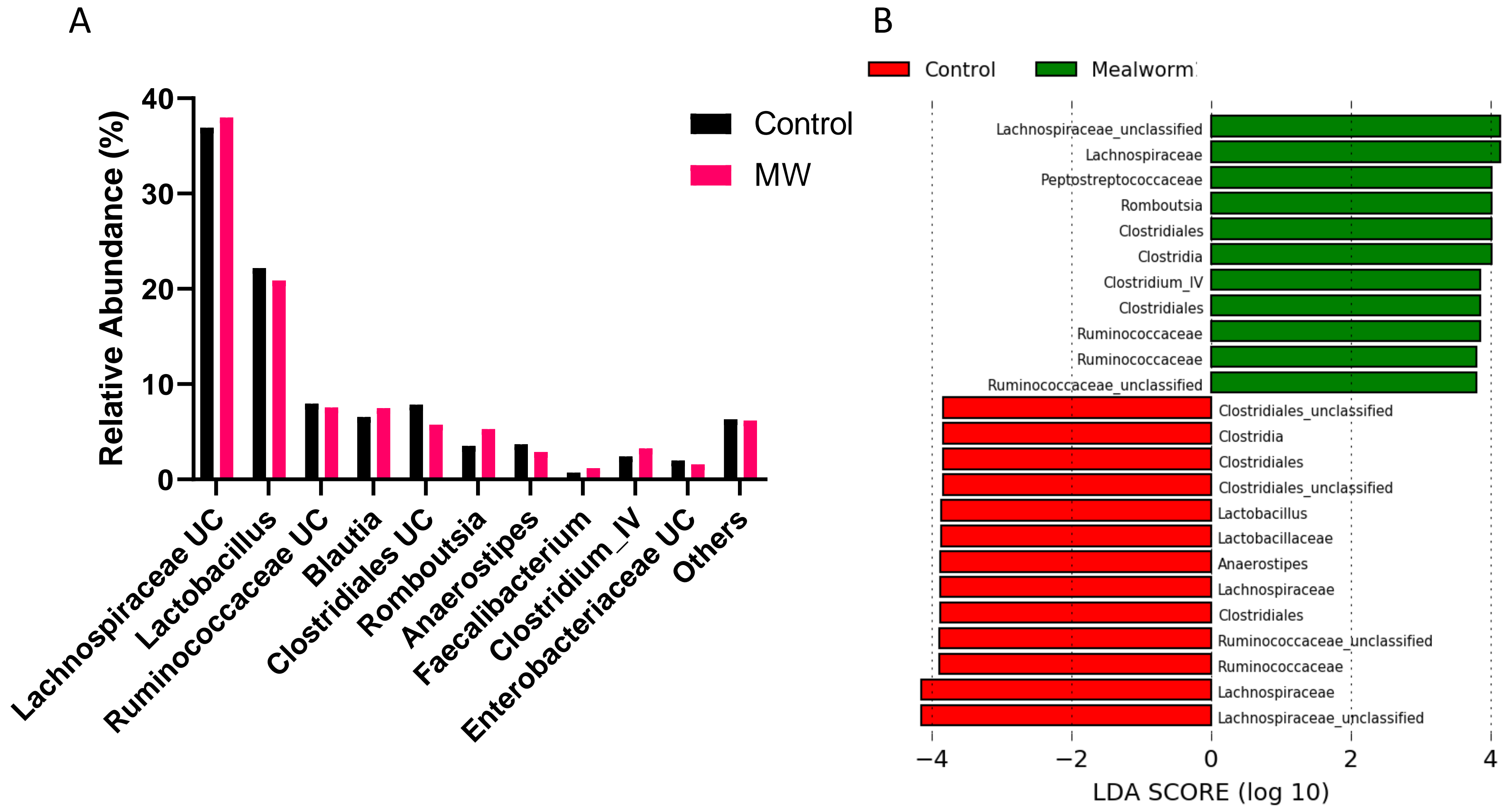


Figure 5.6 *A: Relative abundance of caecal microbiota genera of broilers after 30 days on feeds. B: Differential bacterial species identified in the caecal content of broilers after 30 days on feeds analysed using linear discriminant analysis effect size (LEfSE).*

SBM (soybean based broiler feed), MW (10% soybean replaced with mealworms feed). UC = unclassified Data is presented as relative abundance means. n=40 for both feeds.

Table 5.4 *Discriminant analysis of operational translation units (OTUs) from caecal content of broilers fed either Control or MW feeds over a 30-day period.*

OTU[1]	P Value	Genus on LefSe[2]	Response	% Identity	Species
OTU0002	P<0.001	Lachnospiraceae UC[3]	Increased in MW	96%	Sellimonas intestinalis strain BR72 (Firmicutes, Clostridia, Clostridales, Lachnospiraceae)
OTU0005	P=0.006	Romboutsia	Increased in MW	99%	Romboutsia timonensis strain DR1 (Firmicutes, Clostridia, Eubacteriales, Peptostreptococcaceae)
OTU0009	P=0.004	Ruminococcaceae UC[3]	Increased in MW	92%	Acutalibacter muris strain KB18 (Firmicutes, Clostridia, Clostridales, Oscillospiraceae)
OTU0014	P<0.001	Clostridium_IV	Increased in MW	96%	Acutalibacter muris strain KB18 (Firmicutes, Clostridia, Eubacteriales, Oscillospiraceae)
OTU0007	P<0.001	Lachnospiraceae UC[3]	Decreased in MW	97%	Ruminococcus torques strain VPI B2-51 (Firmicutes, Clostridia, Clostridales, Lacnopiraceae)
OTU0008	P=0.001	Anaerostipes	Decreased in MW	100%	Anaerostipes butyraticus strain 35-7 (Firmicutes, Clostridia, Eubacteriales, Lachnospiraceae)
OTU0016	P<0.001	Clostridales UC[3]	Decreased in MW		Unknown
OTU0017	P<0.001	Lactobacillus	Decreased in MW	99%	Lactobacillus reuteri DSM 20016 (Firmicutes, Bacilli, Lactobacillales, Lactobacillaaceae)
OTU0048	P=0.038	Ruminococcaceae UC[3]	Decreased in MW	98%	Agathobaculum desmolans strain ATCC (Firmicutes, Clostridia, Clostridales, Ruminococcaceae)

[1]OTU = operational translation unit, [2]LefSe = Linear discriminant analysis effect size [3]UC = unclassified.

5.4 Discussion

Partial replacement of soybean meal with mealworms had no effect on growth of broilers or body composition but increased feed intake, increasing feed conversion ratio

As partial replacement of soybean meal with mealworms did not affect broiler growth, mealworms would appear to be suitable for inclusion into broiler feeds. This result was similar to previous studies, which partially or completely replaced soybean meal. For example, Ramos-Elorduy et al., (2002) also replaced 10% of soybean meal and reported no differences in broiler growth, while complete replacement of soybean meal with mealworms has also been reported not to affect broiler growth (Bovera et al., 2015). Similarly, Biasato et al., (2016) observed no changes in growth of broiler chickens when 7.5% maize gluten meal was substituted for mealworm meal. These studies all indicate that mealworms are not having a negative effect on growth of broiler chickens

Feed intake (measured on a pen basis) was increased in the broilers consuming the MW feed compared to the Control (soybean meal) feed. Caution should be taken in interpreting the feed intake data as birds were not raised in commercial conditions. Feed intake in broilers is known to be influenced by both dietary energy and amino acid profile (Ferket and Gernat, 2006). In the current study, the MW feed was formulated to match the protein and oil. There were no differences in the crude protein content of the feeds but there was numerically higher gross energy in the MW feed. Although the feeds were not formulated to amino acids there were no apparent differences in the essential amino acid contents of the two feeds. The decreased calcium content of the mealworm feed may be of concern, therefore commercial formulation of mealworms into broiler feeds needs to ensure

minerals are balanced, with the potential for a supplement to be produced alongside mealworms to ensure optimal minerals are being supplied.

As the exoskeleton of mealworms contains the procuticle, which has microfibers of chitin surrounded by a matrix of protein (Chapman, 2013). Chitin is not degraded and absorbed in the small intestine (Bovera et al., 2015), and thus can negatively affect protein digestibility (Marono et al., 2016). However, the use of pure chitin as a supplement had no negative effects on performance (Razdan and Pettersson, 1994), suggesting that it is the matrix of the chitin-containing cuticle that is potentially affecting digestibility. The digestibility of nutrients in the gastrointestinal tract and the efficiency of conversion of these absorbed nutrients into energy and ultimately growth. It would be expected that a feed providing increased energy would decrease feed intake compared to a lower energy feed. However, energy was measured as gross energy, nutrients which could have provided energy could have been bound by the cuticle and unavailable for absorption. Therefore, the MW fed birds may have been unable to digest and absorb some nutrients (amino acids and energy) required and therefore increased their feed intake to compensate for this.

Since the birds consumed more of the MW feed this suggests that it was palatable, which is perhaps expected, as birds will naturally eat insects. As there were no differences in live weight gain, the increased feed intakes resulted in a higher (i.e. poorer) FCR for the MW fed birds. This would potentially be negative for broiler production, as more feed is required to produce the same weight gain. The significance of this would be dependent on the cost of the feed compared to conventional ingredients but more work may be required to overcome this prior to use in commercial feeds. Additionally, it must be noted that the FCR

recorded in this study was a lot higher than commercial broiler units. Potentially owing to the method for measuring feed consumption, wasted feed by the birds was not weighed as it was mixed with the sawdust bedding on the pen floor. Therefore, the feed consumption may be higher than what was eaten by the birds resulting in a higher FCR.

Previous studies have reported mixed findings concerning insect inclusion on broiler feed intake and FCR. (Biasato et al., 2018) reported similar findings to this study in terms of increased feed intakes with 10 and 15% inclusion of mealworms, but they also reported an increase in body weight. They also fed at 5% inclusion and concluded that low inclusion levels may be more suitable for broiler feeds (Biasato et al., 2018). In contrast, Ballitoc and Sun (2013) reported decreased feed intakes with 0.5%, 1%, 2% and 10% inclusion of mealworms compared to the control, resulting in a lower FCR for the mealworm-fed groups. Bovera *et al.*, (2016) also saw an improvement (decrease) in FCR with complete replacement of soybean meal with mealworms, but the chickens used were older in age than the current study. Ramos-Elorduy *et al.*, (2002) observed no difference in FCR, when replacing 5 and 10% of soybean meal with mealworms in a sorghum based feed, whereas the current study used a wheat based feed.

In the current study, mealworms were ground to a powder, but it was clear when observing the birds eating that they were selecting components (presumably partially intact mealworms missed in the grinding process) out of the mash feed. The increased feed intake could therefore be due to the added interest in the mealworms, which if selected may not completely match their requirements. Furthermore, chicks will increase their pecking behaviour when they see other birds exhibiting this behaviour (Ferket and Gernat, 2006), so if one bird found a larger mealworm piece and then increased its pecking behaviour, this

could have resulted in the other three birds in the pen also increasing their pecking, thereby increasing total pen feed intake. This suggests that inclusion of mealworms in the feed could potentially be a welfare initiative, providing an outlet to remove boredom or associated negative behaviours.

The significant increase in feed intake could also suggest that feeds that include mealworms may be more palatable for the birds compared to the soybean meal feed. In agreement with this, inclusion of mealworms as a complete replacement for maize gluten meal has been suggested to improve palatability (Biasato et al., 2016). To our knowledge, there are no published studies that examine whether broilers prefer a feed with mealworms compared to a traditional feed. Quails subjected to a feed choice experiment with black soldier fly-based feeds did tend to prefer insect-based feed (Cullere et al., 2016). The innate behaviour of chickens to root around for insects suggests that they will actively consume them. Free access to mealworms alongside a normal feed, has been shown to improve body condition score, gait score and footpad dermatitis in cage-reared broilers (Chen 2018). Additionally free dietary choice behaviours have been reported to promote appetite, leading to increased foraging behaviours (Chen et al., 2018).

Partial replacement of soybean meal with mealworms had no effect on body composition of the broilers in the current study. Similarly, a 7.5% mealworm inclusion in female hybrid chickens resulted in no effect on body composition (Biasato et al., 2016). Whereas a lower inclusion of 2% has been reported to improve carcass yield and internal organ weight (Ballitoc and Sun, 2013). Gizzard weight was not recorded in our study, but Ballitoc and Sun (2013) found 10% inclusion of mealworms produced a heavier gizzard, implying that feed was retained there for longer, potentially allowing for increased mechanical digestion.

Partial replacement of soybean meal with mealworms had no effect on apparent ileal crude protein or amino acid digestibility coefficients

There was an increase in crude protein, alanine, glycine, histidine, isoleucine, lysine, methionine, tyrosine and valine contents in the ileal digesta of the mealworm fed birds compared to the control birds, suggesting that there was decreased absorption of amino acids in the mealworm fed birds. However, there were no differences in apparent ileal digestibility coefficients (AIDC) of either crude protein or individual amino acids, suggesting that apparent digestibility of mealworms was similar to that of soybean meal.

The AIDC calculations utilised ileal titanium content measurements to correct for volume of ileal digesta collected and assume that the titanium dioxide was fully recovered (Stein et al., 2007). However, there was a wide range of titanium contents measured in the ileal samples, even though the same quantity of titanium dioxide was mixed into both the control and MW feeds, indicating that the ileal contents should be similar. Some variation in the samples might be expected due to the samples consisting of ileal contents from four different birds. However, it is of note due to equipment access issues caused by COVID-19, the titanium was measured through ICPMS and not the standard method (Short et al., 1996), which specifically measures TiO_2. The ICPMS method measured titanium content after an acid digestion to break down the titanium dioxide. This method may not have been sensitive enough to measure all the titanium in the samples due to the presence of matrix-based spectral interferences, where some of the Ti isotopes measured are overlapped by other mineral isotopes (McCurdy and Woods, 2015). As with all digestibility trials, the methods used to calculate the digestibility values have an impact on the results obtained, therefore limiting comparability between studies (Masey O'Neill et al., 2014).

The calculated AIDC of crude protein (0.92) is higher than previously reported for mealworm inclusion into broiler feeds. Bovera et al., (2016) reported that the complete replacement of soybean meal with mealworms resulted in a lower AIDC crude protein of 0.80 compared to 0.87 for the control feed. Marono et al., (2016) noted that crude protein digestibility was negatively correlated to chitin content when measured *in vitro*. Therefore completely replacing soybean meal with mealworms would create a feed with a higher chitin content compared to the 10% replacement used in our study, possibly explaining the reduced digestibility coefficient described by Marono et al., (2016).

There were no significant differences in the apparent ileal amino acid digestibility coefficients when comparing the mealworm and control feeds. Similarly, Hall et al., (2018) found no differences when using housefly larvae as an alternative protein source in broiler feeds. In contrast, the use of mealworms in pig feeds tended to show increased AIDC of amino acids compared to fish meal, meat meal or poultry meal (Yoo et al., 2019). Amino acids have been noted to be readily available from insects with digestibility values comparable to soybean meal and fishmeal (Hawkey et al., in press 2021). Previous studies investigating the digestibility of insects have normally looked in more detail at the specific digestibility of insect meal, instead of the feed containing insects, as was the case in this study. De Marco 2015 reported that mealworm AIDC of amino acids ranged from 0.80 to 0.93 in broilers.

Chickens have been shown to express chitinase in their glandular stomach, indicating that they have the capacity to digest chitin-containing organisms (Tabata et al., 2017). As there were no differences in the AIDC for crude protein or amino acids, this suggests that either the endogenous expression of chitinase was aiding digestion of the chitin or the broilers

were not utilising the chitin at all and it was passing through undigested or possibly being digested in the caeca. Alternatively using a 10% inclusion of mealworms the chitin concentration is not high enough to effect digestibility.

Caecal microflora was positively affected by mealworm inclusion into broiler feeds as a partial substitution for soybean meal

The caecal microflora was affected by the partial replacement of soybean meal with mealworms, with an increase observed in the phyla, Firmicutes, Proteobacteria and Bacteroidetes. On a genus level there was a decrease in lactobacillus and anaerostipes in the MW fed birds and an increase in Romboutsia and Clostridium_IV. Therefore, the hypothesis that MW inclusion can manipulate the caecal microflora content appears to be correct.

Comparison of caecal microflora between studies is limited as the environment and age of the birds both affect the caecal microflora (Ocejo et al., 2019). The major phyla in this study were Firmicutes with over 96% relative abundance in both feed groups, which is higher than previous broiler studies (Oakley et al., 2014). The Firmicutes phylum is predominant in the gastrointestinal tract of broilers and has been linked to the efficiency of energy harvesting in various animals (Wang et al., 2016). It may significantly influence both feed digestion and host health (Biasato et al., 2019b), therefore the increase in the MW group may be of importance. Biasato et al., (2018) also observed an increase in the relative abundance of Firmicutes with a 7.5% inclusion of mealworm, although a higher inclusion (10-15%) reduced the Firmicutes relative abundance, suggesting that lower inclusion levels may be preferable (Biasato et al., 2019a). The MW fed birds had a higher Bacteroidetes relative abundance compared the control (soybean meal) fed birds, which had none. However, previous work

has found a decreased level of Bacteroidetes in broilers fed mealworm based feeds (Biasato et al., 2018).

The LefSe report highlighted that there were large (log) changes in Lachnospiraceae UC for both feed groups. This bacterial family makes up the core of gut microbiota in humans, and its members are some of the main producers of butyrate and other short chain fatty acids (Vacca et al., 2020) and may have the potential to breakdown chitin (Biddle et al., 2013). Liu et al., (2021) noted the importance in differentiating between Lachnospiraceae species as a few members are positively correlated with broiler feed efficiency but others have the opposite impact. Specific genera were investigated further to give an indication of the potential species being affected by mealworm inclusion.

MW fed birds had increased relative abundance of Romboutsia compared to control birds. The roles of Romboutsia genera are still relatively unknown, but they have a wide metabolic capability to ferment carbohydrates and are adapted to a nutrient rich environment (Gerritsen et al., 2019). There was a significant increase in OTU0005 that aligned 99% to *Romboutsia timonensis strain DR1*. In humans the genus Romboutsia is often associated with a healthy gut (Ricaboni et al., 2016). *Sellimonas intestinalis* strain was increased in the MW fed birds; little is known about this bacteria but it has been noted as a biomarker of gut homeostasis recovery (Muñoz et al., 2020) and an indicator of a healthy gut. There was a decrease in *Ruminococcus torques* strain in the MW fed birds compared to the control. This bacteria has been associated with a decrease in gut barrier integrity through degrading mucus (Deaver et al., 2018), suggesting that the decrease may potentially be a good effect. The changes in these species of bacteria in the MW fed birds may therefore indicate that the inclusion of mealworms is having a positive effect on gut health.

There was an increased in Clostridium IV in the MW fed birds, with a 96% alignment identity to *Acutalibacter muris* strain KB18. This strain was also highlighted to have a 92% identity to another OTU (OTU0009). Clostridium IV genera covers a broad range of functions but are known for ability to alleviate dysfunctions and disorders in the gastrointestinal tract, additionally they can utilise indigestible polysaccharide (Guo et al., 2020). Therefore, chitin could be a polysaccharide which is indigestible by the host (broiler) but is utilised by Clostridium bacteria for growth.

The MW fed birds had a decrease in lactobacillus and increase in Romboutsia genera and it has been suggested that there is a relationship between these two genera. Supplementation of astraglus (Chinese herbal medicine) in hen feeds resulted in an increase in lactobacillus and decrease in Romboutsia, with the suggestion that there was a competitive exclusion relationship between the abundance of the two genera (Qiao et al., 2018). Therefore, the MW could be acting to actively increase the abundance of Romboutsia, which then outcompetes the lactobacillus initiating a decrease in their abundance.

In livestock production Lactobacilli can be used to enhance nutrient digestion and improve feed conversion efficiency by limiting pathogen infection levels (Józefiak et al., 2020), so the decrease in lactobacillus in the MW fed birds may in part be playing a role in the increased FCR. The lactobacillus genus was found to be *Lactobacillus reuteri*, which has been highlighted as a potential probiotic (Hou et al., 2015). Supplementing broilers with *Lactobacillus reuteri* has been reported to regulate tight junctions enhancing mucosal barrier function and helping to prevent against pathogenic infection (Nii et al., 2020). Therefore, a decrease in this species of lactobacillus with the mealworm feed may be negatively affecting the gut mucosal barrier. This species of lactobacillus was also reported

to have a contradicting effect in a study by Józefiak et al., (2020), were inclusion of 0.2% mealworms stimulated the development of *lactobacillus reuteri* compared to the positive control, but was not significantly different.

The changes in lactobacillus may also be linked to the changes in the Anaerostipes. Lactobacillus produce lactic acid which can then be utilised as a feed by Anaerostipes (so called cross feeding). Consequently, a reduction in lactobacillus in the MW fed birds may have led to a decrease in the anaerostipes as well. Further analysis revealed a high percentage identity of the Anaerostipes species to *Anaerostipes butyraticus* strain 35-7, which are butyrate producing bacteria (Eeckhaut et al., 2010). In the MW fed birds there was also a decrease in the Ruminococcaceae, *Agathobaculum desmolans,* which are also butyrate producing bacteria (Ahn et al., 2016). Butyrate stimulates the development of intestinal mucosa, increases mucin production (Eeckhaut et al., 2010) and increases the barrier function of the gut wall (Mátis et al., 2019). The reduction of these two butyrate-producing bacteria in MW fed birds may be negatively affecting gut health.

Bacteria have been reported to have the capability to produce chitinase to meet nutritional requirements (Cohen-Kupiec and Chet, 1998). Furthermore the same bacteria can produce several chitinases to ensure that a range of chitin structures can be digested (Cohen-Kupiec and Chet, 1998). None of the bacteria species highlighted in this analysis has been reported to have chitinase activity, however new developments in caecal microbiota analysis are leading to increased bacteria function understanding. Therefore, it remains to be established whether the chitin component of the mealworms was digested either by chitinase enzymes in the proventriculus (i.e. produced by the host chicken) or by chitinases

in the caecum (i.e. produced by the microbiota), and whether these breakdown products that are potentially modulating the bacteria present in the caecal microbiota.

5.5 Conclusions

This broiler trial was carried out to determine the effects of partial inclusion of mealworms into a broiler feed on performance, apparent digestibility, and the caecal microbiome. There were no effects on broiler growth, but feed intake and subsequent FCR were both increased when mealworms partially replaced soybean meal, demonstrating that the mealworm fed birds had to consume more feed in order to reach the same weight.

There was no effect of mealworm inclusion on the apparent ileal digestibility coefficients (AIDC) for crude protein or individual amino acids, suggesting that a decrease in digestibility was not the reason for the increased feed intake. However due to methodological problems with measuring titanium dioxide this needs to be repeated using the standard method.

Partially replacing soybean meal with mealworms significantly affected the caecal microbiota, with changes at both phyla and genus level. The main changes appeared to be an increase in relative abundance of OTU with high percentage identity to *Romboutsia timonensis and Sellimonas intestinalis,* which are normally associated with a healthy gut. This suggests that inclusion of mealworms may be having a positive effect on the gut microflora. However, the decrease in the lactobacillus and butyrate-producing bacteria are a concern, due to their reported positive effects on gut integrity.

Overall, at 10% inclusion level mealworms appear to be a palatable feed ingredient that maintain broiler growth. However, the reduced feed efficiency and possible adverse effects on the gut microbiome require further investigation. Ultimately, the sustainability and cost of mealworm production may be the drivers of whether they become a commercial alternative to soybean meal.

6 Investigation into the use of chitinase treated mealworms in broiler feeds

6.1 Introduction

Mealworm physiology, particularly the exoskeleton, impacts upon nutrient content and digestibility (Williams *et al*., 2016). The exoskeleton contains chitin, the nitrogen containing polysaccharide that could have both positive and negative effects concerning inclusion into poultry feeds. The insect cuticle contains between 20 and 50% chitin on a dry weight basis (Chapman, 2013). Chitin is a linear polysaccharide composed of N-acetyl glucosamine monomer units (Rathore and Gupta, 2015), linked together by β (1,4) glycosidic bonds (Figure 6.1) (Chapman, 2013). It occurs naturally in three forms, alpha, beta, and gamma and differ by their structural chain arrangements. Alpha chitin has antiparallel chains, beta chitin has parallel chains and gamma chitin has a mixture of parallel and anti-parallel chains (Kaya et al., 2015).

The most predominant structural type in mealworms is the alpha form (Son et al., 2021), as the anti-parallel chain format is the most stable form in nature (Rathore and Gupta, 2015). Within this cuticle there is a matrix of proteins, lipids, chitin and other compounds bound together (Finke, 2007).

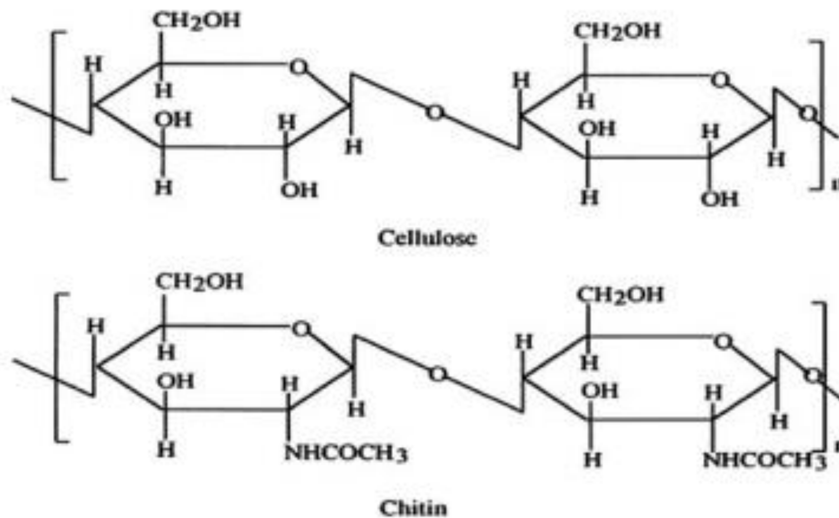


Figure 6.1 *Structure of cellulose and chitin, taken from* (Chawla et al., 2014)

Chitin could be perceived as an anti-nutritional factor, as it is insoluble, has a high viscosity, (Pillai et al., 2009) and binds nutrients in the matrix (Rathore and Gupta, 2015), making them unavailable for digestion. Protein digestibility in insects has been reported to be negatively correlated to the chitin content (Marono et al., 2016), due to its ability to bind to both protein and amino acids (Rathore and Gupta, 2015). Alternatively, chitin has been shown to be beneficial at low inclusions due to its antioxidant and anti-inflammatory properties (Park and Kim, 2010), and could therefore be used to stimulate the immune system or modulate animal gut health. This area warrants further investigation to understand the impact of chitin inclusion in animal feed.

Traditional livestock feeds utilise exogenous enzymes added into the formulation (Bedford and Partridge, 2010) to release nutrients bound by anti-nutritional factors and to improve digestibility of feed ingredients. Chitinases are glycosyl hydrolases which hydrolyse the glycosidic bonds between N-acetyl D glucosamine monomers in the chitin polymer chain

(Rathore and Gupta, 2015), so have the potential to breakdown the chitin to improve the digestibility of mealworms and other insects. They are naturally found in the gut of chickens (Han et al., 1997; Tabata et al., 2017) and also present in bacteria, fungi, plants and insects (Rathore and Gupta, 2015). In plants, chitinases act as a guard against pathogens, while in fungi they increase plasticity of cell walls to ensure maintenance of normal growth and in insects they allow modification of the chitin in the exoskeleton, particularly during growth and development (Rathore and Gupta, 2015).

Additionally, the ability of chitinases to hydrolyse chitin to N-acetyl D glucosamine may have potential health benefits, including restoring the gastrointestinal tract lining to increase blood supply and protect the gut against harmful substances (Chen et al., 2010). N-acetyl D glucosamine is used as a supplement to slow the development of osteoarthritis and increase collagen synthesis in humans (Kubomura et al., 2017), so may have similar positive effects on chicken health, particularly in the gut.

To our knowledge the use of chitinase enzymes to improve the digestion of mealworms in broiler feeds has not previously been reported. This study described in this chapter explored the use of a chitinase on digestion of mealworms both *in vitro* and *in vivo*, focussing specifically on broiler production, the caecal microbiome and digestibility of crude protein and amino acids.

Aims

1. To assess the ability of 7 different chitinases to hydrolyse chitin and mealworms *in vitro*
2. To assess the impacts of adding the most active chitinase to broiler feeds containing mealworms (as a partial replacement of soya) on broiler production and apparent digestibilities of crude protein and amino acids, as well as the caecal microbiome.

Hypothesis

The addition of chitinase into broiler feeds containing mealworms will improve the feed conversion ratio and impact upon the caecal microbiome.

6.2 Materials and methods

6.2.1 In vitro incubation digestions

To determine the volume of enzyme needed for the broiler trials, *in vitro* incubation trials with 7 different chitinases were carried out.

The seven chitinase enzymes to be tested were provided by AB enzymes (Table 6.1). The production host was a genetically modified *Trichoderma reesei* (filamentous fungus), where the chitinase gene was expressed using the strong cellobiohydrolase (cbh1) promoter. Enzymes were prepared as concentrated culture supernatants, where chitinase made up 60% of the protein. However, not all major cellulase genes were deleted from the host so

some background proteins remained, therefore a negative control was also provided to account for the effect of the background proteins. The negative control was the supernatant from the same filamentous fungus, but not transformed with the chitinase gene.

AB enzymes subsequently increased the protein content of the most active chitinase to allow for a smaller volume to be used to pre-treat the mealworms for use in the broiler trial. This is reported as high 9 and high 4 (Table 6.1).

Table 6.1 *Protein content of 8 original chitinase enzymes and 2 concentrated supplied from AB enzymes*

Chitinase Enzyme	Protein content (mg/ml)
4 (-ve control)	20.9
5	25.5
6	25.0
7	25.2
8	24.4
9	25.4
10	21.8
11	22.5
High 9	156
High 4	149

All experiments were conducted under the same conditions but with differing volumes of enzymes, types of substrate and incubation times. For all incubations, 50mM sodium citrate at pH 5.2 was used as the buffer to mimic the average pH along the chicken gastrointestinal tract. Incubations were carried out in a bench top turning incubator (Envirogenie) at 40.7°C (as a representative temperature of chicken gastrointestinal tract).

A stock solution of either chitin or mealworms was re-suspended in the sodium citrate buffer and mixed. Samples for incubation were then removed and put in 1.5ml tubes, the individual enzyme was added and the tubes placed in the incubator as quickly as possible. The 7 incubations carried out are described in table 6.2, each incubation described are batches of incubations, there was a single incubation tube for each time point and enzyme.

Table 6.2 *Details of incubations with chitinase enzymes*

Incubation[1]	Solution	Chitinase Enzyme	Volume of enzyme added (µl)	Sampling Times (hours)
1	10g/L chitin	4,5,6,7,8,9,10	100	20
2	10g/L chitin	9	10, 100	0, 3, 24, 48
3	20g/L mealworm	4 and 9	100	0, 4, 20, 48
4	10g/L chitin	High 9 and 4	1	0, 0.5, 1, 2.5, 4
5	10g/L chitin	High 9	1, 10	0, 3, 10, 24, 48
6	20g/L mealworm	High 9	10	0, 3, 18, 24
7	10g/L	High 9 and 4	1	0, 0.5, 1, 2.5, 4

[1] For each incubation number separate digestions were set up in individual 1.5ml tubes for each chitinase enzyme at each volume of enzyme added and each time point.

6.2.2 Sample Processing for assay

Sample Processing before assay

Following incubation, all samples were centrifuged at 13000rpm for 5 minutes. 500µl of the supernatant were placed in a glass tube with 500µl of 4M hydrochloric acid; the tube was covered with foil and then heated in a heating block at 100°C for 2 hours. After heating the glass tubes where removed, 1ml of 2M NaOH was added to neutralise the acid, and the solution was left to cool for 10 minutes. The purpose of this step was to deacetylate the N acetyl glucosamine to D glucosamine.

Mealworm samples were further treated to remove protein and fat components. The reagents used were Carrez I, II and sodium hydroxide. Carrez I was potassium hexacyanoferrate (II), 3.6g was dissolved in 100ml of distilled water. Carrez II was zinc sulphate; 7.2g was dissolved in 100ml of distilled water. For each 250µl of acid digested neutralised sample 80µl Carrez I, 80µl Carrez II and 160µl of 100mM NaOH were added. A precipitate formed, the sample was centrifuged at 13 000rpm for 5 minutes, and the supernatant was taken for use in the assay.

6.2.3 D Glucosamine Assay

A D-glucosamine assay (Megazyme, Co. Wicklow, Ireland) was used to measure the release of D-glucosamine from chitin and mealworms after digestion with chitinase. The assay is an enzyme coupled assay which uses a series of enzymes to ultimately reduce NADP to NADPH. There is a stoichiometric relationship between NADPH and D glucosamine.

Process:

1. D-glucosamine + ATP → D-glucosamine-6-P + ADP + Pi (Hexokinase)
2. D-glucosamine-6-P + H_2O → Fructose-6-P + NH_4^+ (Glucosamine-6-phosphate deaminase)
3. Fructose-6-P → Glucose-6-P (Phosphoglucose isomerase)
4. Glucose-6-P + $NADP^+$ → Gluconate-6-P + NADPH + H^+ (Glucose-6-phosphate dehydrogenase)

NADPH is measured by the increase in absorbance at 340nm.

To allow for easier pipetting master solutions were prepared using solutions provided in the Megazyme kit.

Amounts per well

Master 1: 78µl water, 10µl Buffer (pH 7.6), 10µl Solution 2 ($NADP^+$, ATP and PVP), 2µl solution 3 (hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase)

Master 2: 2µl solution 4 (glucose-6-phosphate deaminase), 8µl water

Microplate procedure:

20µl sample or standard, 104µl water and 100µl master 1 were pipetted onto a 96-well plate and left for 3 minutes and then read at 340nm using a Bio-Rad Plate Reader. Then 10µl master 2 was pipetted onto the plate and left for 8 minutes, then read consecutively every 4 minutes until the absorbance stopped increasing. Absorbance values for samples were determined by subtracting background values from initial reading. Standard curve equation of lines were used to calculate the D glucosamine unknown samples.

6.2.4 Chitinase Broiler Trial

6.2.4.1 Feeds

It was calculated that 200kg of feed would be required for a 30-day trial period. Of this 200kg of feed, 10% would be mealworms (20kg). As mealworms are reported to contain 5% chitin (Song et al., 2018), this equates to 1kg of chitin in the total 200kg of broiler feed.

Table 6.3 shows an average D glucosamine release from chitin with digestions. These values were used to calculate the volume of chitinase required to pre-treat the mealworms before inclusion into broiler feed. Initial calculations indicated 5 litres of chitinase would be required (Figure 6.2), but this was considered too high, as it may disrupt the physical form of the feed. This value was divided by 2.5 to give a treatment of 2 litres per 1kg of chitin, resulting in a potential 6% release of D glucosamine after a 48-hour digestion (Figure 6.2).

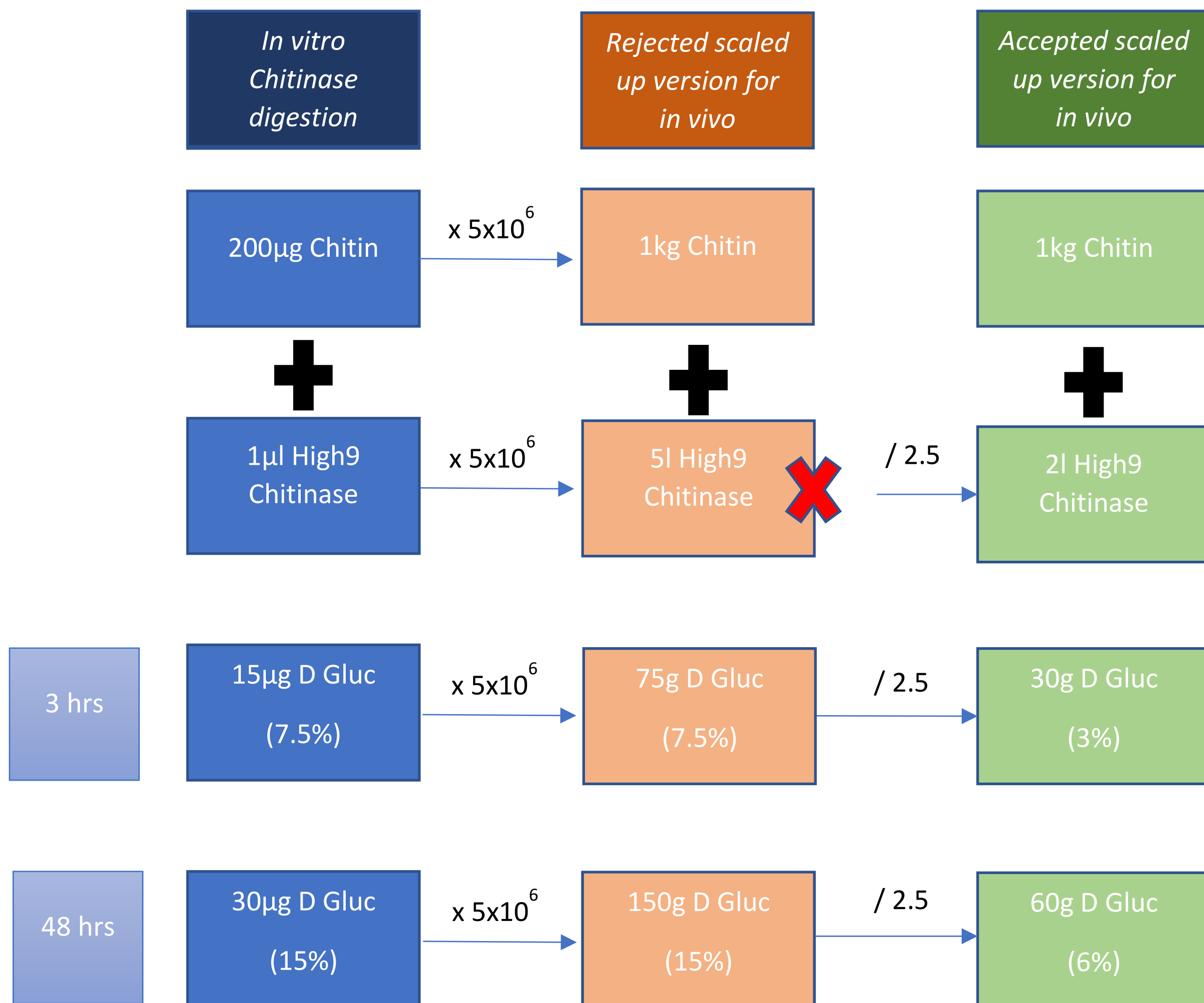


Figure 6. 2 *Calculations for chitinase volume required for pre-treatment of mealworms prior to inclusion into broiler feed.*

Blue column = results of *in vitro* digestions of 3 and 48 hours of chitin with high9 chitinase

Red column = calculated volume of high9 chitinase required to treat 1kg of chitin in the same volume proportions of *in vitro* (values where multiplied by $5x10^6$ (200µg → 1kg). This was rejected as volume was considered too high for feed inclusion.

Green column = calculated volume of high9 chitinase required to treat 1kg of chitin at a lower volume (divided by 2.5) as *in vitro.* This was accepted as volume of high9 chitinase to treat mealworms in broiler trial.

Hrs = hours, D Gluc = D glucosamine

There were two feeds for this broiler trial:

- MW+Neg – basal feed with 10% mealworms pre-treated with negative control enzyme.
- MW+Chitinase – basal feed with 10% mealworms pre-treated with chitinase enzyme.

2 litres of negative control or high 9 chitinase were used to pre-treat 20kg mealworms for 48 hours before inclusion into the basal feed.

The mixing of the enzyme and mealworms was undertaken in batches to ensure equal application of the enzyme. The 20kg of mealworms were split into 2.5kg bags and were treated with 250ml of either negative control or chitinase enzyme. 250ml of chitinase was mixed into 500g of mealworms in a bench top food processor for 2 minutes, a further 500g of mealworms were added gradually and mixed for 2 minutes. The remaining 1.5kg was then added and the total was mixed for 4 minutes. This 2.5kg was then bagged ready for mixing into the basal feed 48 hours later. The same mixing procedure was used for inclusion of the negative control enzyme.

The same formulation for the basal feed from chapter 6 was used in this second chitinase broiler trial. For each bag of 22.5kg of basal feed, 1 scoop of feed was taken out of the bag and mixed in a separate bag with 125g of titanium dioxide. Another 4 scoops were added gradually with continual manual mixing of the bag. The remaining feed in the 22.5kg bag was placed in a screw mixer. After starting the mixer, the mix of titanium dioxide and feed was added, followed by 2.5kg of mealworms per 22.5kg of basal feed. The feed was mixed for 5 minutes and then bagged in two 25kg bags and sealed.

Feeds were stored in cool dry room until needed to feed chicks.

6.2.4.2 Experimental Design

Eighty-day old broiler chicks were acclimatised on control feed from the first broiler trial for 6 days, then allocated to one of 20 pens with 4 birds per pen. There were 10 replicate pens per feed (MW+Neg and MW+Chitinase) each containing 4 birds. Birds were fed the appropriate feed and water *ad libitum* for 30 days. Birds were weighed and feed intakes recorded twice weekly.

6.2.4.3 Sample Collection

On day 35, pens 1-10 were weighed then then culled by Schedule 1 method (Animals (Scientific Procedures) Act 1986) of an overdose of pentobarbitone (anaesthetic) through intravenous neck injection, with pens 11-20 being similarly weighed and culled on day 36. After confirmation of death, the digestive tract was exposed and samples of digesta taken from the ileum, jejunum and caeca of each bird. Alongside these digesta samples, 3cm pieces of tract were also sampled. All digesta and tissue samples were snap frozen in liquid nitrogen before storage at -80ºC.

6.2.4.4 Gut Digesta Sample Analysis

Apparent Ileal Digestibility

Ileal digesta samples collected from each bird were freeze dried until a constant weight and then crushed to a fine powder using a pestle and mortar, then the 4 samples from each pen were pooled. Pooled pen samples (n=10 per feed) were then analysed for crude protein (by

nitrogen analyser, FlashEA 1112 Series, Thermo Fisher Scientific), mineral content via acid digestion and ICPMS (Thermo- Fisher iCAP-Q) and amino acids (LCMS-MS, Thermo Fisher).

Feed samples (n=4 for each feed) were freeze dried until a constant weight then samples were crushed to a fine powder using a pestle and mortar. Proximate nutritional analysis included crude protein by nitrogen analyser (FlashEA 1112 Series, Thermo Fisher Scientific), gross energy content by bomb calorimetry (6300 Calorimeter, Parr), mineral content via acid digestion and ICPMS (Thermo- Fisher iCAP-Q) and amino acids (LCMS-MS, Thermo Fisher).

Apparent digestibility coefficients were then calculated on a pen basis according to the equations described in Chapter 2.

Caecal Microbiome Analysis

DNA was isolated from caecal digesta samples collected from each bird and used individually for microbiome analysis. Initially the V4 regions of the bacterial 16S rRNA genes were amplified by PCR, then the Illumina MiSeq platform was used to sequence amplicons using 2x 250-bp cycles. The Schloss lab in Mothur (online software) was then used to quality filter 16S rRNA sequences and cluster into OTUs. LEfSE plots were then used to analyse changes between feed treatments for specific families.

See chapter 2 for detailed methods on caecal microbiome analysis.

6.2.5 Statistical Analysis

All graphs were drawn using Graph Pad Prism 8. Genstat (20th Edition) was used to perform statistical analysis. The effect of chitinase on the *in vitro* digestion of chitin and release of D glucosamine in incubation was determined by one-way ANOVA, whereas the effect of

chitinase on the *in vitro* digestion of chitin and mealworms over time on the release of D glucosamine was determined by 2-way ANOVA (time x treatment). For the broiler trial the effect of feed on performance indicators (initial live weight, final live weight, body weight gain, feed intake and FCR) were determined by one-way ANOVA, whereas effects on live weight over time were analysed by repeated measures 2-way ANOVA (time x treatment). Similarly, effects of treatment on apparent ileal digestibility coefficients (AIDC) (crude protein and amino acids) were analysed by one-way ANOVA.

For the caecal microbiome, analysis of molecular variance (AMOVA) was calculated within Mothur (Kozich et al., 2013). Linear discriminant analysis effect size (LefSe) was also implemented within Mothur.

Significance was set at $P<0.05$, with trends set at $P<0.1$ but >0.05.

6.3 Results

6.3.1 Chitinase Digestions

Digesting a chitin solution with chitinase significantly increased D glucosamine release compared to the control (1) and negative control (4(-ve)) ($P<0.001$) (Figure 6.3). Enzymes 5-11 hydrolysed significantly more D glucosamine compared to the control and negative control ($P<0.05$) (Figure 6.1). Enzyme 9 released significantly more D glucosamine compared to all of the other enzymes except from enzyme 5 ($P<0.05$) (Figure 6.3).

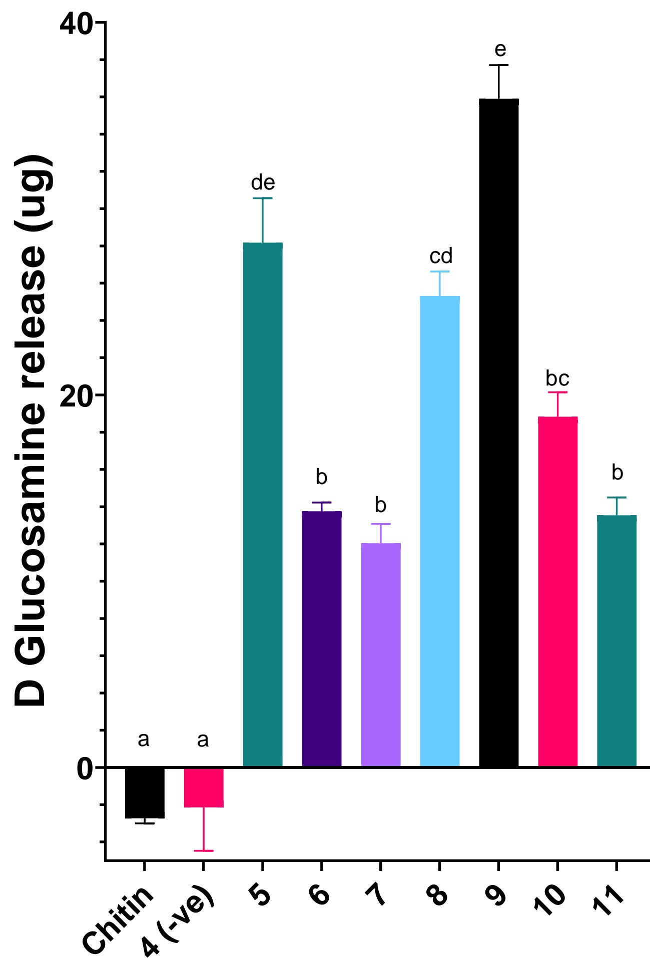


Figure 6.3 *Incubation 1: The effect of seven different chitinases addition on chitin digestion on the release of D Glucosamine. Chitin was digested with differing chitinases (5-11) and a negative control (4) for 20 hours.*

Data is presented as mean±SEM. Different lower-case letters are significantly different (P<0.05), Bonferroni).

Treating chitin solution with differing volumes of chitinase significantly increased D glucosamine release, with a significant interaction between time and treatment ($P<0.001$) (Figure 6.4). There was significantly increased D glucosamine release with 100ul at 24 and 48 hours compared to all other time points and treatments, except 100ul at 9 hours and 10ul at 48 hours ($P<0.05$) (Figure 6.4). There were no differences between D glucosamine release at 0 hours between any of the treatments ($P<0.05$). Using 10ul for 3 hours was not significantly different to 0 hours for any of the treatments but was significantly different to 3 hours using 100ul ($P<0.05$) (Figure 6.4).

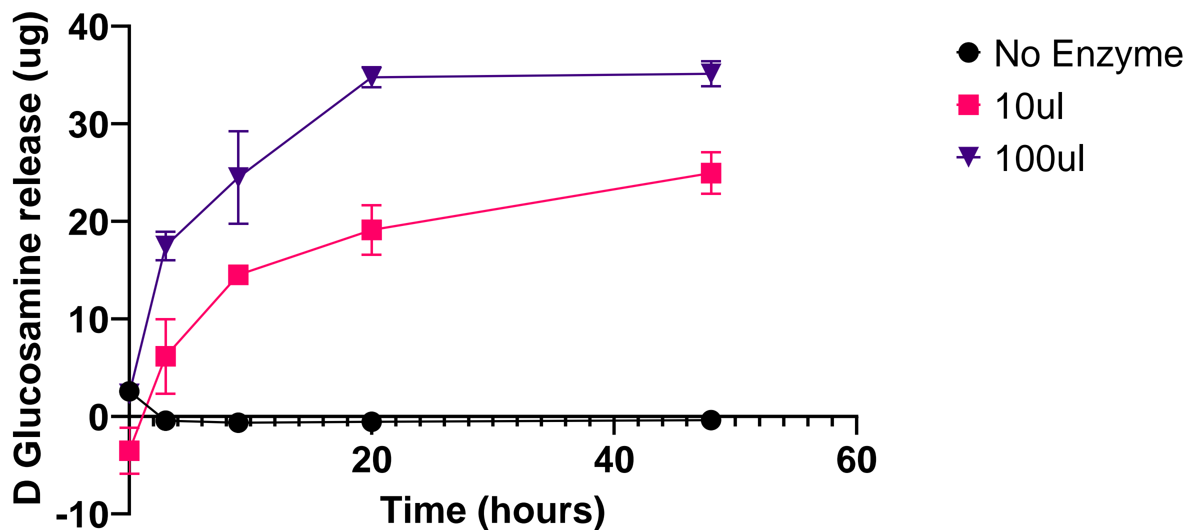


Figure 6. 4 *Incubation 2: The effect of different volumes of chitinase on the digestion of chitin to release D glucosamine.*

Data is presented as mean±SEM

There was no significant effect of treatment (P=0.186) or time (P=0.269) on D glucosamine release from mealworms (Figure 6.5).

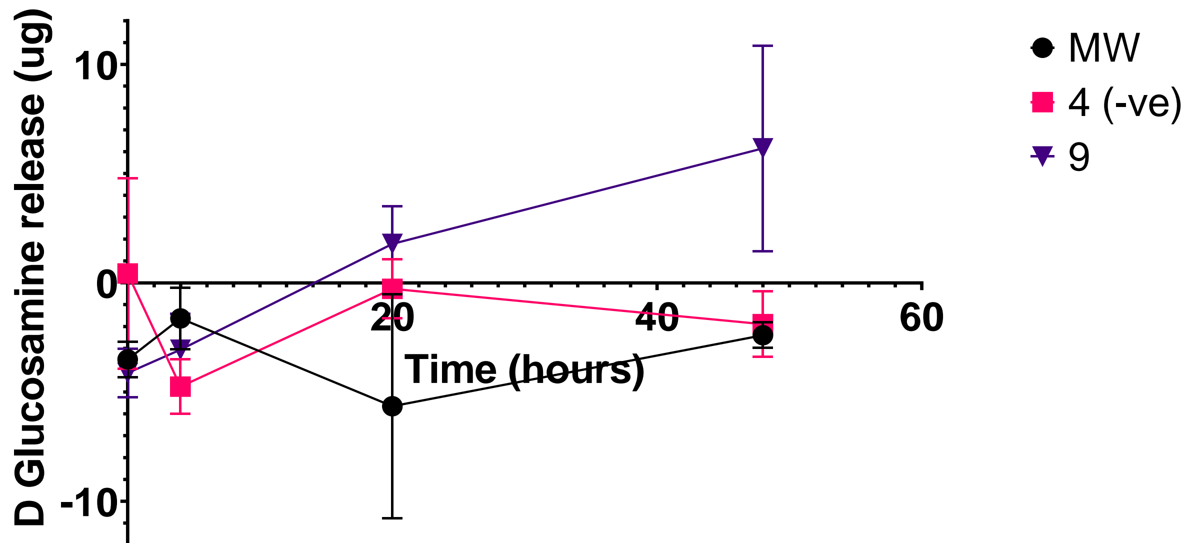


Figure 6.5 *Incubation 3: The effect of 100µl of chitinase (9) or negative control (4) on digestion of mealworm compared to the control of just mealworm to release D glucosamine.*

Data is presented as mean±SEM

There was a significant interaction between time and treatment when chitin was digested with no enzyme, negative control and 1ul of high 9 chitinase ($P<0.001$) (Figure 6.6A). There were no significant differences between treatments of no enzyme or negative control at any of the time points, except no enzyme at 0 hours and negative control at 4 hours ($P<0.05$). There was a significant increase in D glucosamine released with treatment of 1ul of high 9 chitinase at 0.5 and 1 hour compared to all other treatments and times ($P<0.05$) and additionally at 2.5 and 4 hours were significantly increased above 0.5 and 1 hour digestion times ($P<0.05$) (Figure 6.6A).

There was a significant interaction between time and treatment when chitin was treated with no enzyme, 1ul and 10ul of high 9 chitinase ($P<0.001$) (Figure 6.6B). There was a significant increase in D glucosamine released at 10, 24 and 48 hours with both enzyme treatments and at 3 hours with 10ul compared to no enzyme ($P<0.05$) (Figure 6.6B). There were no significant differences between all-time points for no enzyme, 0 hours for 1 and 10ul and 3 hours for 1ul ($P<0.05$) (Figure 6.6B).

There was no significant effect of enzyme treatment ($P=0.243$) or time ($P=0.651$) on D glucosamine release when mealworms were digested (Figure 6.6C). There was large standard error of the mean on the final time point at 24 hours for the mealworm samples, indicating potentially issues with the assay method.

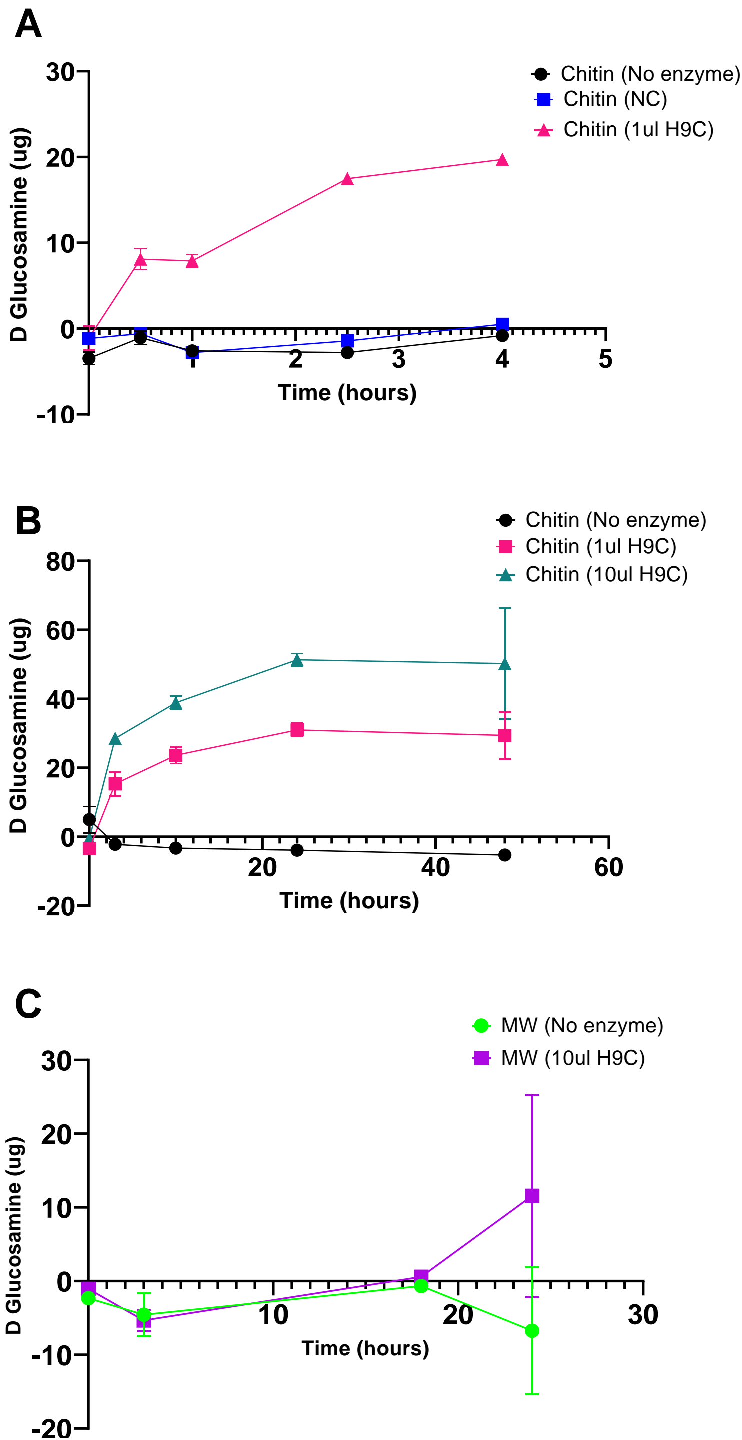


***Figure 6.6** (A) Incubation 4: Digestion of chitin with no enzyme, negative control and 1ul of high 9 chitinase over 4 hours. (B) Incubation 5: Digestion of chitin with no enzyme, 1ul and 10ul of high 9 chitinase over 48 hours. (C) Incubation 6: Digestion of mealworm with no enzyme and 10ul of high 9 chitinase over 24 hours.*

Data is presented as mean±SEM for graphs. NC = negative control, H9C = high 9 chitinase, MW=mealworm

There was similar D glucosamine released with incubation of high 9 chitinase with chitin as shown in Table 6.3. Therefore, the value of 15 and 30 were chosen to calculate the volume of chitinase needed for the broiler trials.

Table 6.3 *Comparison of the amount of D glucosamine released after different incubations*

Incubation	Sample Time (hours)	D Glucosamine (µg)
4	2.5	17.5
5	3	15.3
	48	30.0
7	2.5	15.0

6.3.2 Broiler trial 2

There was no significant difference in initial bird live weight (Table 6.4). Pre-treatment of mealworms with either negative control or chitinase enzyme in broiler feeds had no effect on final bird live weight, body weight gain, total pen feed intake and pen FCR (Table 6.4).

Table 6.4 *Broiler trial performance indicator results after feeding two different feeds for 30-day trial period*

	Feeds[1]			
	MW+Neg	MW+Chitinase	SED[2]	P Value
Initial Bird Live weight (kg)	0.132	0.133	0.003	0.700
Final Bird Live weight (kg)	2.111	2.149	0.080	0.645
BWG (kg)[3]	1.979	2.016	0.078	0.646
Total Pen Feed Intake (kg)	13.52	13.26	0.567	0.654
FCR (pen)[4]	1.708	1.647	0.042	0.166

[1]MW+Neg (mealworm based feed with negative control enzyme), MW+Chitinase (mealworm based feed with chitinase enzyme)
[2] SED – standard error of the differences of the means
[3]BWG (Body weight gain)
[4]FCR (Feed conversion ratio).
Data is presented as means, significance set at $P<0.05$. For Initial bird weight, final bird weight and BWG n= 40 for each treatment, for Total pen feed intake and FCR n=10 for each treatment

There was a significant decrease in the feed conversion ratio in the last 5 days of the 30-day feeding trial in birds feed MW+Chitinase (P=0.048) (Figure 6.7). There were no differences between the two feeds on any of the other measurement days (P>0.05) (Figure 6.7).

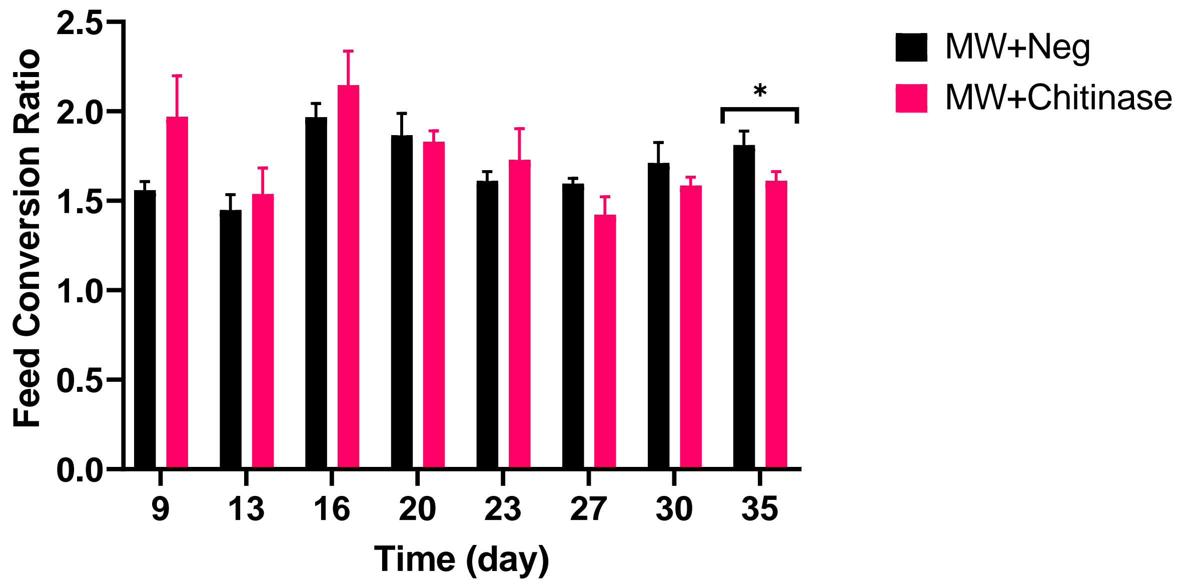


Figure 6.7 *The effect of inclusion of chitinase into mealworm feed on feed conversion ratio biweekly over 30 day trial*

MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme). Data is presented as mean±SEM, n=10, *P<0.05

Bird live weight significantly increased with time (P<0.001), however there was no effect of inclusion either negative control or chitinase enzyme on mealworms on bird live weight (P=1.000) (Figure 6.8).

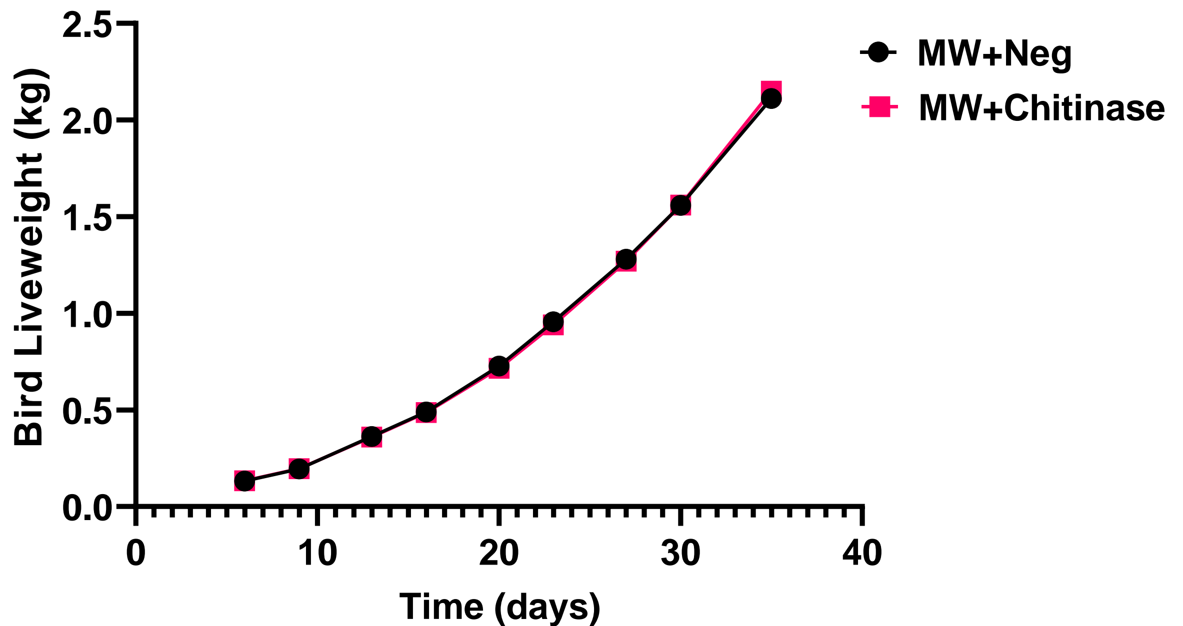


Figure 6.8 *The effect of inclusion of chitinase into mealworm feed on bird live weight biweekly over 30-day trial*

MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme). Data is presented as means±SEM, n=40

6.3.3 Ileal Protein Digestibility

There were no significant differences in the average ileal titanium amount between negative control and chitinase fed birds (P=0.799) (Figure 6.9). There was a large variation in the titanium amounts from the different pens across both feed groups. For the negative control birds the standard error of the mean was 107, which is 21.5% of the mean. For the chitinase birds the standard error of the mean was 195, which is 36.4% of the mean.

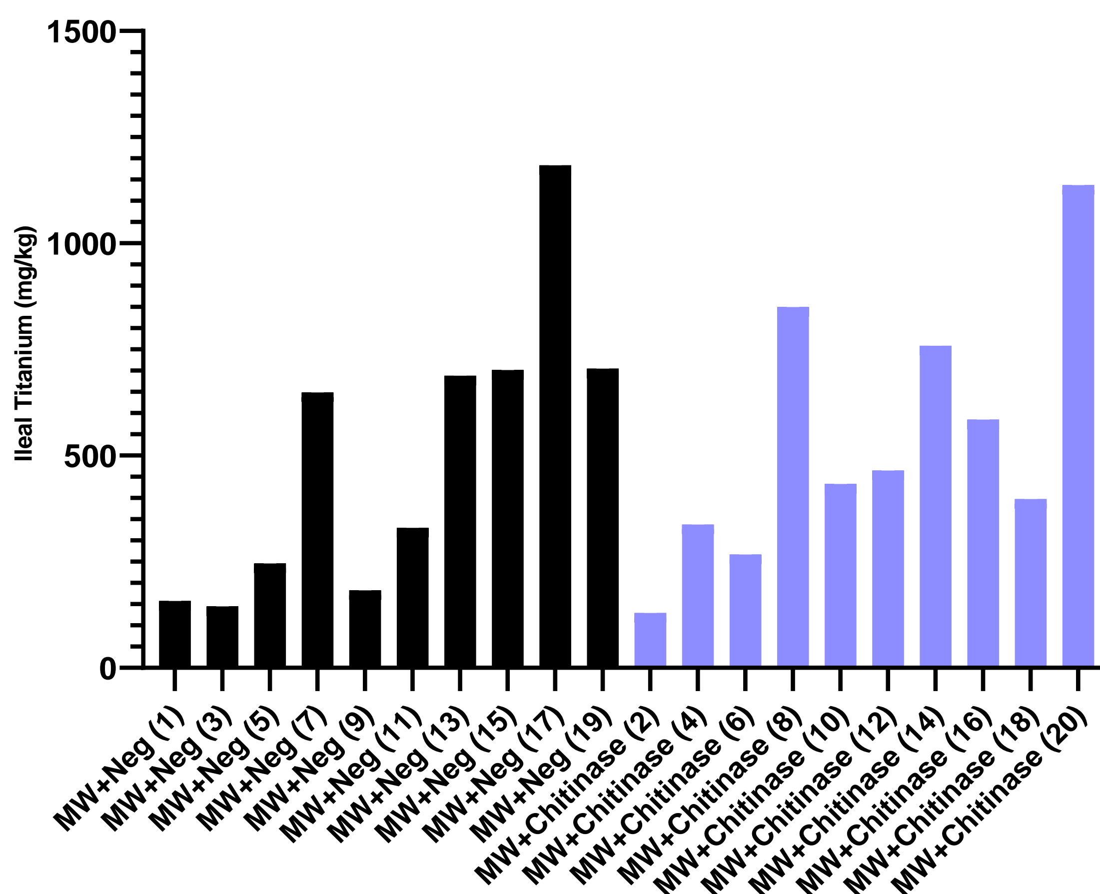


Figure 6.9 *Average pen ileal titanium amount (mg/kg) displayed on a pen basis after 30 days on feeds*

MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme). Data is presented as individual values; 250g of freeze-dried ileal digesta from each of the 4 birds was combined to represent 1 pen.

MW+Chitinase birds had a significantly higher ileal crude protein content compared to MW+Neg fed birds (P=0.01), but there were no differences in apparent ileal crude protein digestibility coefficients (P=0.450) (Table 6.5)

Table 6.5 *Ileal Crude protein content and apparent ileal crude protein digestibility coefficient after 30 days on feeds*

	Feeds[1]			
	MW+Neg	MW+Chitinase	SED[2]	P Value
Ileal CP (g/kg)	119.00	138.94	10.14	0.010
$AIDC_{cp}$[3]	0.91	0.89	0.017	0.450

[1] MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme).
[2] SED – standard error of the differences of the means
[3] $AIDC_{cp}$ – Apparent ileal digestibility coefficient for crude protein
Data is presented as means of pen, significance set at P<0.05. n= 10 for both treatments.

Chitinase fed birds had significantly increased ileal content of all amino acids measured except from aspartate and threonine (Figure 6.10).

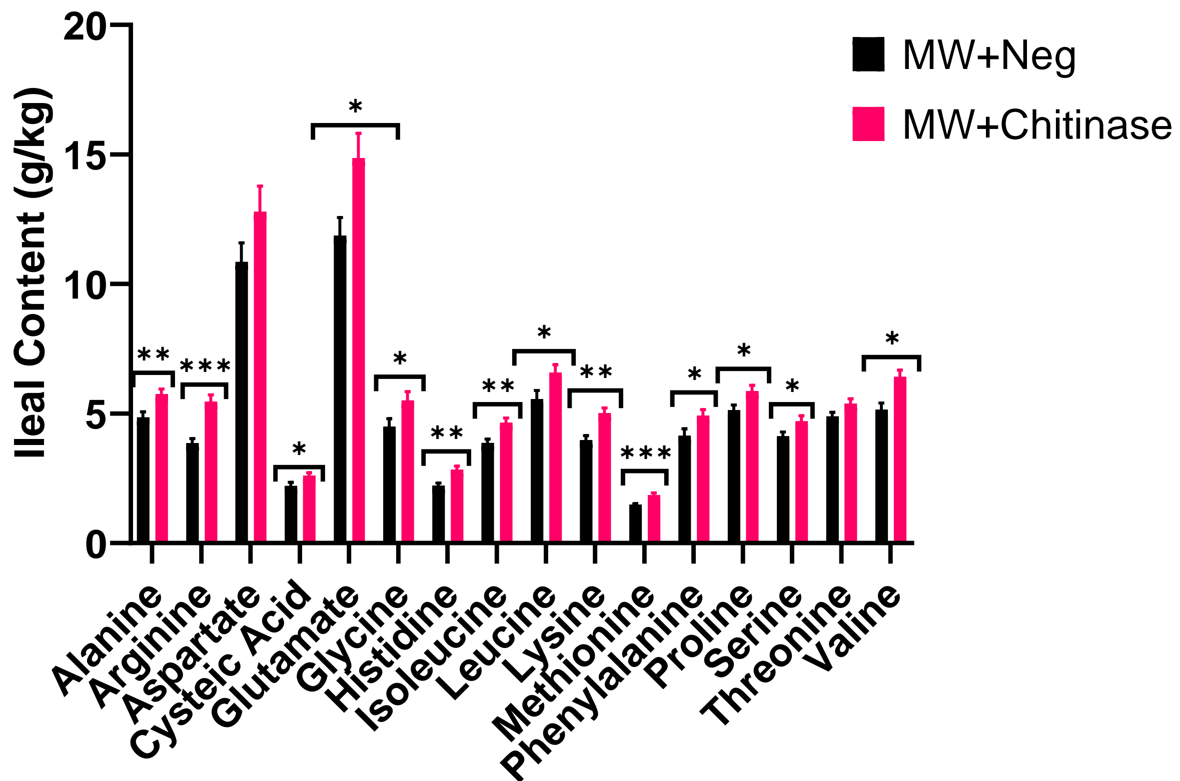


Figure 6.10 *Ileal amino acid content of broilers after 30 days on feeds*

MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme). Data is presented as means±SEM, n=10 per feed treatment. Data analysed through 1 way ANOVA, * P<0.05, **P<0.01, *** P<0.001

There was no effect of chitinase inclusion on apparent ileal amino acid digestibility coefficients compared to the negative control feed (Figure 6.11). Chitinase inclusion tended to increase the apparent digestibility of serine (P=0.095) and threonine (P= 0.090) (Figure 6.11).

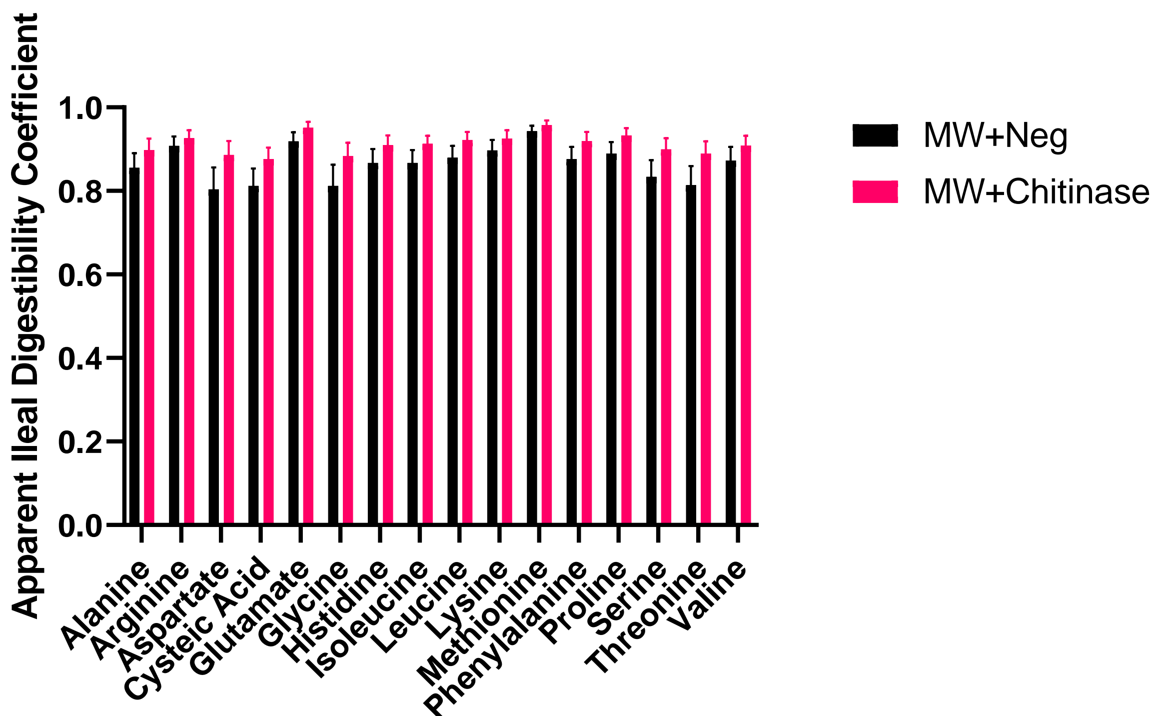


Figure 6.11 *Apparent ileal amino acid digestibility coefficients of broilers after 30 days on feeds*

MW+Neg (mealworm based feed with negative control enzyme). MW+Chitinase (mealworm based feed with chitinase enzyme). Data is presented as means±SEM, n=10 per feed treatment.

6.3.4 Caecal Microbiome

There was a trend for a difference of the pre-treatment of mealworm with chitinase included in feeds as a partial replacement of soybean compared to no pre-treatment of mealworms on the caecal microbiota of broilers (P=0.096) (Figure 6.12). Firmicutes were the dominant phylum accounting for over 95% in the caecal contents of both feed groups (Figure 6.12). The second most abundant phylum was proteobacteria, followed by

actinobacteria, bacteria unclassified and then others (Figure 6.12). The Bacteroidetes phylum was in both treatment groups (MW+Neg 8.12×10^{-5} % and MW+Chitinase 4.54×10^{-5} %) (Figure 6.12). Further analysis into the specific OTU contributing to the Bacteroidetes revealed that the effect was being caused by bird 95, pen 10 (OTU 2196) for the Negative Control group and pen 13, bird 47 (OTU1591) for the chitinase group.

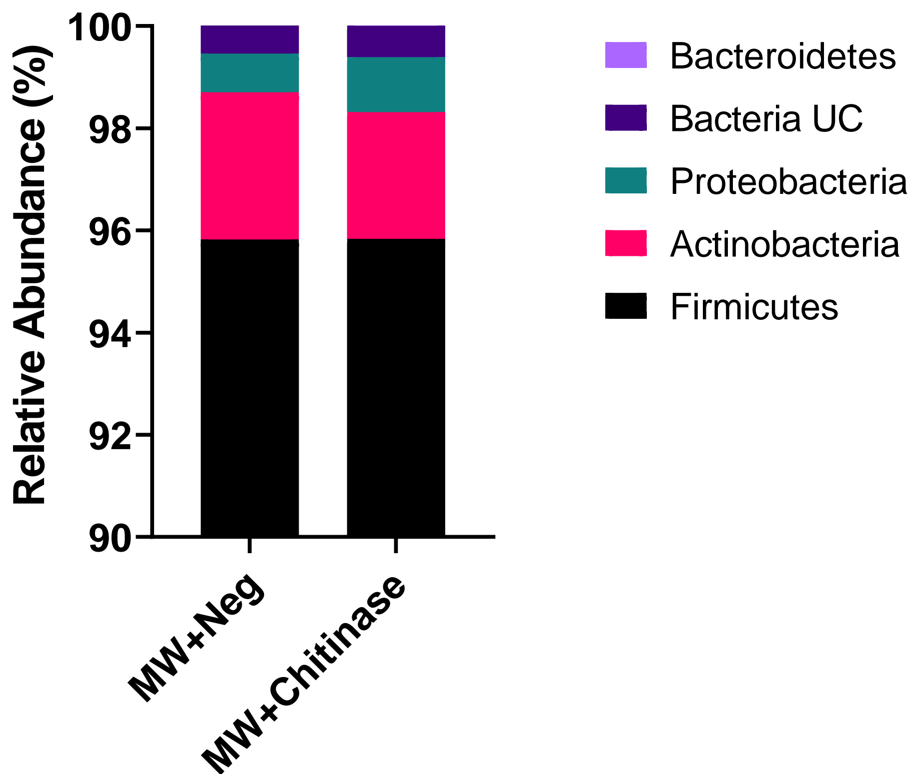


Figure 6.12 *Relative abundance MW+Neg and MW+Chitinase for broiler trial 2 caecal content phyla*

MW+Neg (mealworm based feed with negative control enzyme), MW+Chitinase (mealworm based feed with chitinase enzyme). Bacteroidetes (MW+Neg 8.12×10^{-5} % and MW+Chitinase 4.54×10^{-5} %). Bacteria UC = Bacteria unclassified Data is presented as relative abundance means. n=40 for both feeds.

The top 10 operational taxonomic units (OTU's) with the greatest relative abundances are shown in Figure 6.13A for all birds sampled. The genus with the greatest relative abundance was Lachnospiraceae UC, followed by Lactobacillus and Ruminococcaceae UC, all of these genera have a numerically higher relative abundance in the chitinase fed birds compared to the negative control birds. Figure 6.13B shows the discriminative OTU's between the Negative control and the Chitinase fed birds. The genera, which had significant log changes in the LEfSE, plot in both the chitinase and negative control fed birds was Lachnospiraceae.

Table 6.6 displays further analysis of utilising the base sequences of the OTU sequences, which were changing significantly in the LefSe report, and running through NCBI Blast to identify the potential species of bacteria. Table 6.6 reports the specific OTU's and the potential species causing the changes in either the MW+Neg or MW+Chitinase fed birds.

Lachnospiraceae family features in the LEfSE report in both the MW+Neg and MW+Chitinase birds. In the chitinase fed birds there was a significant decrease of OTU0038 (P=0.004) and OTU0037 (P=0.013), these bacteria may be *Clostridium algidixylanolyticum* strain SPL73 and *Faecalicatena orotica* strain JCM 1429 respectively. The change in the chitinase birds Lachnospiraceae in Figure 6.13B was because of a significant decrease in OTU0019 (P=0.02), which has a high percentage identity to *Blautia hydrogenotrophica* strain S5a36.

In the chitinase fed birds there was a significant decrease in OTU0044 (P=0.012) and OTU0078 (P<0.01) which were potentially identified as *Vallitalea pronyensis* strain FatNI3 and *Ruminococcus lactaris* ATCC 29176 respectively. There was a significant increase in OTU0031 (P=0.02) in chitinase fed birds, which has a high percentage identity to *Negativibacillus massiliensis* strain Marseille-P3213.

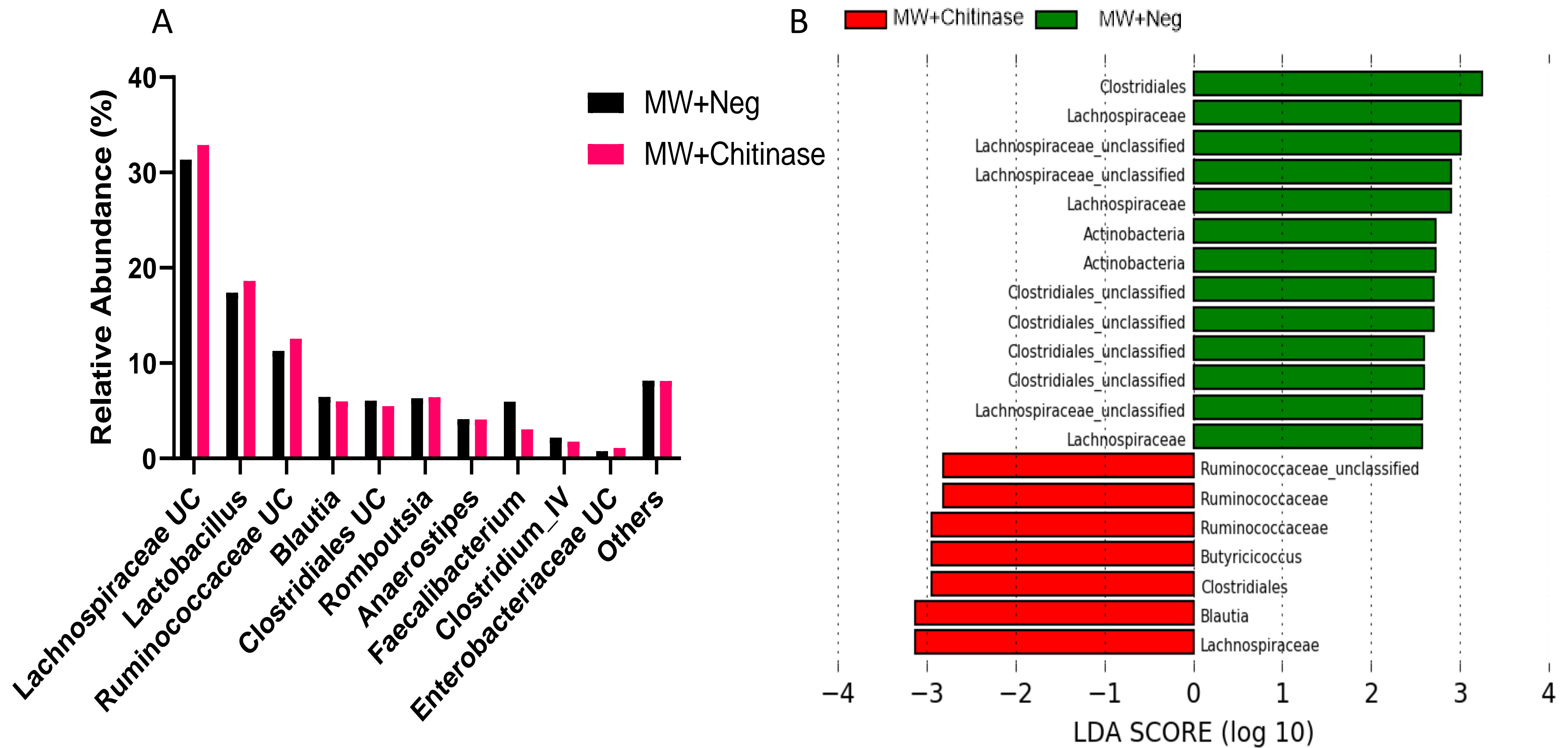


Figure 6.13 *A: Relative abundance of broiler caecal content genus on MW+Neg and MW+Chitinase feeds. B: Differential bacterial species identified in caecal contents from birds fed the MW+Neg and MW+Chitinase feed analysed using linear discriminant analysis effect size (LEfSE).*

MW+Neg (mealworm based feed with negative control enzyme), MW+Chitinase (mealworm based feed with chitinase enzyme). UC = unclassified. A Data is presented as relative abundance means. B Data is presented as linear discriminant analysis score (LDA) n=40 for both feeds.

Table 6.6 *Discriminant analysis of operational translation unit (OTU) from broiler caecal content fed either MW+Neg and MW+Chitinase feeds over 30 day period.*

OUT	P Value	Response	% Identity	Species
OTU0038	P=0.04	Decrease in MW+Chitinase	96%	Clostridium algidixylanolyticum strain SPL73 Firmicutes; Clostridia; Eubacteriales; Lachnospiraceae; Lacrimispora
OTU0037	P=0.013	Decrease in MW+Chitinase	94%	Faecalicatena orotica strain JCM 1429 Firmicutes; Clostridia; Eubacteriales; Lachnospiraceae; Faecalicatena
OTU0044	P=0.012	Decrease in MW+Chitinase	89%	Vallitalea pronyensis strain FatNI3 Firmicutes; Clostridia; Eubacteriales; Vallitaleaceae; Vallitalea.
OTU0078	P<0.001	Decrease in MW+Chitinase	97%	Ruminococcus lactaris ATCC 29176 Firmicutes; Clostridia; Eubacteriales; Lachnospiraceae; Mediterraneibacter
OTU0031	P=0.012	Increase in MW+Chitinase	98%	Negativibacillus massiliensis strain Marseille-P3213 Firmicutes; Negativibacillus
OTU0019	P=0.02	Increase in MW+Chitinase	95%	Blautia hydrogenotrophica strain S5a36 Firmicutes; Clostridia; Eubacteriales; Lachnospiraceae; Blautia

[1]OTU = operational translation uni

6.4 Discussion

Exogenous chitinase degrades chitin in vitro to release D glucosamine.

The first incubation of various chitinase enzymes highlighted that enzyme 9 hydrolysed chitin most efficiently and resulted in the greatest release in D glucosamine compared to all other enzyme except enzyme 5. Further digestions found that there was no significant increase in D glucosamine release when a higher volume was used. The original volumes used would have been too high when scaled up for inclusion into broiler feeds. Therefore, the commercial partners provided a more concentrated version of enzyme 9. The high 9 chitinase hydrolysed chitin to release D glucosamine in lower volumes compared to the original enzyme. Therefore, the hypothesis can be accepted that exogenous chitinase can break down chitin *in vitro.*

Time course investigations of both enzyme 9s showed an initial rapid increase in D glucosamine release in the first 24 hours and then a plateau with no further changes in the subsequent 24 hours. This is in agreement with Jamialahmadi et al., (2011) who had the same plateauing with production of N-acetyl-D-glucosamine by crude chitinase enzyme and with Ilankovan et al., (2006) who reported the majority of N-acetylchitobiose produced by chitin treated with pepsin was in the first 24 hours of a 72 hour incubation. Jamialahmadi et al., (2011) highlighted that a high concentration of N-acetyl-D-glucosamine was inhibitory on the hydrolysis reaction, suggesting that feedback inhibition may have decreased production rate. This could align with the incubations carried out in this chapter with D glucosamine acting as an inhibitory product binding to the chitinase enzyme active site.

Shrimp chitin has an alpha structure, which consists of tightly packed alternating parallel and antiparallel chains (Aam et al., 2010). Therefore, the initial increase in D glucosamine could be from the hydrolysis of the bonds at the edges of the structure. Following this initial increase there may be limited availability to β-glycosidic bonds in the interior structure of the alpha structure that slows down or even stops the hydrolysis reaction.

The reaction progress curve for differing volumes of enzyme 9 resulted in the maximum plateau of D glucosamine reached different values, potentially due to enzyme instability over time (Brooks et al., 2012). Alternatively, the enzyme active site is saturated and held in a saturated form so cannot continue the reactions. As chitin is a relatively insoluble polysaccharide, there could have been incomplete solubilisation of the chitin in the solution for the incubation. Jamialahmadi et al., (2011) also found that there was an increased yield of N-acetyl D glucosamine with increased enzyme amounts, with the same plateauing at 24 hours no matter what the enzyme amount.

Chitinase dependant release of D glucosamine is affected by both pH and temperature (Kuk et al., 2005). The pH and temperature used in the incubations was chosen to be representative of the chicken gastrointestinal tract. The acid step was used to stop the chitinase reaction. However, at 80-90°C temperatures hydrochloric acid can breakdown chitin to N-acetyl glucosamine (Chen et al., 2010). If the acid treatment step was not maintained at 100°C, this could have potentially increased hydrolysis of chitin. All samples were treated with the acid step and using the same hot bloc, therefore any heat changes would have affected all samples within the same digestion. No D glucosamine was detected in the negative control or no enzyme treatments. This confirms that if the enzyme is not

present, the hydrolysis will not take place and that the acid step to remove the acetyl group does not affect the amount of D glucosamine being released.

Exogenous chitinase did not degrade mealworms in vitro to release D glucosamine, potentially owing to assay issues

There was no effect of chitinase on D glucosamine release from mealworms but there was a large range of D glucosamine values recorded, leading to concerns with the methodology. This work was novel and there is very little literature published on the effectiveness of chitinases in hydrolysing chitin in insects *in vitro*.

Chitinases are highly biodiverse, differing in function, mechanism and specificity (Hamid et al., 2013). Each of the components in the incubations were derived from different species (shrimp chitin, insects and fungal chitinase) raising issues regarding enzyme specificity (Oyeleye and Normi, 2018). However, shrimp, insect and fungal chitinases are all structurally similar and classified within the same 'family 18' (Hamid et al., 2013). All chitinases within family 18 should be able to breakdown chitin from within the same family (Rathore and Gupta, 2015). Furthermore insect pesticides utilised fungal chitinases to break down insect exoskeletons (Hamid et al., 2013), so there should be no effect of enzyme specificity.

It was unclear whether the assay carried out was accurate for insect samples. Some insect digestion samples produced results that were comparable to those from the chitin incubation. However, some samples produced only a small increase in the 340nm assay readings, resulting in negative D glucosamine readings. As the *in vitro* incubations using a chitin solution provided clear results with a small error, this indicates that something within the mealworms was disrupting the reaction. The mealworm samples were further treated

with Carrez reagents to remove protein and fat components improving clarification and reducing interference, however the gastrointestinal tract of the mealworms was not removed prior to incubation.

The assay does not measure D-glucosamine directly, therefore external factors from the mealworms could interact with the reactions producing false measurements. The Megazyme assay relied on an inferred 1:1 stoichiometric relationship between NADPH and D-glucosamine released. In order to measure the NADPH four different reactions took place within the assay. A reducing agent present in the mealworm samples could have caused the exogenous NADP to be reduced to NADPH before the final incubation was carried out, limiting the NADP available for the final incubation resulting in a small increase in 340nm absorbance. In future, an alternative method, such as a DIONEX high performance liquid anion exchange chromatography method could be explored as a more accurate measure of D glucosamine released from insects and resulted in reduced error.

There was no overall effect of chitinase inclusion on broiler production, however feed conversion ratio of chitinase fed birds did improve in the last 5 trial days

To our knowledge this is the first trial where mealworms have been pre-treated with chitinase prior to inclusion into broiler feeds. There was no difference in the overall growth, feed intake or FCR between the negative control and chitinase fed birds, suggesting that the chitinase was having no effect on bird performance. There was a decrease in the FCR of chitinase fed birds in the last 5 days of the trial suggesting that the chitinase may be impacting on feed conversion as the birds aged. To date, there has been very limited work looking at the impact of chitinase on digestibility of insects. The current study shows no

negative effect on broiler production and further studies on chitinase inclusion may be required to confirm any positive effects on production. However, caution should be taken in interpreting feed intake data as birds were not raised in commercial conditions. It must be noted that the FCR recorded was a lot higher compared to commercial practise. This is owing to the method for measuring feed consumption, wasted feed by the birds was not weighed as it was mixed with the sawdust bedding on the pen floor. Therefore, the feed consumption may be higher than what was eaten by the birds resulting in a higher FCR.

There are no reported studies where chitinase treated mealworms have been included in broiler feeds. Glucosamine has been included in broiler feeds as glucosamine sulphates supplement to improve bone strength, inclusion resulted in increased weight gain and reduced locomotor problems (Martins et al., 2020). Pure chitosan (deacetylated chitin) has been included in broiler feeds and significantly reduced live weight and feed intakes in comparison to control and chitin containing feeds (Razdan and Pettersson, 1994), whereas other studies have found no differences when chitosan was included into feeds (Arslan and Tufan, 2018). It has also been reported to improved jejunum villous height therefore altering gut morphology (Ibitoye et al., 2019) and to have the health benefits; reducing pathogens in the intestine, improving immune function, and being antifungal and antimicrobial (Park and Kim, 2010).

There was no effect of chitinase inclusion on apparent ileal digestibility

There was increased crude protein and most amino acids, excluding aspartate and threonine, in the chitinase fed birds, suggesting decreased absorption of the amino acids. There were no differences in apparent ileal digestibility coefficients of either crude protein or amino acids suggesting that chitinase inclusion was not improving digestibility. The

apparent digestibility of chitin in chickens has been reported to be low (18-24%, Khempaka et al., 2006), as chitinase hydrolyses chitin into more soluble chito-oligosaccharides, it would be expected to improve digestibility of protein and amino acids.

Inclusion of other alternative exogenous enzymes, such as xylanase, amylase and protease, have been shown to improve apparent ileal digestibility of crude protein and amino acids in broilers (Cowieson et al., 2006; Liu et al., 2013). Additionally, feed supplementation of microbial protease improved apparent ileal crude protein and lysine digestibility (Erdaw et al., 2017). These enzymes are routinely used in monogastric feeds to improve efficiency; therefore the technology of exogenous enzymes works, it may be that chitinase inclusion need to be further refined.

Factors relating to birds (enzymatic activities in the digestive tract) and feed (substrate availability) may have limited nutrient digestibility (Yegani and Korver, 2013). Chitinase is naturally expressed in the upper gut of birds (Han et al., 1997; Koh and Iwamae, 2013; Tabata et al., 2017), so some digestion could be occurring in the gut and the inclusion of exogenous chitinase is having no further effect due to chitin structure. There are three principle stages for the complete lysis of chitin; (i) cleaving of chitin polymer into soluble oligomers, (ii) splitting oligomers into dimers, (iii) cleavage of dimers into monomers (Beier and Bertilsson, 2013). The first two stages utilises chitinase to digest the chitin to trimers and dimers (Koh and Iwamae, 2013). The third stage uses N-acetyl-β-D-glucosaminidase (NAGase) to reduce the trimers and dimers to monomers which can then be absorbed (Koh and Iwamae, 2013). Chickens have high levels of mucosal chitinase reported to be in the proventriculus (Koh and Iwamae, 2013). There are mixed reports that chickens have NAGase activity in the gastrointestinal tract of broilers, Jeuniaux and Cornelius, (1978) found that

NAGase was absent or very low in the tract, except in the caecum but Koh and Iwamae, (2013) reported a high activity of NAGase in the proventriculus. Potentially the exogenous chitinase is hydrolysing the chitin to trimers and dimers but then due to lack of NAGase activity monomers are not being created.

Chitinase was included into feed to get an approximate 6% maximum release of D glucosamine from *in vitro* calculations. There could have only been a partial hydrolysis of the outer β glycosidic bonds in the α structure leading to the matrix still holding nutrients leaving them unavailable for absorption. Furthermore, as the pre-treatment of mealworms was not in a solution (like the digestions), the exogenous chitinase may not have hydrolysed the chitin at all and then the chitinase active site could have been denatured by the acidic proventriculus (Mabelebele et al., 2014).

Effect on the caecal microbiome

Despite there being no significant differences observed in the caecal microbiota between birds fed the negative control or the chitinase feeds, there were significant differences detected at the OTU level through LefSe analysis. The chitinase pre-treatment of mealworms prior inclusion into broiler feeds influenced specific bacteria in the caecum.

As there were no overall changes in the caecal microbiota, the chitin component of mealworms may not have been fully hydrolysed by the exogenous chitinase inclusion to N-acetyl-D-glucosamine. Xylanase supplementation and additional xylo-oligosaccharides in broilers has manipulated the gut microbiome (Pourabedin et al., 2017). A similar process could have occurred where partial hydrolysis of chitin could have produced chito-

oligosaccharides of differing sizes, which may not have been any absorption in the small intestine, but it passed through into the caecum to be used as a fermentable substrate.

The major phyla in this analysis were Firmicutes with over 95% relative abundance in both the negative control and chitinase fed birds. Other studies have reported a lower relative abundance of Firmicutes in Ross 308 broilers (Oakley et al., 2014). It is important to note that the environmental conditions and age of the birds play a vital role in the development of the caecal microbiota (Ocejo et al., 2019). For example, Biasato et al., (2019a) found female broiler chicks had a predominance Bacteroidetes over Firmicutes in both the control feed and a mealworm-based feed. The relative abundance of Bacteroidetes in both the negative control and chitinase fed birds was due to one bird in each treatment, potentially related to the development of the caecal microbiota in these specific birds.

The top three relative abundance of genera were Lachnospiraceae UC, lactobacillus and Ruminococcaceae UC, all of which had higher relative abundance in the chitinase fed birds compared to the negative control. Both the Lachnospiraceae and Ruminococcaceae families are positively correlated with improved feed conversion ratio due to associations with the production of short chain fatty acids and degradation of plant materials (Biddle et al., 2013). However, there was no differences in the feed conversion ratios of the chitinase and negative control fed birds until the last five days. Potentially members of the Lachnospiraceae and Ruminococcaceae families which are positively correlated to feed utilisation developed later in the trial period contributing to the improved FCR in the final 5 days.

Further analysis into the OTU responsible for the increase in Ruminococcaceae UC highlighted high percentage identity with *Negativibacillus massiliensis* bacteria in chitinase

fed birds compared to the negative control fed birds. This species is from the Ruminococcaceae family, which has been reported to have the ability to break down cellulose (Valles et al., 2021). Chitin has been reported to have a similar structure to that of cellulose (Figure 6.1) (Aam et al., 2010), therefore if the bacteria family can breakdown cellulose there is the possibility to breakdown chitin or chito-oligomers. As this increase was in the chitinase fed groups, either the chitinase is not hydrolysing the mealworm chitin or alternatively the chitinase is hydrolysing to chito-oligosaccharides that are then being used as a bacterial energy source.

Lachnospiraceae were noted to be in both the chitinase and negative control on the LefSe analysis. In the chitinase fed birds, there was a significant increase in *Blautia hydrogenotrophica* of the Lachnospiraceae family compared to the negative control birds. This bacteria has been reported to be involved in the Wood-Ljungdahl pathway, responsible for increased branched-chain amino acid fermentation (Liu et al., 2021) and linked to the inflammatory response (Ramli et al., 2020). The Wood-Ljungdahl pathway involves the consumption of hydrogen and carbon dioxide by *Blautia hydrogenotrophica* to produce acetic acid (Liu et al., 2021). Potentially chitinase pre-treatment of mealworms has produced a chito-oligosaccharide which has then been utilised by a carbohydrate fermenting bacteria species, resulting in increased carbon dioxide and hydrogen in the caecum. *Blautia hydrogenotrophica* could then have utilised these via cross feeding.

There was decreased *Clostridium algidixylanolyticum*, *Faecalicatena orotica* and *Ruminococcus lactaris*, all the Lachnospiraceae family in the chitinase fed birds compared to the negative control birds. Little is known about these specific species of bacteria in relation to the role they play in broilers. Although *Clostridium aligidixylanolyticum* is known to

degrade xylan and be present on the caecal mucosa (Gong et al., 2007). A decrease in *Ruminococcus lactaris* has previously been linked to an increase in interleukins and inflammatory response (Shintouo et al., 2020). Additionally, *Ruminococcus lactaris* has been reported to be closely related to *Sellimonas intestinalis* (93.5%), this bacteria has been noted as marker of a healthy gut and of gut homeostasis recovery (Muñoz et al., 2020). Therefore, if these bacteria have a similar function, alongside the associations of the inflammatory response a decrease in the chitinase fed birds may be a negative effect.

6.5 Conclusions

In vitro digestion of chitin with chitinase enzyme released D glucosamine. Enzyme 9 released the greatest amount of D glucosamine so was used to treat mealworms for inclusion into broiler feeds. Due to methodological problems regarding the D glucosamine assay there is a lack of evidence that the chitinase could breakdown insects *in vitro.*

Pre-treating mealworms with chitinase and then including into broiler feeds had no effect on broiler growth, feed intake or feed conversion ratio over 30 day trial period. However, the improvement of FCR in the final 5 days with the chitinase fed birds suggests some potential benefits, at least in older birds. There was no effect on apparent ileal digestibility of crude protein, amino acids or minerals. There was no overall significant effect of chitinase pre-treatment on caecal microbiome, however there was increase in *Blautia hydrogenotrophica* and *Negativibacillus massiliensis* suggesting that trimers and dimers of chito-oligosaccharides are modulating the caecal microflora.

Overall, the results of this study were inconclusive with little effects if exogenous chitin in young birds but with some suggestion of beneficial effects on FCR in older birds. Furthermore, we were unable to demonstrate directly that the chitinase used was active in breaking down insect chitin. Further studies, using an alternative method of determining chitin digestion might be required. However, the current data suggests relatively modest effects of exogenous chitinase on the production of broilers fed on diets containing mealworms.

7 Discussion

Prior to the work carried out in this thesis, it was widely accepted that mealworms could be potentially included into broiler feeds (Hong et al., 2020). However, little had been reported on utilising already available technologies to improve mealworms as nutrient concentrators. In the work described in this thesis, mealworms were initially manipulated and then utilised in broiler feeds to investigate whether they could be used as a nutrient concentrator for sustainable animal feeds. Ultimately, mealworm production and inclusion into animal feeds needs to be commercially viable and sustainable, to at least partially replace traditional protein sources, such as soybean meal.

The first hypothesis that the growth and composition of mealworms could be manipulated by production conditions, feed and commercially available enzymes can be partially accepted. Growth and composition were affected by changes in feed and to a certain extent by commercially available enzymes, but not by production conditions. A major finding was that mealworms could be grown utilising water-soaked cotton wool as a water source with no major differences compared to using carrot. Additionally, there were no benefits of feeding mealworms on chick crumb compared to wheat bran, with no differences in growth performance or proximate nutritional composition. Mealworm composition was not affected by changes in environmental conditions (when comparing an uncontrolled room to a controlled incubator).

To be commercially viable, mealworms would have to be produced at a cost that is comparable to, or ideally cheaper, than soya. Using water-soaked cotton wool as a water source and feeding wheat bran, a by-product of the flour industry, would be cheaper than

using carrot and feeding chick crumb. Both wheat bran and chick crumb can be used for livestock production, but chick crumb is specifically formulated for broiler production and contains soya, which mealworms are potentially replacing. However, it might be commercially attractive to feed mealworms wheat bran, effectively upgrading a fibrous, 17% protein product, into a 50% protein product.

In monogastrics the use of exogenous enzymes has radically improved digestibilities and feed efficiencies and thereby reduced feed costs (Bedford and Partridge, 2010), but use in mealworm feeds needs further investigation into the commercial viability. Although pre-treatment of mealworm feeds with phytase increased the mealworm content of some (but decreased others) minerals use would have to be carefully considered due to the reduced mealworm crude protein content and increased feed processing requirements. Mealworms will not consume wet feeds and the inclusion of dried enzyme had no effects; therefore enzyme inclusion had to be through soaking the feed before drying it out again to feed to mealworms. This would increase the cost and the energy required to produce the feeds, so it may not be viable to include exogenous enzymes in mealworm feeds, given the lack of positive results in terms of growth and reduction in crude protein.

The high death percentage of mealworms seen in many of the experiments described is a cause for concern. Initially, mealworms transferred from the supplier to the University within 3 hours. This changed to an overnight courier, and it was at this point the death percentage increased, suggesting increased travelling time was resulting in the increased death rate. Additionally due to the various laboratory moves that occurred during the PhD, the mealworms were housed in differing locations during the different trials. There were increased deaths in the incubator compared to room, potentially suggesting the conditions

in the incubator were not optimal. This was further highlighted when comparing death rates between studies. The lowest death rate was in study 1 (5 - 11%), which was carried out under non-controlled environmental conditions in the laboratory. The highest death rate was recorded in Study 8, in which 50% died, which was carried out in the environmentally controlled incubator. In all mealworm trials, apart from Study 3, there was no differences in the death rates between the control and treated groups, confirming it was not the treatment causing the deaths. Further work is required to ascertain the specific causes of these large variations in mortality.

Mealworm production conditions are highly important in determining commercial viability. Utilising a temperature and humidity-controlled incubator increased average individual mealworm growth but resulted in higher feed intakes, which would increase the production cost as there was no difference in the growth compared to an uncontrolled environment (laboratory room). Furthermore, incubator use is more expensive and has environmental consideration compared to ambient conditions, due to the associated electrical and water supply. Environmental and financial impacts could be reduced by using renewable energy sources. As the study comparing the incubator and ambient conditions was carried out in the summer, only minor fluctuations in temperatures were seen. Further experiments looking at greater variations in temperature would be valuable.

In traditional livestock systems feed conversion ratios (FCR) are used to indicate production efficiency informing on economic gain (McDonald et al., 2011). FCR for mealworms were not reported in this thesis due to the inaccuracy of measuring feed intake. The introduction of pre sieving wheat bran to ensure that wheat bran was retained within the middle of two sieves allowed for more accurate measurement of feed intake (Studies 3-8). However, this

was still impacted upon by the fact that mealworms can reduce feed particle size, by consuming parts of wheat bran particles and leaving smaller particles, allowing left over feed to drop through the bottom sieve and mix with frass particles. Furthermore, using group mealworm weight can result in a negative body weight change if there is a high death number, which when used to calculate FCR, results in either a negative or very high value. A more robust methodology to determine feed intake in growth trials is required for reliable feed conversion ratio calculations, allowing for more detailed economic analysis of mealworms as an alternative protein source. Even with the issues and variability of FCR measurement in these trials, the value of FCR may not be of up most importance when describing the use of mealworms as an alternative feed source, due to their ability to convert less nutrient dense products into a useful feed source.

The second hypothesis that mealworms will be suitable as a feed ingredient for sustainable broiler feeds can be accepted, although the increase in FCR of the broilers needs to be addressed with respect to being commercially viable. The second broiler trial indicated that chitinase inclusion could potentially improve the FCR in older birds. Both broiler studies looked at mealworm inclusion into broiler feeds, however as the FCR improved as the birds aged when fed the chitinase pre-treated feed, it may be that mealworms may be a more suitable protein source in older birds, such as layers. It has already been shown that mealworm inclusion at 2.5% and 5% into layers, aged 63 -72 weeks, improved FCR, and additionally inclusion improved egg production and mass (Sedgh-Gooya et al., 2021).

As the two trials were carried out on different batches of birds fed different feeds they are not directly comparable. Ideally a trial should have been carried out with all 4 treatment groups (control, MW, MW+Neg and MW+Chitinase) to allow for a direct comparison. This

was not carried out due to time restraints and to allow for a fermentation batch of the higher protein chitinase to be produced by commercial partners. One issue with the second broiler trial is that the effect of chitinase treated mealworms could not be directly compared to a normal broiler feed (no mealworms). Furthermore, both the negative control and chitinase enzyme used to pre-treat the mealworms contained background proteins. There was nothing to compare the effect of these background proteins too; therefore these could have been modulating broiler production, and, more specifically, the caecal microbiome. Depending on the relative cost of mealworm production (relative to soybean meal) an increased FCR in mealworm fed broilers is potentially more expensive to produce compared to traditionally fed broilers. However, if mealworm fed broilers are identified as more sustainable, a premium could be put on the cost of the product. Consumer acceptance would also play a role in this; however as insects are part of the natural diet, feeding mealworms to chickens could be seen as a positive factor by the consumer.

There is a positive environmental impact of utilising mealworms as a partial replacement of soybean meal, so long as the mealworms have been produced sustainably. European poultry compound feed production reached 55.9 million tonnes (Ploegmakers, 2021) in 2018. If the control broiler feed used in chapter 5 is a typical formulation, containing 23% Hipro soybean meal, approximately 12.9 million tonnes of Hipro soybean meal was used in poultry feed. If mealworms could replace 10% of the Hipro soybean meal that is a 1.3 million tonnes reduction in soybean meal use in one year in Europe alone. Reformulating poultry feeds to allow for the fat content of mealworms would also reduce soya oil requirement, additionally reducing soya inclusion. Utilising whole insect meal, as both a protein and energy source, removes the need for processing. This would be beneficial both

environmentally and economically, especially if the insects were being produced using waste products.

One of the aims of this thesis was to try and determine whether any negative impact of insect chitin on broiler production could be offset by the inclusion of chitinase in the feed. The results were inconclusive, but it may become more important at a higher inclusion of mealworms in the broiler feeds, thereby further reducing soya use in compound feeds. Work is also needed to establish the potential role of endogenous chitinases expressed in chicken gut in the digestion of mealworms which could mean exogenous inclusion is not be necessary. Throughout this thesis, mealworms are analysed and discussed as a dry product for inclusion into traditional livestock compound-based feed. Free-range chickens will eat live insects when rooting around outside, therefore live mealworms could potentially be used as a supplement or an enrichment factor as a welfare initiative. This however, would require consideration of the storage of live mealworm

Main conclusions from the thesis

Ultimately, the price of sustainably produced mealworms and consumer acceptance are going to be the major challenges that need to be overcome for mealworms to be used as a nutrient concentrator in sustainable animal feeds.

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

- Water can be supplied to mealworms via water-soaked cotton wool, but not directly to wheat bran, as a viable alternative to vegetables, such as carrot.
- Individual average mealworm weight was increased when grown in a temperature and humidity-controlled incubator, but there was also an increase in death rate.

- Mealworms can be fed readily available by-product feeds and concentrate a 17% crude protein fibrous feed (wheat bran) into a 50% protein product (mealworms)
- Mealworm composition changed with age, with an increase in fat and decrease in protein content observed the closer to pupation.
- Pre-treatment of mealworm feeds with phytase had some effect on mineral content but reduced the protein content of mealworms. Use for commercial production of mealworms would have to be carefully considered.
- Xylanase negatively affected mealworm growth and increased total fat content of mealworms.
- There were no effects of partially replacing 10% soybean meal with mealworms on broiler growth, but feed intake and subsequent FCR were both increased. This suggests that the birds had to consume more mealworm feed to reach the same weight. Partially replacing soybean meal with mealworms had no effects on crude protein and amino acid apparent ileal digestibility but significantly affected the caecal microbiota, with changes at both phyla and genus level.
- Exogenous chitinase successfully hydrolysed chitin *in vitro* but results *in vivo* were inconclusive. Mealworm pre-treatment with chitinase did improve FCR in the final 5 days of the study. Again, there were no effects on crude protein or amino acid apparent ileal digestibility but specific bacteria in the caecum were significantly altered.

Potential future work

Future work that should be considered to improve knowledge in this area:

- Further investigation into the optimal growing conditions for mealworms to address the high death rates observed in the incubator.
- Continued exploration of the effect of mealworm inclusion on both digestibility and caecal microbiome by sampling at different ages of birds. Pairing these two types of analysis together, alongside production data allows for a more complete viewpoint of what inclusion of mealworms is doing to the whole digestive tract.
- Further analysis of caecal microbiota using quantitative PCR to confirm the bacterial species changing with mealworm inclusion.
- Determine the titanium dioxide content in the ileal digesta using the traditional method to allow for comparisons to the ICP-MS method and checking of the apparent ileal digestibility coefficients
- Impact of exogenous chitinase at high mealworm inclusion levels could be further explored. Potentially pairing with NAGase to improve the utilisation of chitin in broiler digestion.
- A standardised method for the quantification of chitin needs to be established to help inform on the amounts in feed and potentially to formulate to. This would help provide a more accurate measure of protein content for feed formulation.

Final Conclusion

Mealworms can be reared on relatively low quality feeds and represent a source of high quality protein for livestock feeds. Replacing a proportion of soybean meal with mealworms had no significant detrimental effects on broiler production apart from the increase in FCR. The ultimate value of mealworms as a source of protein in livestock feeds depends on the economic and environmental costs of production and consumer acceptance. The work in this thesis suggests that work should continue to evaluate mealworms, alongside other novel protein sources, as an ultimate replacement for environmentally unsustainable conventional ingredients.

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9 Appendix 1

Appendix 1: Selected Reaction Monitoring transitions for amino acid analysis

Compound Name	Workflow	Precursor m/z	Product m/z	m/z	Collision Energy (V)
ALANINE	TargetPeak	90.055	44.08	44.08	10
ALANINE	Confirming	90.055	90.055	90.055	5

Alanine 13C15N	TargetPeak	94.13	47.1	47.1	10
Alanine 13C15N	Confirming	94.13	93.99	93.99	5
ARGININE	TargetPeak	175.119	70.071	70.071	22
ARGININE	Confirming	175.119	116.042	116.042	14
Arginine 13C15N	TargetPeak	185.17	75.06	75.06	22
Arginine 13C15N	Confirming	185.17	125.09	125.09	14
Asparagine 13C15N	TargetPeak	139.16	59.1	59.1	10
Asparagine 13C15N	Confirming	139.16	77.08	77.08	10
ASPARATATE	TargetPeak	134.045	74.05	74.05	15
ASPARATATE	Confirming	134.045	88.06	88.06	10
Aspartate 13C15N	TargetPeak	139.16	77.05	77.05	15
Aspartate 13C15N	Confirming	139.16	92.15	92.15	10
CYSTEIC ACID	TargetPeak	170.12	106	106	16
CYSTEIC ACID	Confirming	170.12	124	124	16
Cysteine 13C15N	TargetPeak	126.01	79.12	79.12	10
Cysteine 13C15N	Confirming	126.01	126.01	126.01	10
Cysteine 13C15N	Confirming	126.18	97.07	97.07	10
CYSTINE	TargetPeak	241.31	74.2	74.2	27
CYSTINE	Confirming	241.31	152.03	152.03	13
GLUTAMATE	TargetPeak	148.06	84.08	84.08	15
GLUTAMATE	Confirming	148.06	129.917	129.917	10
Glutamate 13C15N	TargetPeak	154.17	60.07	60.07	10
Glutamate 13C15N	Confirming	154.17	136.1	136.1	10
Glutamate13C15N	TargetPeak	154.17	89.07	89.07	10
Glutamine 13C15N	TargetPeak	154.16	60	60	10
Glutamine 13C15N	Confirming	154.16	89.8	89.8	10
GLYCINE	TargetPeak	76.039	30.18	30.18	10
GLYCINE	Confirming	76.039	76.11	76.11	5
Glycine 13C15N	TargetPeak	79.09	32.2	32.2	5
Glycine 13C15N	Confirming	79.09	79.08	79.08	10
HISTIDINE	TargetPeak	156.077	83.1	83.1	24
HISTIDINE	Confirming	156.077	110.11	110.11	13
Histidine 13C15N	TargetPeak	165.17	100.08	100.08	13
Histidine 13C15N	Confirming	165.17	118.13	118.13	24
ISOLEUCINE	TargetPeak	132.066	69.09	69.09	16
ISOLEUCINE	Confirming	132.066	86.13	86.13	10
Isoleucine 13C15N	TargetPeak	139.21	74.1	74.1	16
Isoleucine 13C15N	Confirming	139.21	92.15	92.15	10
LEUCINE	TargetPeak	132.066	43.2	43.2	24

LEUCINE	Confirming	132.066	44.09	44.09	22
LEUCINE	Confirming	132.066	86.054	86.054	10
Leucine 13C15N	TargetPeak	139.21	46.12	46.12	24
Leucine 13C15N	Confirming	139.21	47.13	47.13	22
Leucine 13C15N	Confirming	139.21	92.13	92.13	10
LYSINE	TargetPeak	147.113	84.13	84.13	16
LYSINE	Confirming	147.113	130.11	130.11	10
Lysine 13C15N	TargetPeak	155.2	60.1	60.1	10
Lysine 13C15N	Confirming	155.2	90.21	90.21	16
METHIONINE	TargetPeak	150.058	104.1	104.1	10
METHIONINE	Confirming	150.058	133	133	10
Methionine 13C15N	TargetPeak	156.17	60.08	60.08	10
Methionine 13C15N	Confirming	156.17	109.01	109.01	10
METHIONINE Sulfone	TargetPeak	182.21	56.08	56.08	15
METHIONINE Sulfone	Confirming	182.21	136.06	136.06	15
Norvaline	TargetPeak	117.91	58.089	58.089	10
Norvaline	Confirming	117.91	71.988	71.988	10
PHENYLALANINE	TargetPeak	166.086	103.1	103.1	27
PHENYLALANINE	Confirming	166.086	120.11	120.11	11
Phenylalanine 13C15N	TargetPeak	176.19	111.11	111.11	27
Phenylalanine 13C15N	Confirming	176.19	129.11	129.11	11
PHENYLALANINE 13C6	TargetPeak	172.14	126.09	126.09	10
PHENYLALANINE 13C6	Confirming	172.14	155.12	155.12	10
PHENYLALANINE 13C6	Confirming	172.14	172.13	172.13	10
PROLINE	TargetPeak	116.071	43.2	43.2	28
PROLINE	Confirming	116.071	70	70	15
Proline 13C15N	TargetPeak	122.18	75.1	75.1	15
Proline 13C15N	Confirming	122.18	122.12	122.12	28
SERINE	TargetPeak	106.05	60.3	60.3	10
SERINE	Confirming	106.05	87.97	87.97	10
Serine 13C15N	TargetPeak	110.16	63.04	63.04	10
Serine 13C15N	Confirming	110.16	83.12	83.12	10

THREONINE	TargetPeak	120.066	74.09	74.09	10
THREONINE	Confirming	120.066	102	102	10
Threonine 13C15N	TargetPeak	125.17	78.12	78.12	10
Threonine 13C15N	Confirming	125.17	107.11	107.11	10
Tryptophan 13C15N	TargetPeak	218.18	127.1	127.1	10
Tryptophan 13C15N	Confirming	218.18	156.18	156.18	10
TYROSINE	TargetPeak	182.081	136.1	136.1	12
TYROSINE	Confirming	182.081	165.042	165.042	10
Tyrosine 13C15N	TargetPeak	192.19	145.21	145.21	12
Tyrosine 13C15N	Confirming	192.19	174.17	174.17	10
VALINE	TargetPeak	118.086	55.08	55.08	19
VALINE	Confirming	118.086	72.11	72.11	10
Valine13C15N	TargetPeak	124	59.1	59.1	0
Valine13C15N	Confirming	124	77.12	77.12	0

10 Appendix 2

Primers used for caecal microbiome work

i5

v4.IA501: AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA502: AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA503: AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA504: AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA505: AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA506: AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA507: AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IA508: AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB501: AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB502: AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB503: AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB504: AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB505: AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB506: AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB507: AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IB508: AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC501: AATGATACGGCGACCACCGAGATCTACACACGACGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC502: AATGATACGGCGACCACCGAGATCTACACATATACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC503: AATGATACGGCGACCACCGAGATCTACACCGTCGCTATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC504: AATGATACGGCGACCACCGAGATCTACACCTAGAGCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC505: AATGATACGGCGACCACCGAGATCTACACGCTCTAGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC506: AATGATACGGCGACCACCGAGATCTACACGACACTGATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC507: AATGATACGGCGACCACCGAGATCTACACTGCGTACGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.IC508: AATGATACGGCGACCACCGAGATCTACACTAGTGTAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID501: AATGATACGGCGACCACCGAGATCTACACAAGCAGCATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID502: AATGATACGGCGACCACCGAGATCTACACACGCGTGATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID503: AATGATACGGCGACCACCGAGATCTACACCGATCTACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID504: AATGATACGGCGACCACCGAGATCTACACTGCGTCACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID505: AATGATACGGCGACCACCGAGATCTACACGTCTAGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID506: AATGATACGGCGACCACCGAGATCTACACCTAGTATGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID507: AATGATACGGCGACCACCGAGATCTACACGATAGCGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.ID508: AATGATACGGCGACCACCGAGATCTACACTCTACACTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA

i7

v4.IA701: CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA702: CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA703: CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA704: CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA705: CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA706: CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA707: CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA708: CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA709: CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

v4.IA710: CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA711: CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IA712: CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB701: CAAGCAGAAGACGGCATACGAGATAAGTCGAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB702: CAAGCAGAAGACGGCATACGAGATATACTTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB703: CAAGCAGAAGACGGCATACGAGATAGCTGCTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB704: CAAGCAGAAGACGGCATACGAGATCATAGAGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB705: CAAGCAGAAGACGGCATACGAGATCGTAGATCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB706: CAAGCAGAAGACGGCATACGAGATCTCGTTACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB707: CAAGCAGAAGACGGCATACGAGATGCGCACGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB708: CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB709: CAAGCAGAAGACGGCATACGAGATGTATACGCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB710: CAAGCAGAAGACGGCATACGAGATTACGAGCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB711: CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IB712: CAAGCAGAAGACGGCATACGAGATTCGCTACGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC701: CAAGCAGAAGACGGCATACGAGATACCTACTGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC702: CAAGCAGAAGACGGCATACGAGATAGCGCTATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC703: CAAGCAGAAGACGGCATACGAGATAGTCTAGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC704: CAAGCAGAAGACGGCATACGAGATCATGAGGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC705: CAAGCAGAAGACGGCATACGAGATCTAGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC706: CAAGCAGAAGACGGCATACGAGATCTCTAGAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC707: CAAGCAGAAGACGGCATACGAGATGAGCTCATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC708: CAAGCAGAAGACGGCATACGAGATGGTATGCTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC709: CAAGCAGAAGACGGCATACGAGATGTATGACGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC710: CAAGCAGAAGACGGCATACGAGATTAGACTGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC711: CAAGCAGAAGACGGCATACGAGATTCACGATGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.IC712: CAAGCAGAAGACGGCATACGAGATTCGAGCTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID701: CAAGCAGAAGACGGCATACGAGATACCTAGTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID702: CAAGCAGAAGACGGCATACGAGATACGTACGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID703: CAAGCAGAAGACGGCATACGAGATATATCGCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID704: CAAGCAGAAGACGGCATACGAGATCACGATAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID705: CAAGCAGAAGACGGCATACGAGATCGTATCGCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID706: CAAGCAGAAGACGGCATACGAGATCTGCGACTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID707: CAAGCAGAAGACGGCATACGAGATGCTGTAACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID708: CAAGCAGAAGACGGCATACGAGATGGACGTTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID709: CAAGCAGAAGACGGCATACGAGATGGTCGTAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID710: CAAGCAGAAGACGGCATACGAGATTAAGTCTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID711: CAAGCAGAAGACGGCATACGAGATTACACAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.ID712: CAAGCAGAAGACGGCATACGAGATTTGACGCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Read 1 primer for V4 region

TATGGTAATTGTGTGCCAGCMGCCGCGGTAA

Read 2 primer for V4 region

AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Index primer for V4 region

ATTAGAWACCCBDGTAGTCCGGCTGACTGACT