INVESTIGATION INTO THE NUTRITIONAL VALUE OF BLACK SOLDIER FLY LARVAE (HERMETIA ILLUCENS) AND MEAL WORM (TENEBRIO MOLITOR) FOR BROILER CHICKENS

By

NOURHAN Z.S. ABDELAAL

A thesis submitted in fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

December 2023

Abstract

The aim of this project was to investigate the effect of feeding substrate on nutritional profile of insects, the project also aims to introduce insect larvae meal as an alternative to soyabean meal for poultry production. In addition to that, aims to evaluate the effect of using low doses of insect larvae meal on poultry gut health.

The first study investigated the effect of different feeding waste substrates on mealworm survival rate, substrate use and nutritional profile over two insect feeding trials (T1, and T2). In both trials the type of substrate significantly affected mealworm survival, and protein content, where in T1 trial, mealworm raised on banana peels substrate did not survive the first week of trial with 100% mortality rate recorded at the first week, DDGS substrate on the other hand have positively affected mealworm protein content and yielded 566.2g/kg DM protein. For T2 trial, mealworms fed food waste, sea waste, and sausage waste have yielded, 572.2 g/kg, 635.2 g/kg, 554.5 g/kg protein on dry matter basis respectively. The second study was performed in the form of two trials T3, and T4. T3, and T4 trials investigated the effect of black soldierfly larvae, mealworm larvae graded meal inclusions (20%, 40%, and 60%), both BSFL, and mealworm were raised on different substrates, on the amino acid digestibility, apparent metabolizable energy, and nitrogen retention of broilers against a control soya protein graded inclusion. Different insect feeding substrates have affected the protein content as well as the amino acids content of the insect larvae of both mealworm and BSFL, while the insect larvae meal when fed to broilers, improved amino acid digestibility as broilers fed BSFL raised on fruit waste, bran, and brewery waste showed 94.7%, 89.3%, and 89.3% COD respectively compared to those fed soya bean meal that showed 75.1 COD, while broilers fed mealworm raised on bran and another raised on DDGS showed 94.3%, and 94.9% respectively compared to those fed soyabean meal showed 87.3% COD. However, nitrogen retention values differed

between study groups due to the difference in protein content between the investigated larvae meals. The third study meant to investigate the effect of insect meal low doses on broilers performance, digestibility, jejunum histology, metabolizable energy up to 35 days of age. The study was performed over two trials (T5, and T6). In trial T5, three doses of mealworm (0.3%, 1%, and 5%) from EXT source and a 5% mealworm dose from a competitive source (COMP5) were investigated against a 100% soya bean meal diet as control. This study clearly showed that mealworm supplementation had a positive effect on bird performance that showed clearly in increased body weight gain in the first 10 days of the study, where broilers fed 5% mealworm inclusion gained approximately 177 g compared to broilers fed total soyabean inclusion and showed 151g body weight gain. T6 trial investigated the effect of black soldierfly larvae dietary inclusions (0.3%, 1%, and 5%) on the performance, nitrogen digestibility, apparent metabolizable energy, nitrogen retention, and jejunum histology of broilers to 35 days of age. Generally, the results of this trial showed the viability of using BSFL as partial replacement instead of using a complete BSFL meal. It could be concluded from the studies in the current thesis that:

Insects can utilise variety of waste substrates, however, different feeding substrates have different effect on insect nutritional profile, and protein yield. Also, the same substrate can have a different effect on different types of insects.

Insect meal inclusion significantly enhance amino acids digestibility of broilers compared to conventional soyabean inclusion.

Mealworm low dose in the broilers diet up to 5% can increase feed intake and body weight gain of broilers, that showed in T5 study where EXT0.3, and EXT5 increased chicken palatability and that was evidenced through increased feed intake. Therefore, insect meal can be used to replace up to 5% of soya with no deleterious effect on broilers performance or

digestibility and therefore can work as alternative protein source to soyabean as direction towards less carbon footprints strategy in poultry sector.

Acknowledgment

First of all, all praises to Allah for giving me the resilience and strength to overcome all the obstacles I have encountered in my life and giving me the patience to reach the point where I am now.

I would like to express my deepest appreciation to Professor Emily Burton, my supervisor, and director of studies for her endless support, guidance, and help whenever needed, she has been absolutely amazing, and I learned a lot from her determination and hard work.

I would love to thank Dr Dawn Scholey, for her help in constructing the amino acid chapter and helping to learn the amino acids preparations, analysis, and calculations and really made the amino acids chapter a very interesting chapter to write and investigate, not to mention the personal support and encouragement.

Also special thanks to all the Poultry unit tech team Ben, Agne, Ashley, and John for their great help during the performing of the trials and getting the performance, energy, and Nitrogen results for me in time because they knew how stressed I was about getting the results.

Last but not least I would like to express my infinite gratitude for my parents who have been absolutely supportive of my decisions, and how they have helped me with my daughter Maleka, how they have removed a huge burden off my shoulders so that I can focus and make progress in my PhD.

Dedication

Finally, I would like to dedicate this thesis to my dear sister Nadine who is like a daughter to me, and my daughter Maleka who I have missed so much, and the memory of her face would keep me going, without her being in my life I wouldn’t have reached that far.

Nourhan, December 2023

Contents

1. Chapter 1: Review of literature

1.1. Introduction

Sustainability challenges in crop production are dominated by two factors, firstly the competition between food, feed and fuel for plant-based crops such as soybean and maize, and secondly the over exploitation of land resources such as deforestation to create agricultural land (Foley *et al.*, 2011). In feeding European production animals such as poultry, a further sustainability issue is the intercontinental transport of feed materials. Most European imports of animal feed are of plant protein sources such as soybean and low-phytate maize, as the estimated EU plant protein yield is around 3% of EU's cultivable land (Vankrimpen *et al.*, 2013).

The most well-known and extensively exploited protein-rich constituent for animal feeds is soybean meal. Soybean meal plays a crucial role in animal feed being the highest used vegetable protein compound for livestock (Vankrimpen *et al.*, 2013). The consistent request for protein-rich ingredients of plant origin have led to escalated market prices over the last 5 years. Moreover, feed costs represent 60–70% of total production costs (Van Huis *et al.*, 2013). Therefore, the quest for economically viable alternative protein sources that could replace soybean for livestock is becoming crucial.

Insects are widespread food sources for both humans and animals in many regions of the world (Darfour, 2019). Compared to soybean meal, some forms of insect are equal or even a richer protein meal as a source of amino acids (Finke *et al.*, 1989).

The regulations and legislations of many countries are being developed towards relieving the restriction of insect use as a protein source for poultry, with legislation in 2017 (EU regulation No. 2017/893) reprieving the strict prevention implemented in 2000 (EU regulation No.

999/2001). This is due to the increasing global demand for increase in livestock production to cover the needs for human food.

The adult forms of meal worm (*Tenebrio molitor)* of the order coleoptera are considered as pests on grains, while in contrast, the larval forms commonly valorise organic wastes and are considered as an exotic pet food (Henry *et al.,* 2018). Many recent studies investigate the anti-inflammatory effects of feeding *T. molitor* to carnivorous fish such as European sea bass (Henry *et al.,* 2018) and rainbow trout (Belforti *et al*., 2015). In addition, a suite of studies by Makkar *et al.* (2014) also assessed both fatty acid and amino acid profile of *T. mollitor*.

Black soldier fly larvae (BSFL; *Hermetia illucens)* and meal worm (MWL; *Tenebrio molitor)* were investigated by De Marco *et al*. (2015) for their protein content and ileal digestibility in broiler chickens. Although the scope of this study was limited to evaluating growth performance and metabolizable energy of the diet, the authors considered both larvae form as candidate alternatives, as both fat and protein sources to replace soybean meal. The ability of BSFL to convert excess manure nutrients into valuable by-products and maggot mass for feed was investigated by Sheppard and Newton (2000), who showed through feeding studies of BSFL on swine manure, that the manure mass was 56% reduced, and in a later pot study, the plant growth was enhanced after the introduction of the digested manure residue to the soil.

Several studies suggest that the nutrient content of insect larvae can be modified according to the given feeding substrates (Lock *et al*., 2016; Makkar *et al*., 2014). Consequently, these investigations showed that the insect's fat content can be altered by modifying the cholesterol level in their diet, as insects don't naturally possess cholesterol, they acquire it through their feed (Ritter, 1990). As a result, we can conclude that, the manipulation of insect feeding substrate may have a substantial effect on larval nutrient content which will be the base of our study.

Insects could play a crucial part in sustainable development in terms of food security and preservation of agricultural land (Nischalke *et al*., 2020). However, in order for insect meal to be included in a future large scale to serve as high quality protein, Insect meal must be optimised as a poultry feed material in terms of enhanced nutritional profile and less costly source of feed.

1.2. Meat poultry production

1.2.1 The meat poultry industry

In 2017, the world population reached around 7.5 billion, and is expected to increase to 9.8 billion by 2050 (Garcia *et al*., 2019). Therefore, the world is constantly searching for more sustainable food resources to overcome the struggle for food. Alongside the global population increase, awareness of the need to secure sustainable food resources, and provide healthy food choices is also increasing (Vander Aar, 2016). If these recognised needs are acted upon in balance with the rising population, the outcome will be an improvement of human health and the potential to overcome the global food security problems. Over the past decade, poultry meat has gained popularity due to its comparably lower cost to red meat, and its healthy characteristics in terms of high protein and low-fat content (Murphy *et al*., 2022). According to government reports from the UK and USA, poultry are considered to be healthy protein source (Murphy *et al.*, 2022). Meat poultry also offer a universally acceptable meat protein source to maintain good health status, and to overcome red meat-related animal protein shortage in some countries. Therefore, pressure is increasing to reach higher rates of animal protein production without any negative effects on the environment and without increasing the product price.

Poultry meat consumption is also increasing as a preferred choice, as traditionally there are no cultural or religious constraints involved. In response to this pressure, the poultry industry is

rising rapidly within the global animal production. It was reported in 2017 that, the production of broiler chickens in the UK each year reached 875 million (AHDB, 2017). In 2019, the total world poultry production reached 27.9 billion birds (FAO, 2020). Accordingly, between 2021 and 2023 the world poultry production increased by 1.3% (Fig.1) (FAO,2023). The increased demand for poultry meat has driven breeding companies to utilise genetic selection for better carcass yield and faster growth rate (Havenstein *et al*., 2003). However, this has come at the expense of lower bone density and increased lameness in broiler birds (Duggan *et al.*, 2015). Companies now breed for a wide number of traits that include leg health in order to mitigate this (De Jong, and Van Emous, 2017).

In terms of greenhouse gas emissions, poultry meat is considered to have a relatively low carbon footprint, as poultry meat production contributes 10.8% of greenhouse gas emissions compared to cattle production which produces 62.2% greenhouse gas emissions (FAO, 2017). However, the previous figures demonstrating the size and sector of global production place the poultry industry under immense pressure to further reduce their carbon footprint. A large part of the GHG production associated with animal protein is related to feed, and therefore dependent on the type of feed used. This has attracted attention to investigating low GHG protein sources for poultry feed.

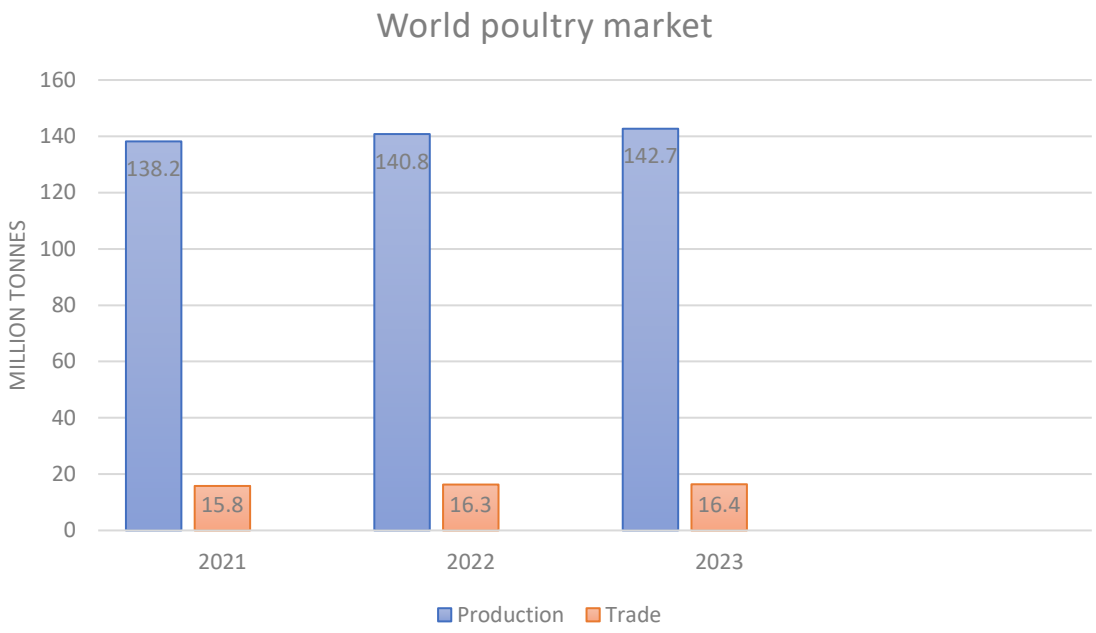

Figure 1.1- The world total Poultry production and trade between 2021, and 2023 (Source: FAO stats, 2023)

1.2.2 Projection of sustainability challenges for poultry production

Despite the lower environmental impact of poultry production compared to other livestock products, the poultry industry has its share in adding to acidification, global warming, and eutrophication phenomena. Factors responsible for the negative environmental impact of poultry industry are feed production and manure management (Leinonen and Kyriazakis, 2016).

Ruminant production has more environmental impact due to the emission of high amount of greenhouse gases (GHG) that mainly resulted from the fermentation process of gut bacteria (Leinonen and Kyriazakis, 2016). In addition to that, the ruminant industry tends to occupy more land space leading to increase of GHG emissions through deforestation.

The major provenance of global warming is CO_2 emissions. Accordingly, the sum of Global warming potential per functional unit which is the measure of GHG can also be called carbon footprint (Leinonen and Kyriazakis, 2016).

Methods like life cycle assessment methods (LCA) have been developed particularly in UK to assess the effects of poultry production systems, subsystems (factors like feed production), and overall impact (like nutritional practices that should have positive impact on the environment).

Although poultry industry has proven to have a relatively minor effect on the environment (Williams *et al.*, 2006), poultry feed provision accounts for 82% of GHG and 80% of total energy use. When taking into consideration that non-ruminant production depends enormously on imported protein sources especially soya that UK alone imports around 3.3 million tonnes annually from Argentina, Brazil, US, and Paraguay, almost 60% of which is used by the poultry industry (www.poultrynews.co.uk/2019). This has resulted in an increase in concern regarding environmental and feed sustainability globally (Eriksson *et al*., 2005; Pelletier, 2008). This is due to the fact that raising crops needed for feed, consumes large amount of energy in addition to transportation, which contributes to the emissions into the environment (Leinonen and Kyriazakis, 2016). Many resolutions are now in progress, working towards the development of optimised nutrient composition of poultry feed and finding protein alternatives to soya bean meal. This could aid in reducing the effect of nutrient emissions that sometimes can be in the form of ammonia (NH3) or carbon dioxide from fossil fuel or nitrous oxide which all contribute to the global warming potential (GWP)(Leinonen and Kyriazakis, 2016). It is worth mentioning that Kieronczyk *et al*., 2022 suggested that insects may have a useful environmental effect, by comparing the GWP of both *H. illucens* and *T. molitor*. The authors found that insects were responsible for about twenty-eight times less GWP than any other conventional protein used in the feed production of poultry, pigs or other livestock (Been, 2020). It is estimated that GWP

for insects is 12 to 13 GWP/ kg protein, compared to conventional protein for poultry and beef of about 50 or 335 GWP/kg, respectively.

1.3 Soya bean in poultry nutrition

1.3.1 History of soybean use as feed.

Soybean originated in China and was transported to Africa through the Chinese African trade in the early 19th century (Khojely *et al*., 2018). Soybean is commercially important crop worldwide, originating in Asia and processed to produce soybean food derivatives consumed as soy sauce, tofu, and miso (a soybean-based paste). In addition to that, soybean is used as a high-quality protein feed for cattle, poultry, pigs, and other livestock, with the amount of protein in soybean reaching 44% (Hymowitz, 2004). In the USA, soyabean was first introduced in 1920 and been used until nowadays in industry. In the United States, in 2015, more than 70% of the soya bean meal production is used in animal feeds. the soybean industry in U.S. has flourished, reaching the production of 50.9 million metric tons/year in 2018 (www.soygrowers.com/2019). Considering that the animal feed industry worldwide is becoming increasingly dependent upon vegetable protein sources. That situation is associated with declining fish stocks and has been dramatically underlined by the recent problems associated with use of animal offal meals in Europe (Rodehutscord *et al*., 2002), problems encountered due to the spread of mad cow disease (a neurological that affects cattle) (Abramson, 2004). As a major vegetable commodity, soya (both as oil extracted meal and the unextracted full fat product) is becoming even more important as a dietary raw material. Soybean is a heavily cultivated crop across the globe. In 2020, the worldwide production of soybean was estimated to be around 336.47 million tonnes (World Soybean Production, 2020), with Brazil, United States, Argentina and India accounted as its major producers (Voora *et al*.,

2020), which exceeded 80% increase growth rate from 2014 to 2018 (Voora *et al*., 2020). By 2023/2024 Soybean meal production in US is expected to reach 399.5 million tons which means a 7.9% increase from the previous year (370.24 million tons) and 80% increase from year 2018 (www.worldagriculturalproduction.com/2023). This strongly increasing soybean production urged the need for extensive investigation for its nutritional value in terms of amino acid digestibility and protein content, as well as investigating the antinutritional aspects of soybean (Ruiz *et al*., 2020).

1.3.2 Characteristics of soya bean (The nutritional value, and biochemical composition of soybean.

The soya bean belongs to the family *Leguminosae*, subfamily *Papilionoideae*, and the genus *Glycine L*. The cultivated form, *Glycine max L. Merrill*, grows annually. Its plant is bushy with height ranging from 0.75 to 1.25m, branching sparsely or densely, depending on cultivars and growing conditions.

The seeds of soybean contain copious amounts of protein and have an excellent amino acid profile which is on par with animal proteins. This allows it to be preferred as a substitute to meat proteins. This “king of beans” has been historically primarily used to extract soy oil. Soybean oil is the second- most consumed edible oil after palm oil (Voora *et al*., 2020), and consequently, a substantial amount of remnant by-product is generated following oil extraction. Once the oil from the seeds is extracted, either through solvent- based or mechanical extraction methods, what is left behind is defatted soybean meal which can be substituted into many edible and nonedible products, such as vegan foods, animal feeds, biodiesel production and other nutraceutical and industrial applications. Prior to the extraction of oil, the seeds are dehulled and the hulls may be added back later if required. These previously mentioned information highlights the worldwide importance of soya beans. However, there are well known problems associated with feeding soya beans. Central to any decision relating to the increased use of a

raw material is the constraints of dietary inclusion within diet formulation due to antinutritional factors, and these are a known issue for soya beans.

1.3.3 Antinutritional factors affecting livestock when using soybean as feed.

Trypsin inhibitors

Trypsin inhibitors are group of peptides with specific inhibitory characteristics depending on the target enzyme, and they are widely distributed in plants (Birk, 1989). The wide variety of inhibitors with different action mechanisms are sulphydryl-, serine-, acid- and metallo-proteases (Xaviera-Filho and Campos, 1989). Serine protease inhibitors are known to be found in soya beans and they act as anti-nutritional factors. Kunitz inhibitor (KSTI) is one of the most abundant trypsin inhibitors in soya bean. It was the first plant inhibitor in soya to be isolated and characterized (Kunitz, 1947), with a molecular weight of about 21 KDa including two disulphide bridges. Trypsin inhibitors function in plants by regulating and protecting against unwanted proteolysis in plant tissues, while also acting as defence mechanism against attack from diseases, insects and animals (Xavier-Filho and Campos, 1989). It can be concluded that they act as defence mechanism to protect the plants against disease, which means that any attack or damage in the plant triggers the accumulation of the proteinase inhibitor proteins (Ryan, 1990).

The anti-nutritional effect of trypsin inhibitors

In the study by Liener and Kakade (1980), trypsin inhibition (TI) contributed to a reduction in growth in rats. However, further research on the role of TI showed that the effect extends to the hyper secretion of pancreatic enzymes by enhancing cholecystokinin (CCK) production

(figure 1.2) leading to growth reduction as a result of loss of essential amino acids and decreased intestinal proteolysis (Schneeman *et al*., 1977).

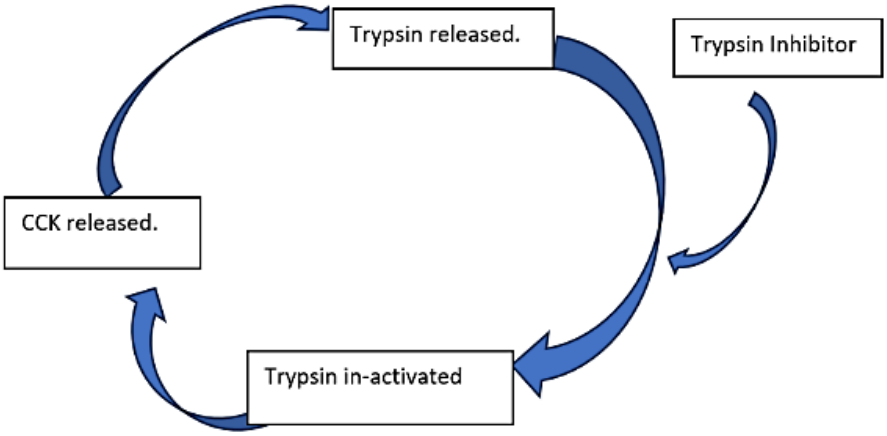

Figure 1.2- Trypsin inhibitors block the negative feedback loop in trypsin release and promoting CCK production.

Phytic acid

Phytic acid is the primary phosphorus reserve in the seed (Reddy, 1989) and is believed to protect plants against oxidative damage during storage and protection against moulds (Kumar *et al*., 2010). Phytic acid is in the form of a ring structure consisting of an inositol ring with six P(OH)3 groups attached (Figure 1.3).

Phytate anti-nutritional effects

Phytates form chelates with minerals like calcium, copper, molybdenum, iron, and manganese, resulting in difficulty in digestion of these complexes even at low PH levels (3 or 4) (Beleia *et*

al., 1993). In addition, phytate can bind to proteins leading to a decrease in their solubility and functionality (Derahm and Jost, 1979). Phytate has very low availability in non- ruminants, however they are extremely abundant and persistent in soyabean. To solve the anti-nutritional effect of phytate in soybean and thereby enhance the soybean nutritional profile, phytase enzymes are being synthesized in seeds of transgenic soybean, reducing the phytate availability (Li, 1997), but more commonly, exogenous phytase enzymes are added to poultry diets to release phytate phosphorus from all dietary sources (Scholey *et al*., 2018).

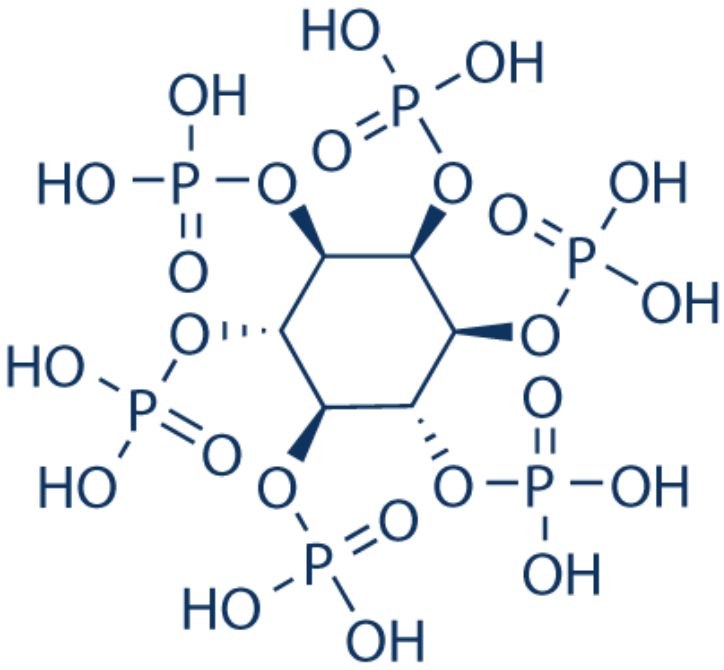

Figure 1.3- Phytic acid ring structure consisting of an inositol ring with six P(OH)3 groups attached (www.selleckchem.com).

Soybean as a feed is full of anti-nutritional factors that must be mitigated before it can be offered to non-ruminants, and hence new feed products that require less processing would be beneficial to the industry.

1.3.4 Soya bean feed alternative

The soybean industry is facing many challenges as mentioned previously, in addition to antinutritional characteristics, there is a detrimental environmental impact which leads to increasing carbon footprint. Nowadays the world trend is mainly focused towards decreasing carbon footprints while providing sustainable feed source for life stock. Two key criteria to be considered when proposing feed alternatives in poultry production, are low feed conversion and high growth. Another criterion when proposing feed alternatives, is the ratio of energy cost to amino acid profile. Therefore, the protein alternative that should replace Soybean as feed, should fulfil the previously mentioned criteria. The conclusive factor should be the suitable balance between energy cost and amino acid profile (Selaledi *et al*., 2020).

Protein feed alternatives cover a wide range, some are from various plant sources which include peas, beans, lupins however, since most plant protein except for soya, is not a match to animal protein, they tend to be characterized by deficient amino acid profile. Lum *et al*. (2013) worked on the idea of producing algae from biodiesel production then conducted research on the viability of replacing soybean with defatted microalgae. These authors stated that microalgae had a relatively consistent amino acid profile. Another high-quality protein by-product is yeast protein concentrate (YPC), which is separated from distillers dried grains with solubles (DDGS), the end-product of bioethanol production. YPC is then dried, powdered, and can be introduced into diets either as a feed ingredient or as a feed additive (Scholey *et al*., 2011). DDGS is the organic by-product of the ethanol production industry and has had substantial attention as a feed ingredient over the past few years. It needs to be taken into account what the abundance of such by products is and their relative cost and availability, before deciding if they can be of use as feed ingredients.

In the past decade, insects have emerged as animal protein alternatives in fisheries and more recently for poultry feed, although legislations in Europe and UK are starting to consider

allowing use of insects as feed. However, more research is needed to develop an understanding of insects that are fed waste products and optimum insect meal inclusion rates. (Vander Aar *et al*., 2019). Azagoh *et al*., 2016, mentioned the future need for nutritious protein sources for both humans and animals. Insects are of increasing interest as an alternative protein source for feed which may be one of the key solutions for the future challenges of providing feed for livestock.

1.4 Insects as a protein source (potentials and challenges)

Insects have proven to be very rich in nutrients, high-quality proteins, and important fats (Rumpold and Schluter, 2013). In 2013, a study by Rumpold and Schluter compared the content of different nutrients in the body of insects in terms of protein, fat, and fibre. The results showed the highest nutrient proportion was protein. Among different orders of insects, the highest protein content is in the order orthoptera with over 60% protein content (dry matter). This is not surprising, as one of the most known orthopteran members are locusts, which are used as food in some parts of Asia like China and Saudi Arabia. The issues are that locusts are wild insects which are difficult to breed in closed facilities and their feeding style on agricultural crops can cause huge losses which stops them being introduced globally as food. This leads to a requirement to find other insect candidates for feed. Order Coleoptera (beetles), Lepidoptera (caterpillars), and Orthoptera (locusts) comprise about 80% of insects which are adequate for feed while the other 20% belongs to the other orders (Lavalette, 2013).

1.4.1 Use of insects as feed alternative for poultry

Studies investigating insects as alternative feed ingredients for poultry have been recently heavily published (Allegretti *et al*., 2018; El-Hack *et al*., 2020; Kim *et al*., 2019). This shows the great interest in this area, due to the reduced environmental impact and the rich amino acid

profile compared to soybean. However, insect production can be met by many constraints, some of which are a) insect rearing for feed is still a maturing sector which may still need to be subjected to development, b) the absence of an organised system of food waste management, and c) the high cost of insect production for feed (Kieronczyk *et al*., 2022). However, studies showed that Insects possess high nutritional value (*De Marco et al*., 2015), antimicrobial properties as they contain plenty of antimicrobial peptides (AMPs) (Bulet *et al*., 1999), in addition to that, the chitin found in invertebrates including insects, could improve gut microbiota, overall immunity and therefore improve poultry growth (Gasco *et al.*, 2020; Jozefiak and Engberg, 2017). Insects also proved their importance being added as full fat meal for poultry to increase performance, with no adverse impact on feed conversion rate, Benzrtiha *et al.*, 2020a, studied the effect of 0.3% fullfat meal inclusions of two insects (*Tenebrio molitor* and *Zophobas morio*) and the results showed that both insects increased body weight gain (BWG) and had no adverse effect on feed conversion ratio (FCR) compared to control.

1.4.2 Insect breeding and processing for feed.

Insects as feed ingredients may lack certain nutrients. This may be enhanced by supplying insects with certain nutrients in their food. Some studies showed that fat content can be altered by modifying the cholesterol level in their diet (Yhoung-aree, 2010) as insects do not naturally have cholesterol, and acquire it through their feed (Ritter, 1990). These studies show that we can potentially obtain insects as feed with enhanced nutrient yield by altering their diet content. Therefore, to obtain an adequate nutritional profile from insects for feed production, attention to the nutrient details within their feeding substrates is needed (Kieronczyk *et al.*, 2022). However, there is little information about the effect of substrates on the insect nutritional profile. Nevertheless, studies on the nutritional aspects of various insects' species such as protein and fat content, vitamins, carbohydrates and minerals were performed (Rothman *et al*., 2014). For example, it was suggested that Gombe chimpanzees hunt on certain type of termites

(Macrotermes subhyalinus) daily which is assumed to be just 2.36g of 38.8g of their whole daily protein requirements (Food and Nutrition Board, 2005) where they acquire the rest of required protein from other sources (O'Malley and Power, 2012), therefore, it was suggested that this insectivory behaviour was to acquire certain micronutrients like minerals and vitamins (Tennie *et al*., 2009; O'Malley and Power, 2012). Mealworm (*T. molitor*), and black soldier fly larvae (*H. illucens*) are among insect candidates to provide alternative feed choice for poultry (*De Marco et al*., 2015; Hong *et al*., 2020). however, further studies on the nutrient needs of *T. molitor* and *H. illucens* are required, especially if they are to be considered as potential candidates for feed replacement of soybean (Cammak and Tomberlin, 2017).

Distillers' dried grains with solubles (DDGS)

DDGS are by products from ethanol production using wheat or maize. After the extraction of starch, DDGS are the unfermented product of ethanol production. DDGS contains high energy and protein (Belyea *et al*., 2010). It has also been discovered that the elimination of starch in feed has a positive effect by increasing the number of digestible fibres (Belyea *et al*., 2004; Cromwell *et al*., 1993; Nyachoty *et al*.,2005; Spiechs *et al*.,2002; Widyaratne *et al*., 2006; Weigel *et al.*,1997). The most common type of DDGS in Europe is maize DDGS while in UK, the most common feedstock is wheat (Scholey, 2012).

However, when wheat DDGS is considered for direct feeding for poultry, this poses a number of challenges including product variation from source of wheat as well as the processing method, and also the high fibre content which negatively affects digestibility especially in monogastric animals (Scholey, 2012). Therefore, DDGS is currently being evaluated as feed for insects especially mealworm that thrive on grain (Langston *et al*., 2023) and black soldier fly larvae that feed indiscriminately on variety of by-products and waste products (Hopkins *et*

al., 2021). Therefore, the use of wheat DDGS to create a protein mass of insect larvae with high nutritional values are important areas to investigate.

Legislations and barriers for insect use as feed globally. The available protein sources for feed are of either animal origin such as fish meal, or of plant origin like soybean meal. The demand on fish meal for feed is in constant increase because the of its high-quality protein and amino acids profile, compared to any protein source of plant origin (Tschirner and Simon, 2015). Accordingly, the costs of fishmeal production increased. Insects are used worldwide as both feed and in recycling of organic wastes, converting the food wastes, slaughter wastes and manure into larval biomass for feed and by-products. For example, in south Africa, the mass production of BSF larvae has led to the production of insect-based meals, extracted fat and oil for industrial purposes. The potential to use insect meals as a replacement to fish meal could save costs estimated at 117 million USD (Klonick, 2017).

Dipteran insect larvae as houseflies, and black soldierflies, as well as larvae of beetles of order coleoptera like mealworm have a great role in organic wastes valorisation and production of insect protein mass, in addition to that the post-feeding substrate (frass) is an excellent soil fertilizer (Poveda, 2021). In Canada, about 6 million black soldier flies are utilized in organic wastes conversion, production of natural fertilizer, and extraction of natural oils from larvae defatting process (Enterra feed, 2016). In Kenya, black soldierfly larvae are utilized in addition to other types of insects on a small scale, in managing different waste streams like food wastes, brewery waste and manure. In accordance with the development of legislation and policies, insects are being used as a high-quality protein for feed instead of high-cost fish meal. However, this is not the case in the EU where regulations are strict. According to EU animal health law regulation (EU No. 2016/429) on transmissible animal diseases, it is forbidden to use slaughterhouse wastes, manure and catering food waste in feeding of livestock to prevent the spread of disease. EU regulation No.1143/2014 limits the types of foreign insects brought

to EU for farming purposes. Also, approval for insects destined for feed as protein meal is complied with processing methods determined (as mentioned in regulation No. 142/2011) in terms of managing particle size, heat and pressure applied. However, by 2017 the EU listed seven insect species that are approved for feeding as a protein meal including black soldierfly (*Hermetia illucens*), mealworm (*Tenebrio molitor*), Jamaican field cricket (*Gryllus assimilis)*, Indian house cricket (*Gryllodes sigillatus*), housefly (*Musca domestica*), southwestern house cricket (*Acheta domesticus*) and darkling beetle (*Alphitobius diaperinus*) (EU regulation No.2017/893). Nevertheless, the regulations and legislations of other countries are currently being developed towards relieving the restrictions of use of insects as a protein source for poultry in 2017 after their strict prevention in 2015 (EU regulations). This is due to the global demand for increase livestock production to maintain food supply. On the other hand, the costs for providing high quality protein of plant origin such as soybean and maize are increasing each year due to competition for plant-based protein between food and feed (*De Marco et al*., 2015).

1.4.3 Legislations on insects farming for animal feed purposes.

Feeding substrates are the most important element affecting the nutritional profile of insects, and if these insects are destined for feed use, then in return, there will be a potential chemical and biological effect on livestock health (Lahteenmaki-Uutela *et al*., 2017). The feed marketing regulations in 2009, in its Annex III regarding substrates for insects, stated that, insects destined for feed should not be fed either constituents of gastrointestinal tract or excrement (Regulation (EC) No 767/2009). The EU regulations in 2011 also prevents use of any unprocessed fish or meat products except for fishmeal (Lahteenmaki-Uutela *et al.,* 2017). Along with that, animal by-products regulations prevent any sort of excreta or serving wastes as feed for insects. These previous regulations are to overcome any contamination that might be caused by harmful pathogens. Therefore, the proposal of new substrates for insects should be processed by the

European food safety authority to ensure the elimination of any risks around using insects as feed (Lahteenmaki-Uutela *et al*., 2017).

1.5 Mealworm *(Tenebrio molitor)* and Black soldierfly (*Hermetia illucens*)

The two insect species mealworm, and black soldierfly have drawn a lot of attention in the animal feed sector, where studies have been investigating viability of mealworm as an alternative to fish meal on variety of aquaculture fish (Gasco *et al*., 2016) as well as black soldierfly especially the defatted meal (Renna *et al*., 2017). Not to mention several studies on either partial replacement (*De Marco et al*., 2015; Schiavone *et al*., 2017) or total replacement (Biasato *et al.,* 2016; Bovera *et al*., 2016) of soya bean meal (SBM).

1.5.1 Mealworm

Tenebrio molitor, known commonly as mealworm belongs to the order coleoptera (beetles). The order coleoptera comprises about 400,000 discovered species. Beetle species vary in size, mode of feeding and lifestyle.

Adult structure, behaviour and life cycle

Adult beetles have a hard elytron forewing, a shield like front wing covering the whole body, and the hind wing is a membranous wing. Adult sizes vary from exceptionally large, the 200-mm-long *Titanus giganteus* cerambycid long-horned beetles of South America, to the smallest forms measuring 0.4-mm-long, *ptiliidae;* feather-winged beetles of North America (Encyclopedia of insects, 2009). *T. molitor*, belongs to family Tenebroinidae which includes 47 species. The name stands for Darkling beetles presumably most species in that family are

between dark brown to black. *T. molitor* adults are small in size (15mm), and black (Su Yean Ong et al., 2018). The life cycle of *T. molitor* is complete, comprising four forms including egg, larvae, pupae and adult. The time it takes to develop from one stage to the next is completely dependent on temperature and humidity. Optimum conditions for a typical 10–12-week cycle, will be 25°C to 28°C and 50 to 60% RH respectively (www.breedinginsects.com, 2020).

Eggs

Eggs are laid on hidden surfaces, in some cases the substrate itself, adult beetles may lay up to 200 eggs at once. Eggs are small in size (2mm) with a distinctive white colour.

Larval forms

Mealworm is a commonly known pest for stored grains and as food for pets. Mealworms have a uniform cylindrical shape and a brownish white colour, with size ranges between 10 to 28 mm in length according to larval stage. The larval stage may last up to 3 to 4 months before development into the next stage (pupae), depending on temperature, humidity and feed availability. The fully grown meal worm is golden brown in colour and reach from 2.5 to 3 cm. In order to obtain a suitable load of insects that are destined for feed and with good protein yield, insects are reared in large numbers with the available substrate (www.breedinginsects.com/2020) (Figure 1.4).

Figure 1.4- Yellow mealworm *T. molitor* (www.breedinginsects.com/2020)

Pupae

The pupal stage is a typical non feeding stage with a type of exarate pupa, with the appendages free and not cemented to the body, while the mouth parts are firmly attached to the head to prevent movement till the adult stage is reached (Britannica, 2022).

1.5.2 Black soldierfly

Adult structure, behaviour and life cycle

Hermetia illucens (Linnaeus, 1758) is one of the insects mentioned frequently when it comes to waste bioconversion, recently *H. illucens* larvae have been associated with pig and poultry facilities for bioconversion of manure. BSF belongs to the order Diptera with typical two wings and another pair modified into a balance organ (halter), resembling members of order Diptera of flies and mosquitoes. Specifically, they are like the family Stratiomyidae with appearance

of discal cell in the nervation of the wings. *H. illucens* are non pest insects and not known to transfer any diseases.

Life cycle

The life cycle of *H. illucens* is complete (holometabolous) with four developmental stages of egg, larvae, pupae, and adults.

Eggs

Eggs of all individual females are oviposited closely in batches (Tomberlin *et al.*, 2002), the egg is characterized by an oval shape and size is about 1mm with beige colour that lightens into off white during development. Eggs develop within 4 days under 27° and 3.5 days under 30° of temperature respectively.

Larvae

Larval active is the only feeding stage and lasts the longest, remaining for two weeks under an optimum temperature of 27°C (Tomberlin *et al*., 2002). Larvae are a typical apodous with neither thoracic nor abdominal appendages. Larvae are saprophagous feeding on organic wastes (rotten vegetables and fruits, animal manure) (Tomberlin and Sheppard, 2002). Larvae pass by five instars and the early larval instar is white in colour (figure 1.5) then the colour darkens until it reaches very dark brown in the final instar (figure 1.6), the last larval instar is about 12 to 25 mm long (Ibadurrohman *et al*., 2020). Larvae of *H. illucens* feed on the substrate until reaching the final larval instar and gets into the prepupa which empties the gut after feeding. That is considered as an advantage in BSFL behaviour to prevent any pathogens in the gut (Spranghers *et al.*, 2017).

Figure 1.5- Early larval instar of *H. illucens*

Figure 1.6- Different larval stages of *H. illucens* (the lighter colour indicates earlier stage (on the right) the darkest colour (on the left) indicates the latest larval stage.

Adults

Adults are dark coloured typical dipterans with one pair of well-developed front wings and the second pair modified into a halter (balance organ). Females are larger than males in size (Tomberlin *et al*., 2002), with a type of long geniculate antenna (resembling members of order Hyemnoptera, wasps). Adults are about 13 to 20 mm size, and do not feed, but they need excess to water and nectar to attract them for egg oviposition (Tomberlin *et al.,* 2009).

1.5.3 Rearing conditions of Black soldier fly larvae (*Hermetia illucens*) and mealworm (*Tenebrio molitor*)

Insect larvae in general and specifically BSF and mealworm, have the ability to transform waste material into a nutritious feeding substrate and large larval biomass which can then be used as feed for livestock (Derler *et al*., 2020). Black soldier fly larvae also have the ability to transform any feeding substrate into a nutrient rich medium which can be used later as a fertilizer. In a study performed by Gao *et al*. (2019), BSFL was raised on maize straw as a feed substrate. This affected the BSFL in terms of prolonged larval stage duration, and decreased female's fecundity. On the other hand, the amount of polyunsaturated fatty acids was 25.37% which is more than that of BSFL raised on wheat bran 16.57% (Gao *et al*., 2019). Yellow mealworm can be raised on plastic byproducts as they are able to ingest the plastic foams of polyethylene and polystyrene (Yang *et al*., 2018). In addition, they have the ability to feed or utilize wheat and corn straws depending on their ability to ingest lignocellulosic products (Yang *et al.*, 2019).

In an experiment performed by Broekhoven *et al*. (2014), mealworm feeding on cookie remains as a source of starch expressed a high mortality rate whereas those fed on potato peels had a normal life cycle pattern (Broekhoven *et al*., 2014).

Humidity is considered an important yet not dependent factor on insect growth, feeding and survival, however, extreme humidity conditions affect insect survival and reproducibility (Norhisham *et al*., 2013). According to Morales-Ramos *et al*., (2018) *T. molitor* larvae should be sprayed with water as they have the ability to absorb moisture in humid air. In a study performed by Norhisham *et al.*, in 2013, they found that Bambo borer (*dinoderus minutus*) responded positively to the increase in humidity at constant temperature of 30°±2°in terms of

oviposition, egg development and hatchability and the higher the humidity (75%) the shorter the oviposition period (Norhisham *et al*., 2013).

1.5.4 Role of black soldier fly in the elimination of food wastes and manure

BSFL mainly feed on food wastes and livestock manure, being able to convert these into usable fertilizers (Siddiqui *et al*., 2022). The accumulation of organic wastes results in biohazard volatile emissions which can compromise the environment leading to pollution. A study in 2018 by Beskin *et al*. showed that black soldier fly larvae (BSFL) feeding on were able to minimize these emissions to a great by more than 87%. That finding is and evidence that BSFL is a sustainable source for reduction of air pollution resulting from wastes volatile emissions.

Tomberlin *et al.*, (2017), investigated the feeding of dipteran larvae on the substrate medium (wounds, corpses, livestock manure), where, As the larvae feed, they secrete useful antimicrobial enzymes. These enzymes work on the enhancement and modification of substrate medium and bacterial microbiota, which if the same concept is applied on wastes as to feeding substrates, then BSFL might induce the same antimicrobial effect, leading to a change of waste substrate into larval biomass and high-quality organic. That would make a self-sustained system for converting waste material into a useful clean medium and larval biomass for feed. A trait when BSFL is feeding on any type of food substrate (any type of manure) is the reduction of both phosphorus and nitrogen in the substrate (Wu *et al*., 2017; Myers *et al*., 2008; Oonincx *et al*., 2015a; Zhou *et al*., 2013). BSFL feeding on soil substrates can reduce the amount of both phosphorus and nitrogen in the soil by 30-80% (Myers et al., 2008). In addition, insects feeding on a substrate in general can reduce the volume of organic wastes by converting it to a valuable protein mass (Oonincx *et al*., 2015). Leonardo *et al*. (2019) investigated the ability of BSFL to supress the activity of harmful bacteria such as *S. minor* in a soil medium, in the complete absence of *E. coli, Salmonella* and *clostridia ssp.* This could have an impact on future agricultural sustainability. In addition, BSF adults are not considered a threat to

human health due to the lack of feeding at this stage and the deficiency of adequate mouth parts for feeding (Rehman *et al*., 2019; Caligiani *et al*., 2018). *H. illucens* adults do not feed depending on their body fats stored from the feeding larval stage, do not approach any wastes (neither human wastes nor animal manure) removing the risk of disease transmission among humans or animals (Diener *et al.*, 2011).

1.5.5 The Use of black soldier fly as source of protein for feed

The continuous global search of sustainable, low-cost high-quality protein source for feed that would replace the soybean meal and be as favourable as fish meal, became a subject of investigation by Hale, 1973, and Newton *et al*.,1977. For example, in 2020, Rawski et al. investigated the possible use of BSFL as a source for high quality protein feed for broiler chicken to replace the fish meal based on some similarity of the amino acid composition (Table.1.1).

Table 1.1- Nutritive value and amino acid profile of black soldier fly full fat larvae meal and fish meal (Rawski *et al*., 2020).

Nutrient	**BSFL**	**FM**
	g/1000 g of dry matter	
Crude protein	350	618
Crude fat	298	165
Crude fibre	79.0	0
Crude ash	53.0	175
Nitrogen free extract	221	42.0
Amino acid	g/1000 g of dry matter	
Aspartic acid	7.30	9.40
Glutamic acid	13.1	14.5

Serine	4.88	4.17
Glycine	6.15	6.41
Histidine	3.25	2.09
Arginine	5.47	6.07
Threonine	4.43	4.10
Alanine	8.21	6.87
Proline	6.68	4.28
Tyrosine	6.71	3.00
Valine	6.79	5.79
Methionine	2.12	2.53
Cystine	0.76	9.59
Isoleucine	4.73	4.24
Leucine	7.83	7.48
Phenylalanine	7.76	3.07
Lysine	6.82	6.63

1.6 Types of feed for Insects

Insect protein is very rich, and the amino acid profile is comparable to soybean which makes them an ideal candidate for feed. The amino acid profile of plant products is typically poor whereas other by- products might have adverse effects on digestibility for poultry. Insects' valorization of low value waste feed into high nutritional value insect protein is a potentially viable way to make use of waste products. Various studies have investigated the effect of

byproducts resulting from different industrial processes on mealworm and BSFL on nutritional profile, weight yield and development rate, survival rate and overall performance.

1.6.1 Viability of bakery byproducts and wastes as feeding substrate for *H. illucens* and *T. molitor.*

In a study by Taglieri *et al*., (2021), to assess the effect of adding flaxseed cake to bread to increase shelf life and compared the effect of feeding the flaxseed cake with and without yeast extract to normal wheat flour bread on the growth performance and mass yield of meal worm (*T. molitor*). The results showed a positive effect for flaxseed treated bread in terms of relatively increased larval mass compared to normal wheat flour bread. In addition to that, meal worm valorisation in the flaxseed treated group was increased by 23% and 89 % compared to sourdough bread and baker's yeast bread respectively. There was minimal to none waste processing strategies utilised on the feed before introduction to the mealworm. However, the long-life cycle of *T. molitor* in some countries requires reconsidering another type of insect with less life span as Taglieri *et al*., (2021) investigation lasted for only three days while rearing mealworm to the last larval stage under certain conditions of temperature and humidity especially in less humid countries (between 29% to 35% RH) may take over 12 weeks as mentioned previously. Nevertheless, this investigation aimed to decrease food waste by feeding to mealworm and establishing larvae mass to be used as feed meanwhile, accomplishing a zero-waste strategy (Taglieri *et al*., 2021). In another study performed by Mattioli *et al*., (2021), mealworm was raised for a year using a collection of bakery products, in 5 treatment groups. Three of diets each had only one ingredient either cookie wastes, or bread spent grains while the other two treatments, one had cookie mixed with bread, and the other, have cookie mixed with spent grains. The results showed a consistency of the fatty acid profile among the treated groups but, some of the treatment groups failed to develop to the prepupal stage (100% cookie wastes, 100% bread and combination of 50% cookie waste +50%

bread). The results of the investigation showed that mealworm could be a good protein alternative to conventional animal feed with rich fatty acids profile, however, mealworm rearing conditions are costly due to their long lifecycle, and feed substrates require processing to reduce the moisture of the feeding substrate. No studies were found for use of bakery wastes or by-products as a substrate for feeding black soldierfly larvae.

1.6.2 Rearing *T. molitor* and *H. illucens* on agricultural by-products

Rearing insects on different diets can affect the insects' nutritional profile (Oonincx *et al*., 2015). The use of less favourable agricultural products for insect feed can in turn produce quality protein feed for livestock, and reduce the insect rearing costs, by using by-products that are less viable as feed for livestock to produce insect mass (Stull *et al*., 2019).

Stull *et al*. (2019) used stover as a feed for mealworm to investigate the protein yield, amino acid profile and iron content of mealworm. Stover is a nitrogen and protein poor agricultural by product, as 60% of nitrogen in maize grain is retained in the rest of the plant (Hoeft *et al.*, 2000). In this study, *T. molitor* larvae were reared for 32 days on three diets (control, mixed maize grain with stover and soy, and a 100% stover diet). The results showed that mealworm of all tested diets yielded had all the essential amino acids with concentrations comparable to other protein sources, and larvae reared on the mixed treatment feed resulted in amino acid profile compatible to that of the control diet. However, although the 100% stover diet had a high survival rate, the amino acid profile was poorer than the rest of the diets. In a second study, mealworm survived in several generations raised on 100% stover diet. The iron content of

100% stover group was the highest. Table 1.2 shows reported essential amino acid composition of T. molitor larvae compared to three traditional protein sources by Stull *et al*., (2019).

Table 1.2- Essential amino acid composition (mg/g) of *T. molitor* larvae compared to three traditional protein sources.

	HIS	ILE	LEU	LYS	MET	PHE	THR	TRP	VAL
					mg/g				
Beef and veal (Bos taurus) edible flesh	6.03	8.52	14.35	15.73	4.78	7.78	8.12		8.86
Chicken (Gallus gallus) edible flesh	5.25	10.69	14.72	15.9	5.02	8	7.94	2.05	10.18
Cow's Milk (pasteurized)	0.92	1.62	3.28	2.68	0.86	1.85	1.63		1.99
Mealworm* (*T. molitor*) (Diet A: Control)	5.92	9.36	15.31	11.78	3.1	7.47	8.11	2.22	13.16
Mealworm* (*T. molitor*) (Diet C: Stover)	3.27	6.87	10.53	8.53	2.1	4.79	5.56	1.43	9.91

Blank cells indicate no data available. Amino acid abbreviations are as follows: HIS (histidine), ILE (isoleucine), LEU (lucine), LYS (lysine), MET (methionine), PHE (phenylalanine), THR (threonine), TRP (tryptohan), VAL (valine)

1.6.3 Vegetables as a supplementary diet for insects with wheat bran

The effect of different types of vegetables as a supplementary diet together with bran as the main substrate has been investigated (Liu *et al*., 2020). In this study, 3 treatment groups were

provided, with bran as the main substrate and the supplementary diet for treatment 1, 2, and 3 were 20g carrots, 20g orange, and 20g red cabbage respectively in addition to 50 wheat bran added to each treatment. There was no significant difference between the experimental groups regarding the survival rate, however, the growth rate and overall weight of larvae fed on bran supplemented with either carrots, or, orange or cabbage inclusions, after four weeks of treatment were significantly higher than the control (only bran diet) (Liu *et al*., 2020).

Feeding substrates have an enormous impact on the growth and nutritional content of larvae destined for feed, in terms of growth rate, protein content, fat content, minerals and amino acid profile.

1.7 Gastrointestinal tract health, structure and microbiota in poultry

1.7.1 Persistence of pathogenic bacteria and antibiotic resistance

Since 1950, the chicken industry (including poultry meat and egg production), has emerged to meet consumption needs in UK and chicken production has increased accordingly (Godley and Williams, 2007). In order to ensure healthy, efficient production and eliminate diseases affect poultry caused by harmful bacteria, antibiotics were extensively applied to overcome the effect of bacterial diseases effecting poultry and the livestock production sector in general. However, while antimicrobials are an effective means to preserve the health of livestock and reduce with disease, they must be used wisely to overcome the antimicrobial resistance phenomenon (FAO, 2019). Concerns over the use of antibiotics as antibiotic growth promoters were noted in the UK House of Lords Swann Report (Kirchhelle, 2018), within 15 years of Jukes and Williams (1953) recognizing their potential usage to promote efficiency in animal production. However, concerns about development of antimicrobial resistance and about transference of antibiotic

resistance genes from animal to human microbiota, led to withdrawal of approval for antibiotics as growth promoters in the European Union since January 1, 2006 (Castanon, 2006).

Livestock diseases directly affect humans when consuming the diseased animal, causing the food borne pathogens to persist in the alimentary canal (Santini *et al*., 2010). For example, *Campylobacter jejuni* are a persistent pathogenic bacterium mainly associated with poultry meat (Santini *et al*., 2010), which have a harmful impact on human health when infected poultry meat is consumed. The persistence of this Campylobacter strain comes specifically from their ability to survive in the alimentary canal and overcome high salinity, elevated temperature and low PH (Santini *et al*., 2010). Therefore, the long-term encounter of antibiotics can lead to the persistence of such pathogen which in turn leads to antibiotic resistance on the long run.

To be able to overcome the challenging of maintaining a healthy gastrointestinal tract without reliance on antibiotics, we must understand the avian gut anatomy and the mechanisms of food consumption and digestion. We should also be able to understand the nature of chicken gut microbiota. Through that we can be able to explain the reason of persistence of such harmful bacteria in the gut and be able develop more effective ways to increase poultry production.

1.7.2 Digestive system of chicken

The digestive tract of poultry, particularly broiler chickens, is comprised of the oesophagus that contains the crop, and terminates in the proventriculus, and gizzard, followed by the small intestine (duodenum, jejunum, and ileum), then finally the caeca and large intestine i.e., the colon and cloaca (Pan and Yu, 2014).

Mouth

The avian mouth is structured to form a beak that replaces the muscles and bones to instinctively pick the feed (seeds or live worms) from the ground (Duke, 1986), and the gastrointestinal tract wis equipped accordingly. The beak is a keratin sheath grown and extended from the mandible and it is a renewable part of the mouth. The attachment of the sclerotized part of the beak to the bird skull is somewhat loose, creating a gap that has the ability to assimilate large size particles of feed. Following the beak, a tongue is placed in the oral cavity with the ability to manipulate the feed and separate the hull from the seed in case of grains (Kirk and Klasing, 1999). The tongue is connected to the hyoid bone which facilitates the food movement towards the oesophagus (Homberger, 2017). Chickens are characterized by very limited number of taste buds, about 316 (Roura *et al*., 2012). This suggests that nutrients requirements are the factor that affects ingestion of feed not taste. The buccal cavity comprises mucus which aids in loosening the feed moving down the oesophagus (Samar *et al*., 2002).

Oesophagus and crop

The oesophagus is characterized by numerous folds with mucous glands in the lining layer and this gives it an expansive ability for large feed particles (McLelland, 1979). The oesophagus opens into a large crop for ingested food storage. This crop is the main storage compartment since the rest of alimentary canal (proventriculus and gizzard) is designed for other functions (Jackson and Duke, 1995). The crop function is utilized when there is a large amount of feed ingested, as the capacity of gizzard is restricted to 5 to 10 g of feed (Svihus, 2014).

Proventriculus

The proventriculus (first portion of the stomach) can be found between end of oesophagus and the gizzard (second portion of the stomach). The proventriculus is where the digestion begins

and the site of the mucosal glands that produce pepsin and hydrochloride for digestion (Rossi *et al*., 2005). It is relatively small in diameter compared to the next part of stomach (the gizzard).

The gizzard

The gizzard is the second portion of the stomach and its main function besides digestion is the manipulation of food particles to prepare for further digestion. The gizzard is supported by a relatively strong muscular layer in addition to two pairs of muscles called thin pairs and thick pairs (Denbow, 2015). The mucosal gland in the gizzard forms a cuticle layer lining the gizzard, which functions as protection against any damage during food manipulation or from proteolytic enzymes secreted from the proventriculus (Denbow, 2015). Between the gizzard and the small intestine, there is a pyloric groove to control the food movement.

The intestine

The structure of the intestine (Figure1.7) is consistent with its function being mainly digested food absorption. In order to fulfil this function, the intestine is equipped with villi and intestinal crypts along the lining of the epithelium (Yamauchi and Isshiki, 1991). The villi are further folded into about 10^5 microvilli for every square millimeter forming a large surface area for proper food absorption. The structure is equipped with intestinal goblet cells for mucous secretion.

The digestive tract of birds has two large ceca (Fig 1.7). The gastric ceca ferment uric acid and carbohydrates into ammonia and volatile fatty acids, after reabsorption of water and salt from retreated digestive fluid and urine (Svihus *et al.*, 2013). The digestive tract widens at the end to form the cloaca after passing by the smaller in diameter rectum.

The cloaca functions in storage of both urine and faeces, however, there is muscular fold located between urodeum (which is the end of both the oviduct and the ureter) and the coprodeum for the storage of faeces. This structure helps in separation of female eggs or male semen to reduce contamination by feces during egg laying or ejaculation (Klasing, 1999).

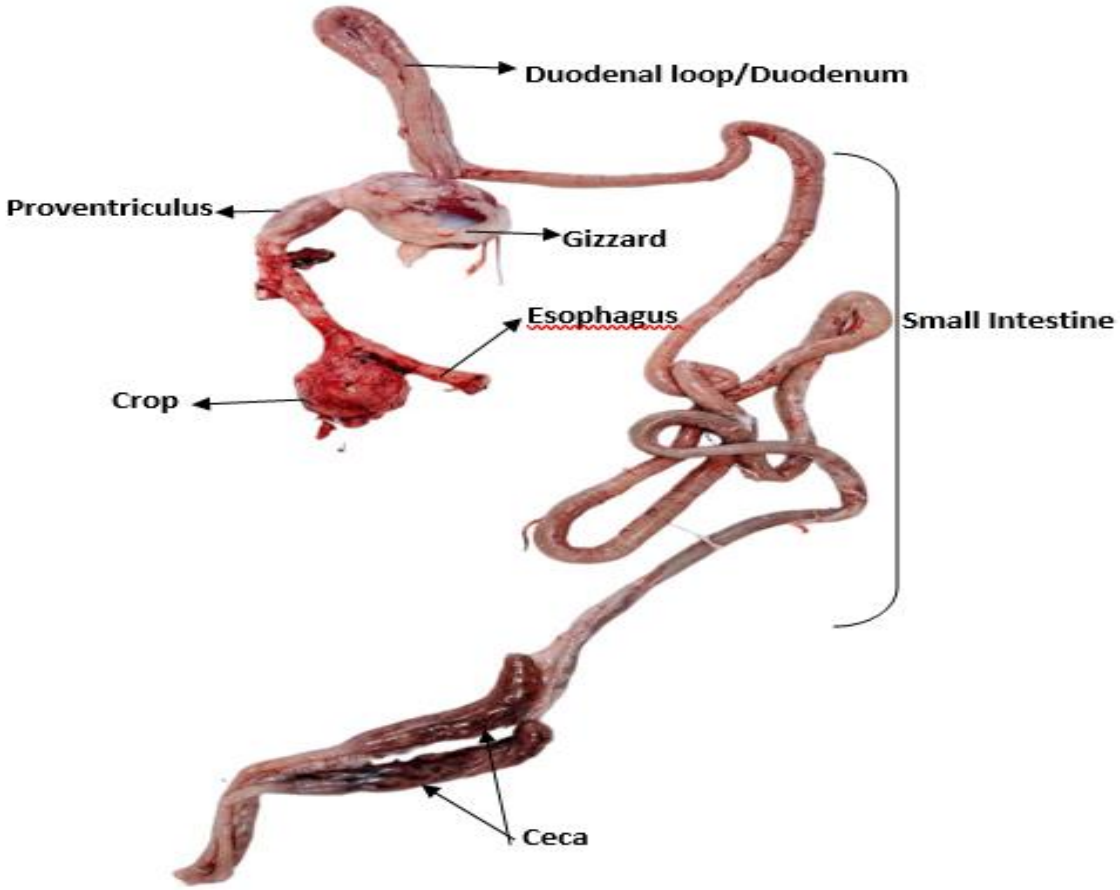

Figure 1.7- Different parts of chicken digestive organs

1.7.3 Gut loading.

Nutrients deficiency of animal feed is one of the crucial problems that are facing the livestock industry. Although insects have been of great interest in animal feed industry, due to their

nutrient profile that is rich in high quality protein, however, there were reports of nutritional deficiencies in animals that were fed insects only diets, for example, some wild animals that feed on invertebrates or insects tend to diversify its diet to compensate minerals deficiency by ingesting soil along with the insects (Hunt *et al.,* 2002). Two methods have been developed to provide insects with needed supplements especially calcium where most insects are deficient with (Hunt *et al.*, 2001).one of the methods is dusting insects destined for feed with the needed minerals like calcium in the form of powder, however this method proved to be not very efficient, as the dusted insects should be fed to the animals instantly to avoid loss of the supplemented powder as insects tend to groom themselves for time to time and thus the amount of supplement provided will not be uniform each time added due to the loss of undetermined amount of the supplement during the grooming process. The second method used is gut loading, where insects destined for feed are being fed the needed supplement with high concentration in the diet until the gut is completely filled in order to increase the supplement concentration for the insects to be consumed (Hunt *et al.*, 2001), therefore, gut loading method seems to be more effective method to provide the insects destined for feed with the appropriate nutrients that is very important for the animal health. A study by Kimberly et al., 2020, showed that calcium content in BSF could be increased by providing the larvae with a fat-soluble vitamin like vitamin A through gut loading method. The results showed the importance of precisely determining the adequate concentration that should be provided to the insect in the food and also the time frame needed to gut load in order to reach a uniform gut loading process.

1.8 Feed and Amino acid digestibility determination in poultry

Amino acid availability is the quantity of amino acids in the intestine that are available for use by the body.

1.8.1 Supplement on digestible amino acid basis instead of total amino acids

Protein consists of a variety of amino acids that differ in both digestibility, importance, and bioavailability. For example, cysteine and lysine are considered crucial amino acids but also have the least digestibility among other amino acid protein constituents (Parsons, 2019). In addition to that, amino acid digestibility can differ between different feed types and sometimes between different inclusion levels of same ingredients of feed. That being said, poultry feed is more viable when formulated on a digestible amino acid basis rather than based on total amino acids (Parsons, 2019). Using the digestible amino acid system to determine the AA digestibility of feed product is useful in the expected food shortage in the near future, feed will be formulated with certain expected amino acid digestibility, and this will also help to differentiate low protein (but high non-protein nitrogen) content feed from truly high protein sources (Lemme *et al*., 2004).

1.8.2 Excreta digestibility against ileal digestibility

Several studies have compared excreta and ileal digestibility to determine the amino acid availability (Ravindran *et al*., 1999). Excreta AA provides rapid determination of the AA digestibility (Lemme *et al*., 2004). However, this method completely ignores the hindgut bacteria, which has its share in the amino acid profile yield. Also, excreta AA usually evaluates the excreta of experimental birds after force feeding directly through the crop. However, the technique changes the whole digestive system physiology, as the test animal will be subjected to long-term fasting and then force feeding which may result in misleading calculations (Lemme *et al*., 2004). Therefore, there is mainly agreement among researchers that ileal digestibility measurement gives a more precise calculation for amino acid availability (Lemme *et al*., 2004; Ravindran *et al.*, 2009). In both systems, amino acid digestibility is affected by basal endogenous losses, which should be taken into account when performing an experiment.

This basal endogenous amino acid loss is dependent on the feed intake not on the nature of the feed itself and there are several potential methods utilised to account for this (Stein *et al*., 2007).

1.8.3 Difference between apparent AA digestibility and true AA digestibility.

If a comparison is made between the true and apparent AA digestibility, we can say that true AA is the expression of AA digestibility with regards to corrections for endogenous AA secretions. On the other hand, the apparent AA digestibility disregards the endogenous loss value. True digestibility can be more viable with high protein content diets. However, in case of feed with low protein content, endogenous amino acids may constitute large share of amino acids in the digesta (McNab, 1989).

1.8.4 Methods for measuring ileal digestibility.

Techniques for measuring digestibility have developed from the most basic growth assay which is not considered a digestibility assay, however the growth assay was considered fixed gold standard assay which monitors true amino acids availability across the different development stages of a living organism by supplementing certain AA in investigation to a basal diet that is known to be deficient with these two particular AA then deriving a slope ratio to see if these particular AA included in the basal diet are bioavailable across stages including digestion, absorption and use for synthesis of protein. Despite the viability of the growth essay, however, it proved to be time consuming, too costly since, only one AA can be tested during the essay (Parsons, 2020). Afterwards, a digestibility assay based on faeces collection was developed by Bragg *et al*., (1969). However, parsons *et al*., (1982), showed that there was at least a 25% overestimation of digestibility using excreta, due to ignoring the digestibility values of hind gut bacteria. Recently, the most frequent used digestibility assay is the ileal digestibility assay

for poultry (Ravindran and Bryden, 1999a; Parsons., 2002; Lemme *et al.*, 2004) which depends on including an inert marker within the feed (of determined amount). In the standardized ileal digestibility assay (SIAAD), digesta is collected from birds at post euthanasia from the ileal region between Meckel's diverticulum and the ileo-cecal junction. The SIAAD utilises a regression method, that involves ad libitum feeding of birds with a diet where the only protein source is the source under investigation (Scholey, 2012). Also, 3 graded levels of protein inclusions are required (usually 20, 40, and 60%) (Rodehutscord *et al*., 2004).

1.8.5 Titanium as an indicator of digestibility

Indicators for digestibility are inert, indigestible markers, that are used to determine digestibility at a specific point in the intestine, with no need for including excreta yield or feed intake (Short *et al*., 1996).

There are three frequently used indigestible inert markers. These are chromic oxide, titanium dioxide and acid insoluble ash. The acid insoluble ash marker requires measurement using a gravimetric method which requires a separation procedure and quantitative assessment for the sample (Parsons, 2019). Therefore, that requires larger sample size for obtaining precise results. On the other hand, both chromic oxide and titanium oxide marker require small sample volumes so are more suitable for studies based on digesta samples.

The use of chromic oxide as digestibility marker, has a practical advantage in that treated diets show clearly due to the resulting dark green colour of the diet while titanium dioxide is in the form white powder that does not noticeably tint the diet. This advantage confirms that the marker has been added to the diet and how intense the green colour can be, we can estimate whether the correct amount has been added or not (Parsons, 2020). However, despite the clear colour resulting from digestibility analysis, safety concerns were raised over the components

of the chromic oxide digestibility analysis (Parsons, 2019). This does not apply to titanium dioxide quantification method which does not raise any health concerns, although the nanoparticles of titanium dioxide are currently being considered as a potential health hazard in the human food chain (EFSA, 2021).

1.8.6 Chicken gut health and microbiota

It has been reported that the nature of gut microbiome affects the health and natural immunity of all living organisms (Aruwa *et al*., 2021). One of the fundamental parameters of gut health of living organisms is the gut microbiota, where the variety and integrity of gut microbes are responsible for the protection of gut lining (epithelium), immune-function and protection against pathogens (Dunkley *et al*., 2007a). Additionally, different diets have different effects on the format of gut microbiota. Where in some instances (Hird *et al*., 2014).

The integrity of gut microbiota plays an important role in gut health and elimination of pathogens. This role takes place through several mechanisms, some of which are, the occupation of adjunct sites in the epithelium lining the gut, preventing harmful bacteria from persistence in the gut, production of short chain fatty acids and other antimicrobial compounds (Kogut, 2013). The latter are responsible for gut haemostasis and reduction of inflammation or stress response resulting from any invasive microorganisms. Therefore, the metabolic and digestion processes go hand in hand with immunity functions and maintaining good health status. but the gut microbial community controls the pH levels and mucus production of the digestive tract.

First incidence of bacterial gut colonization in birds occurs at the time of hatching, where both Enterobacteria and Streptococci are the first appearing family of bacteria (Smith, 1965).

However, the full development of bacterial communities along the small and large intestine takes up to 2 weeks (Barnes *et al*., 1972).

1.8.7 The development of gut microbiota

As the microbial community develops it also changes in structure across the gastrointestinal tract with main groups of bacterial family representing each part of digestive tract (Gong *et al*., 2002). The variation of bacterial density along the gastrointestinal tract, depends on physiological and functional factors. We can find the highest number of microbial communities concentrated in both the crop and caeca, based on the evidence that the physiological conditions and level of antimicrobial resistance are more suitable in these areas. On the other hand, parts of the intestine, for example, the duodenum has lower microbial density due to the elevated levels of bile salts and enzymes leading to a less conductive environment for microbiota (Gabriel *et al*., 2006). Data on bacterial taxa and their distribution across the GIT are represented in Figure 1.8., (data from; Yeoman *et al*., 2012; and Gong *et al*., 2002).

In the crop region (10^8-10^9/g) there are higher bacterial community than the gizzard region, however there is a common presence of Firmicutes, Lactobacillus, in the two regions (figure 1.8). The caeca is the most rich region in microbial community as in terms of variety we can notice that Firmicutes comprises (44-56%) of population, Bacteroidetes represents from 23 to 46%, 1-16% of Proteobacteria showed (0.81%) presence. Finally, candida family were among fungi present, the abundant variety in the caecum might be due to it being a repository of microbes in chickens which highlights the importance of caeca microbial community, and this enables us to also recognise the pathogenic bacteria like campylobacter (Yan *et al*., 2019), small and large intestine contained two main bacterial communities, the Firmicutes and Proteobacteria (Yeoman *et al*., 2012).

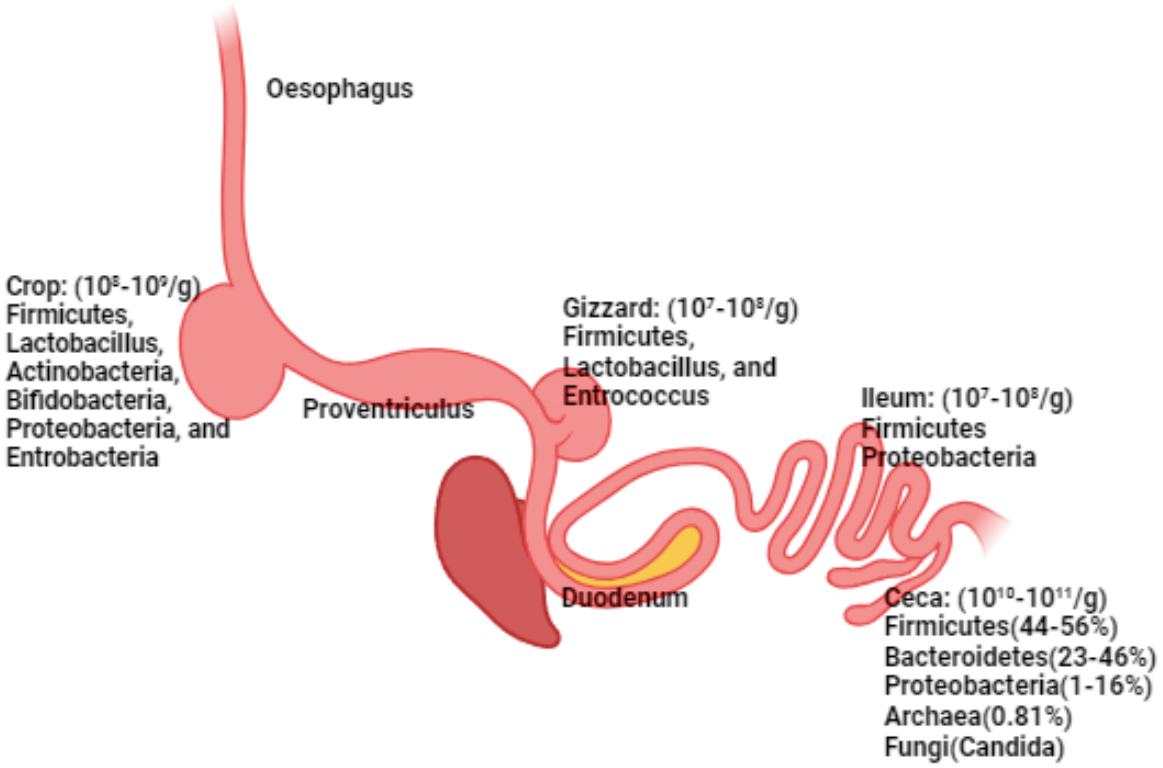

Figure 1.8- Bacterial community in the main portions of Chicken GIT, Created in BioRender.com

1.8.8 Bacterial biofilm formation

Bacteria tends to construct a firm structure through gathering and clustering, this complicated structure is hard to demolish, and this is a bacterial mechanism through which pathogenic bacteria can survive in host system and cause chronic damage to the host organs.

This structure is also resistant to known antibiotics and through this mechanism, bacteria can gain survival and persistence through the host as well as the next host being transmitted to (Sharma *et al*., 2019).

The well-known Gram-positive biofilm forming bacteria are staphylococcus aureus while that of Gram-negative strains are *K. pneumoniae, E. coli and P. aeruginosa* (Maniello *et al*., 2021).

So, at a certain point, antibiotics will fail to achieve the purpose and later on, a new type of antibiotics should be manufactured and clearly meanwhile, new costs for clinical trials are being spent and more time required to confirm the safety of use, deaths due to more bacterial infections will continue.

1.9 The effect of age on immune function in modern commercial broilers

The continuous increase of the world demands for poultry meat puts more pressure on increasing production. However, with the advancement in biogenetics and the development of new strains through genetic selection, resulting in fast growing chickens that are ready for slaughter earlier than the expected standard slaughter age (Kokoszyński *et al*., 2017). The historic, exclusive focus of the genetic technology towards more efficient feeding, leading to better carcass yield, delivered the aims very effectively. Nevertheless, these acquired advantages have come on the expense of less investment in the chicken digestive and overall immunity, especially with modern commercial broilers that are subjected to stress caused by the rapid rate of growth (Cheema *et al*., 2003).

The development of immune organs continues from the embryonic phase prior to hatch, sexual maturity of the chicken when immune organs are fully mature and cease to develop (Gordon and Manley, 2011). The central immune organs are responsible for the formation and transport of lymphocytes to the peripheral immune organs (Song *et al*., 2021). The overall immune function of birds is governed by the growth and development of immune organs, with a strong influence from environmental factors on maturation (Song *et al*, 2021). While immune organs of poultry greatly differ from those of mammals, they may be similarly categorised into peripheral immune organs (such as the spleen and caecal tonsils) and central immune organs

(such as the thymus, bursa and bone marrow). Initially, maternal antibodies play an important protective role for chicks against infectious disease challenge, but this protection is lost around 3 weeks post hatch. s non-specific cellular immunity, specific cellular immunity, specific humoral immunity in the peripheral blood and mucosal immunity continue to increase from d 1 to 34.

The central immune organs initially develop during the embryonic stage and continue developing after hatch until sexual maturity [Gordon and Manley, 2011]. The central immune organs can cultivate mature functional lymphocytes without antigen stimulation and then export these lymphocytes to the peripheral immune system to participate in immune reactions. Lymphoid-like stem cells from bone marrow mature in thymus and bursa, and then migrate to peripheral immune organs through blood and lymphatic circulation. Throughout the life of the bird, these immune cells will proliferate and differentiate when exposed to foreign antigens [Dekruff *et al*., 1975]. Early development of the immune organs directly determines bird resistance to various antigens and stresses in their living environments [Naukkarinen and Hippelãinen, 1989] but the intestinal mucosa plays an important role in the initial activation of the immune response and the subsequent regulation of its maturation [Hrncir *et al*., 2008]. Alongside the intestinal mucosa, maturation of the immune system occurs early in life and is heavily influenced by the intestinal microbiota [Tlaskalovahogenova *et al*., 2004]. More recent research into the specific timeframe for the development of the immune system of broilers indicates low peripheral blood cytokine levels and intestinal mucosa cytokine expression within d 6 to 13 and that the peripheral blood cellular immune system reaches maximum functionality between d 30 to 34 (Song *et al*, 2021).

Diet is one of the key external factors influencing the development of immune function in broilers. A study by Hrncir *et al*., 2008 showed that the diet can probably stimulate the immune system by stimulating the gut mucosa, due to the various microbial content of the diet.

Therefore, the diet can manipulate the concentration of produced cytokines, for example, the LPS-rich diet increases the manufacture of interleukin-12 and supress the manufacturing of interleukin-4 (Hrncir *et al.*, 2008).

1.9.1 Immune biomarkers

Immune function is a highly complex interaction of multiple body systems occurring at both localised and systemic levels. Despite this complexity, there is a need for some relatively simple, quantitative measure of immune modulation. Immune biomarkers are a set of parameters that have been broadly agreed as offering some representation of immune response. In particular, research that aims at investigating the effects of feed additives or vaccines on the immunological response of animals rely on immune biomarkers to determine the positive and negative effects of these additives. Commonly, immune biomarkers are simply cytokines circulating in the blood to regulate the bird immune response. The effect of cytokines is profound and is almost a reflection of body status including majority of body organs. Cytokines are categorised into tumour necrosis factors (TNF), interleukins (IL) and interferons (IFN). These cytokines are responsible for either proinflammatory or anti-inflammatory responses and their abundance or scarcity could indicate whether the body condition is normal or going through stress or even can be indication of pathological disorders (Llibre and Duffy, 2018). In particular for poultry important immune biomarkers are IL-6, IgA, and IgY.

IL-6 is a soluble moderator that has an effect on more than one phenotypic trait. IL-6 is instantly produced in response to body infection or stress, being subjected to transcriptional mechanism, IL-6 over-expression may indicate or could cause malignant chronic inflammation as extensive expression of IL-6 could lead to amyloid A amyloidosis (Gillmore *et al*. 2001). Which in turn leads to amyloid fibril deposition that results in retrogression of different body organs (Tanaka *et al*., 2024). Therefore, high expression or concentration of IL-6 is indicator of either living

organism is subjected to high stress level or negatively affected by a certain additive, drug, or diet etc.

IgA is a very important biomarker that is responsible for mucosal immunity in which it is associated with physical stress or health and in some instances can be related to the animal psychological welfare. Long term stress can cause IgA concentration to drop and even to shut off, while animal prosperity results in IgA becoming fixed at high concentrations (Staley et al., 2018).

1.10 Insect Immunity and defence against pathogens

Insects have persisted over millions of years, they also showed great variation as well as their presence in every environmental niche (air, water, land, swamps etc., ….). That was the result of enormous resistance towards infectious pathogens from protozoans to more advanced nematodes (Kaya, 2002), thanks to a powerful immune system that insects possess. Therefore, it can be concluded that, innate immunity of insects acts as a powerful defence system against pathogenic organisms. Insect immunity includes two types of response, operated by haemocytes, those two responses are cellular, and humoral response as described below.

A- Cellular response

which includes several types of methods as: Encapsulation, Phagocytosis and nodulation (Figure 1.9).

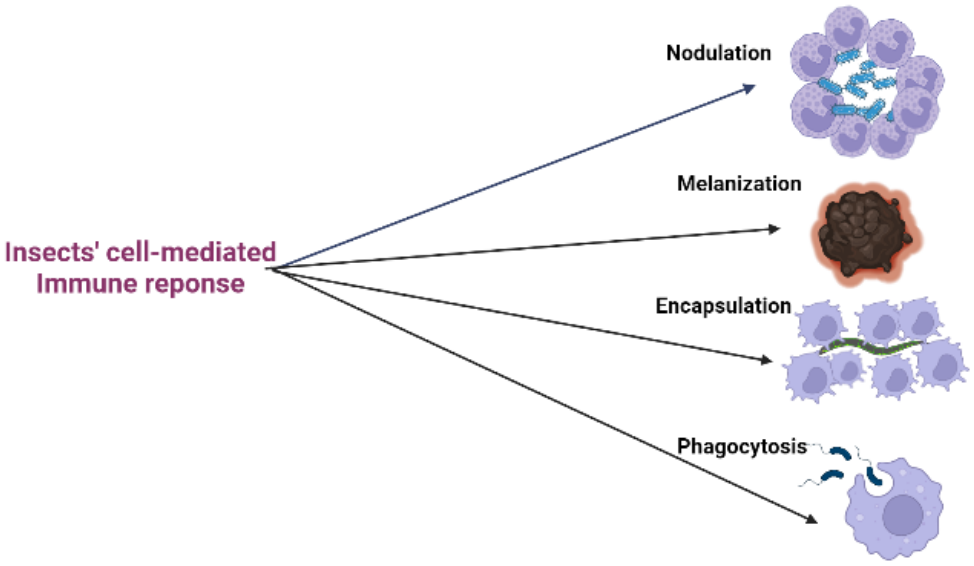

Figure 1.9- Types of cell-mediated Immune response in insects, Figure created in BioRender.com.

Phagocytosis

Phagocytosis can be simply described as cell eating. In this process, the pathogenic cell is engulfed by the host immune cell, and the lysosome is included to digest the pathogen cell. In insects, phagocytosis is reported to occur in various ways, for example, formation of pseudopodia as reported in *Calliphora* sp. In this process, blood cells formed protrusions and surround part of the haemolymph, assuming the engulfed vacuole in the micrograph contained a bacterial cell (Crossley,1964). Another mechanism is by forming pinocytotic vesicles, as shown by Leutenegger (1967) in his micrograph of *Galleria* sp. blood cells, forming vesicles to engulf *Sericesthis iridescent* virus particles (Figure1.10).

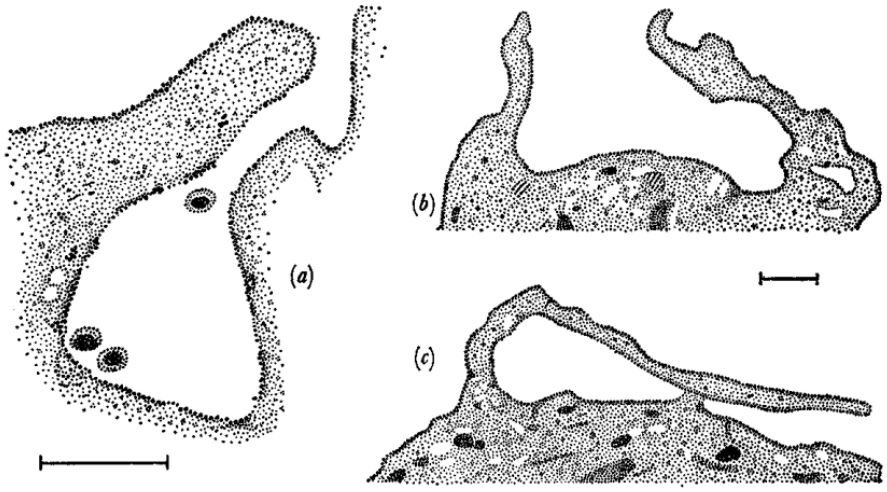

Figure 1.10- Micrograph showing three different types of phagocytosis by Insects' haemocytes (a) pinocytotic vesicle engulfing *S. iridescent* virus particles, (b, c) Pseudopodia formed by insects' blood cells (Leutenegger, 1967).

Encapsulation

Sometime phagocytosis fails with large pathogen particles. For example, eggs of parasitoid wasps which are inserted into lepidopteran larvae, due to large size of the parasitoid egg compared to the size of host immune cell. This is when encapsulation becomes more viable. The encapsulation mechanism in insects is a harmonical interaction between insects' haemocytes where, their interaction with one another forms a capsule of overlying cells around the invading pathogen (Hillyer, 2016). Whether there are specific types of haemocytes responsible for encapsulation process in insects, remains unknown, according to previous literature, in lepidopterans, specifically *Galleria mellonella*, suggests that granulocytes degranulation elicits the binding of plasmatocytes to initiate encapsulation (Schmit and Ratcliffe, 1977). However, in *Melolontha melolontha*, a type of beetles of order Coleoptera, encapsulation takes place directly through plasmatocytes without any stimulation from any

other type of haemocytes (Brehélin and Boemare, 1988). Nevertheless, plasmatocytes remain the common type of haemocytes in insects responsible for encapsulation.

Nodulation

Nodulation is simply the aggregation of immune cells, mainly granulocytes, towards a group of invading pathogenic cells, forming a cluster by which plasmatocytes surround and melanise. This type of immune-response is among the common cell-mediated responses in insects; however, it seems that nodulation depends on stimulation by nodular proteins and eicosanoid fatty acids (Gandhe *et al*., 2007; Kim *et al*., 2018).

Melanisation

Melanisation as an immune response, derived by the infection of bacteria, and its strength is controlled through tyrosine enzyme. The whole process involves a series of chemical reactions triggered by tyrosine and lead through by phenol oxidases. The end product is formed by the interaction of indole-5, 6-quinone with hemolymph proteins, forming a melanin layer around the invading pathogen. This layer is in the form of a dark spot that isolates the pathogen from the surrounding hemolymph preventing the pathogen from obtaining essential nutrients needed for survival. An example of the melanisation process in insects is the PRO- PO of *Armigeres subalbatus* forming melanin around invasive filarial worms (pathogenic nematodes) (Tsao *et al*., 2015).

B- Humoral response

Humoral response is mediated by the insect's fat cells and haemocytes. The three most recognised pathways for immunity in insects are the IMD, Toll and Jak/Stat pathways. Immunity response is initiated upon pathogen infection by the pattern recognition receptors

(PRRs) which are bound with pathogen associated molecular patterns (PAMPs) found only in invading pathogens and not in insects. Upon infection, PRRs through the Toll pathway, activates Spatzle, an extracellular cytokine which in turn, binds to the Toll receptor in the cell membrane and this initiates a series of reactions leading to the stimulation of transcription factor NF-KB to activate the antimicrobial peptides (AMPs) transcription along with the immune effector genes (Cao *et al*., 2015; Clayton *et al*., 2014) (Figure 1.11).

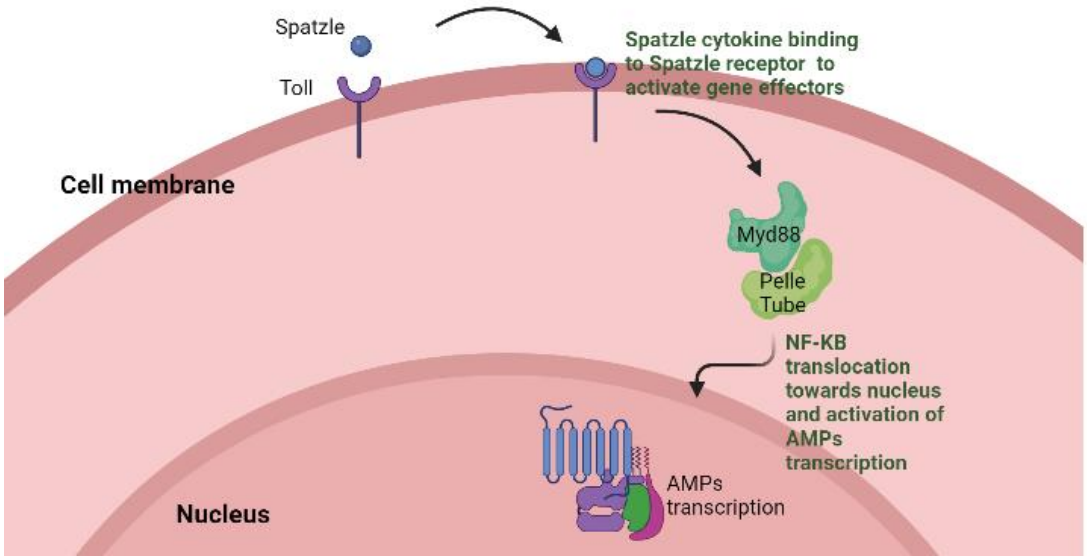

Figure 1.11- Showing Toll pathway and initiation of AMPs transcription upon recognition of infection by PRRs, Illustration created in BioRender.com

1.10.1 Insects' antimicrobial peptides

Insects AMPs have been shown to supress an extensive range of Gram-negative bacteria such as, *Salmonella Entritidis*, *S. polurum*, and *Entobacter aeronegnes*, in addition to Gram positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis*, and *Bacillus subtilis* (Kieronczyk *et al.*, 2022). AMPs are released as a humeral immune response to infection. AMPs are diverse and with varied structure, where any change in this unit structure

results in a completely different type of AMP with respect to biology and function. This diversity results from adaption to changing environments or changes in bacterial communities, a term known as “Adaptive immunity” (Zasloff, 2002).

AMPs’ mechanism of action

The release of AMPs is mainly triggered by infection. AMPs target bacterial cell permeability by the electrostatic reaction between cationic peptides and negative charges of lipids in the lipid bilayer on the surface of bacterial cells, leading to disturbance of cell permeability through pores formation and eventually disruption and leakage of the bacterial cell (Zasloff, 2002). AMPs do not attack normal animal or plant cells, probably because the negative charges of lipid bilayer in the normal cell membranes are situated in the inner layer facing the inner cell cytoplasm, unlike the negatively charged lipids in the outer cell layer. Another possibly is that the presence of cholesterol particles within the phospholipid bilayer of animal or plant cells may decrease the interaction with the AMPs (Matsuzaki, 1999).

The review shows how dominant soyabean is as a feed material for poultry, but the review also highlights the negative environmental issues associated with use of soy. These include the high carbon cost associated with land use change and transportation from soya production areas such as USA, Brazil, and Argentina to Europe and also the biodiversity loss associated with some land use change in the Amazonian regions of South America. Insects represent one alternative to soya as protein source that could be used to increase the environmental sustainability of poultry production. However, production on a large scale is hampered by many factors such as the precise environmental conditions required for larvae growth, lack of knowledge on which insects are most appropriate for farming, and which substrates are most suitable in terms of waste valorisation, larvae growth and resulting nutritional profile.

The aims of this PhD thesis are as follows:

1. Determine the effect of low economic value {waste} rearing substrates on yellow meal worm development and nutritional profile.
2. Determine the effect of rearing substrate on digestible amino acid and metabolizable energy of yellow meal and black soldier fly for meat poultry.
3. Investigate the effect of differing dietary inclusion levels of yellow meal worm on broiler performance, health, immune status and nutrient utilisation.
4. Investigate the effect of differing dietary inclusion levels of black soldier fly on broiler performance, health, immune status and nutrient utilisation.

Chapter 2: Materials and Methods

2.1 Introduction

This chapter describes the general materials and methods used in this thesis and how they correspond to subsequent thesis chapters. A total of 6 studies comprising 4 bird trials and 2 insect trials were conducted as summarised below in (table 2.1) at the Poultry Research Unit (Nottingham Trent University, Brackenhurst). Trial 1 and Trial 2 were insect feeding trials conducted to study the effect of different feeding substrates on overall performance and the nutritional profile of mealworms (*Tenebrio molitor*). Trial 3 and Trial 4 were bird trials and were conducted to investigate the effects of Black soldierfly larvae (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) on the amino acid digestibility of broiler chickens. Trial 5 and Trial 6 were dose response trials conducted to investigate the effects of incremental inclusion of Black soldierfly larvae (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) (0.3%, 1%, and 5% inclusion) on performance and gut health parameters of broiler chickens.

Table 2.1- Description of individual trials in the study

Study	Areas investigated	Chapter
Trial1 (T1) Insect trial	Effect of two substrates (DDGS and banana peels) on mealworm performance, weight, survival rate and nutritional profile.	3
Trial2 (T2) Insect trial	Effect of three unconventional substrates (food waste, sea waste, and sausage waste) on mealworm performance, weight, survival rate and nutritional profile.	3
Trial3 (T3) Bird trial	Effect of BSFL meal inclusions (20%, 40%, and 60%) on amino acid digestibility of broilers	4

Trial4 (T4) Bird trial	Effect of mealworm meal inclusions (20%, 40%, and 60%) on amino acid digestibility of broilers	4
Trial5 (T5) Bird trial	Effect of mealworm meal inclusion (0.3%, 1%, and 5%) on performance in meat poultry	5
Trial6 (T6) Bird trial	Effect of BSFL meal inclusion (0.3%, 1%, and 5%) on performance in meat poultry	6

Institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986) were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group and logged as Trial1: ARE192045, Trial2: ARE202148, Trial3: ARE212248 Trial4: ARE1743013, Trial5: ARE212203, and Trial6: ARE1628825. All bird trials used Ross 308, male broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxfordshire and transported to the trial locations by NTU poultry research staff.

Bird trials

2.1.1. Birds and husbandry

All animal trials used Ross 308, male broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxon. A total of 144 birds were used for each T3, and T4 trials and a total of 320 were used for each T5, and T6 trials. The chicks were grouped in preheated 0.64m2 pens in a purpose built, insulated poultry house. Any unusually sized birds were not allocated to trial pens. The birds were bedded on clean wood shavings (approximately 3 cm thick) and fresh shavings were added into the pens as required. Feed and water were always

available ad libitum. Dates and weights of dead birds and reasons if culled were recorded. When it was time to start the treatment diets, Birds were always allowed *ad libitum* access to the treatment diets and water for the duration of the trial. Commercial guidelines for the care and husbandry of Ross 308 broilers were followed in all studies (Aviagen, 2018). The room was thermostatically controlled to produce an initial temperature of 32°C reduced to 21°C by day 21 using heating fans. The lighting regime used was 24 hours light on d1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached, and this was maintained throughout the remainder of the study. Birds were checked twice daily to monitor the environmental conditions; heating and ventilation were adjusted accordingly. Any mortalities were recorded along with the date and weight of the bird and reason if culled. All birds sampled were euthanised by cervical dislocation as determined by DEFRA (DEFRA, 2015).

2.1.2 Diet formulation

Birds were fed commercially available chick starter crumb from GLW feeds, Shepshed, for the amino acid digestibility trials (T3, and T4), prior to the trial diets. For T4, and T6 trials, starter, grower and finisher diets were prepared onsite. The composition and analysis of all the trial diets are detailed in the appropriate chapter. Diets were mixed onsite. The ingredients were individually weighed and mixed dry for 5 minutes in a ribbon mixer (Rigal Bennett, Goole, UK) before oil addition. The diets were then mixed for a further 5 minutes with an intermediary brush down within the mixer to remove oil clumps. Titanium dioxide was carefully incorporated into the diets as an inert marker by mixing with other minor ingredients prior to inclusion in the dry mix to ensure homogeneity. Titanium dioxide content (5g/kg) was selected to ensure the digesta samples would contain sufficient titanium for laboratory measurement. Diets for all studies were allocated randomly to pens within the study room to reduce any effect of room position. Bags were topped up with feed as required and added feed weights recorded.

Diet was manufactured on site and fed as mash for both trials. The particle size of each diet was uniform, consistent and typical for broiler diets, averaging at approximately 1 mm. The composition and analysis of the trial diet are detailed in the corresponding chapters. When manufacturing the diets, each ingredient was individually weighed out and mixed dry for 5 min in a ribbon mixer (Rigal Bennett, Goole, UK) before addition of oil. The diet was then mixed for further five minutes. The mixer was brushed down at various stages throughout the mixing process to ensure oil clumps were removed. Diets were randomly allocated to pens within the room by block, to eliminate any effect of room position. A grab sample was taken during the feed weighing prior to the trial to allow for proximate analysis at a later date. Diets were weighed into bags (new individual bags for each feeding phase; starter, grower and finisher) for each pen to allow feed intake to be measured. Bags were topped up with feed as required and added feed weights recorded.

2.1.3. Feeding procedure and feed intake

Weighed feed from the correct bag for each pen was added to the feeders when required to ensure fresh feed is available *ad libitum* at all times. Feed troughs were positioned horizontally to minimise spillage. On sampling days remaining feed in the trough and bag, and any spilt feed if able to be collected, were weighed. although the depth of food in the lips of each feeder should be kept low to avoid wastage. Feed intake was measured per pen on day 0, 17, and 20 for T3, and T4 and on day 0, 10, 21, 28 and 35 For T5 and T6. Feed intake was measured as total intake per pen then the average amount consumed per bird calculated for each time period. Birds were fed on an *ad libitum* basis; with the amount consumed over the test diet feeding phase recorded for performance trials T5, and T6. A set recorded volume of feed was weighed into individual bags (one per pen) at the start of the test diet feeding phase.

2.1.4. Bird weights

Chicks were weighed on arrival, and any outside the selected weight range were not included in the trial. Birds were distributed into pens based on average weight per pen, ensuring there were no significant differences in starting pen weight between dietary treatments. Birds were weighed on day 0, 17, and 20 for T3, and T4 and birds were weighed on day 0, 10, 21, 28 and 35 for T5 and T6. Bird weights were measured by weighing the whole pen, and then calculating the average bird weight, unless stated in the specific trial methodology. The increase in average bird weight was used (Bodyweight gain), alongside the average feed intake value, to calculate the average feed conversion ratio (FCR) per pen.

2.1.5. Sampling

For trial T3, and T4, on day 17, all birds were pulled for measurements of weight and feed intake, on day 20, all birds per were pulled to measure weight and feed intake and then were culled to for digesta for digestibility measurements. In trial T5, two birds per pen were sampled on day 21 and 35. In trial T6, three birds per pen were sampled on day 21 and 35 (Table 2.2 and Table 2.3). Birds were euthanized in a separate room via cervical dislocation by trained NTU staff. On each sampling occasion, blood, digesta and Jejunum tissue were collected as described in sections below. Birds will be euthanized by cervical dislocation by a trained operative.

Table 2.2- Postmortem tissue sampling for trial5

Sample	Requirements	Number of birds/pens
Serum		2 birds per pen D21 and D35
Ileal digesta	N and GE, Ti	2 birds D21 pooled per pen, and 2 birds pooled D35
Litter samples	DM, N	Per pen, D35
FPD score	Visual score	All birds at weighing D35
Ceca	Store for microbiome	5 reps per treatment, D21, D35 Pen 1-25
Gizzard+ Gut lesion score	Visual score	2 birds per pen D21, D35
Carcass yield	Breast, thigh, drum	1 bird per pen D35
Histology	Fix Jejunum 5cm	1 bird per pen D21

Table 2.3- Postmortem tissue sampling for trial6

Sample	Requirements	Number of birds/pens
Plasma		2 birds per pen D21 and D35
Ileal digesta	N and GE, Ti	3 birds D21 pooled per pen, and 3 birds pooled D35
Litter samples	DM, N	Per pen, D35
FPD score	Visual score	All birds at weighing D35
Ceca	Store for microbiome	5 reps per treatment, D21, D35
Gizzard+Gut lesion score	Visual score	2 birds per pen D21, D35
Carcass yield	Breast, thigh, drum	1 bird per pen D35
Histology	Fix Jejunum 5cm	1 bird per pen D21

2.1.5.1 Blood plasma collection

For performance trials (T5, and T6), Postmortem blood samples were collected immediately post euthanasia into EDTA coated tubes. Samples were centrifuged at 3000 rpm for 5 min to separate the plasma which was collected in sterile Eppendorf tubes and stored at -20^{o}C.

2.1.6. Analytical procedures for feed analysis

2.1.6.1 Dry matter determination

Dry matter content of the diet for performance trials (T5, and T6), and raw materials for trials T3, and T4, were analysed by accurately weighing approximately 5-10 g of finely ground sample into pre-weighed crucibles. The crucibles were then dried in a drying oven set at 105°C for approximately 4 days, until a constant weight was reached. The dried samples were cooled in a desiccator and reweighed.

2.1.6.2 Ash determination

Ash content of the diet for performance trials (T5, and T6), and raw materials for trials (T3, and T4), were analyzed by accurately weighing approximately 2-5 g of sample into a pre-weighed ceramic crucible. The crucibles were then placed in a muffle furnace (Nabertherm, B180) for on a program that brought them from room temperature up to 650°C over a two-hour period, then maintained them for 14 hours at 650°C, before automatically shutting off and allowing them to cool back to room temperature. The ashed samples were then cooled in a desiccator and reweighed. Ash percentage was calculated by the following formula:

$$(\boldsymbol{Fresh_{wt}} \div \boldsymbol{Ash_{wt}}) \times \mathbf{100}$$

2.1.6.3 Crude protein determination

Protein content of each diet was analyzed using the Dumatherm Nitrogen Analyzer (Gerhardt, UK). The instrument works according to the principle of Dumas method which is a quick combustion of liquid or solid samples in pure oxygen atmosphere, followed by analyzing the resulting gases. The measurement of the thermal conductivity with a TCD detector gives a signal which corresponds to the amount of nitrogen in the combusted sample. Results are

expressed as mg of nitrogen, then converted into percentage of protein present in the sample. Protein content was calculated by nitrogen content × 6.25 (standard multiplier).

$$\%\ \boldsymbol{crude\ protein} = \mathbf{6.25}\ \times\ \%\boldsymbol{Nitrogen}$$

To run the analysis, 0.5g of the sample was weighed in a tin foil. The tin foil was then placed in the shaping tool provided with the instrument. The tin foil along with the sample was compressed in the form of an air-tight tablet by pressing and turning the closing cap clockwise. the tablets made were placed in the sample tray which is then inserted in the sample loader in the Dumatherm.

2.1.6.4 Extractable fat determination

Fat content of the diet for performance trials (T5, and T6), and raw materials for trials (T3, and T4), were analysed for extractable fat content by The Soxtherm fat extraction system (Gerhardt, UK) which is based on the same principles as the conventional Soxhlet fat extraction. Clean dry extraction flasks with boiling stones were accurately weighed at the start of the fat extraction process. 5 g of dried diet was accurately weighed and inserted into extraction thimbles which were then placed in fat extraction beakers. The fat extraction process took a total of 2 hours and constituted of the following programmable steps:

a. Hot extraction phase: 170ml petroleum ether (CAS 64742-49-0; Fisher Scientific, UK) was poured into the extraction flask containing dried samples and brought to boil at 150oC for 30 minutes. Fat was liberated from the sample during this process.

b. Evaporating phase A: the level of the solvent was lowered below the extraction thimble. Excess solvent was collected in the rear solvent recovery tank.

c. Extraction phase: petroleum ether was refluxed to further extract fat from sample for 1h.

d. Evaporating phase B: the remaining solvent was distilled and collected in the rear solvent recovery tank.

e. Evaporating phase C: a further recovery of the remaining solvent which was distilled and collected in the rear solvent recovery tank. The extraction flasks with remaining petroleum ether and boiling stones were placed on a hot plate to evaporate off the solvent.

Flasks were then placed in an oven for 2h set at 105°C until 70 constant weight was reached. Flasks including contents (fat and boiling stones) were weighed after cooling down in a desiccator. Fat was determined using the following formula:

$$\% \, \boldsymbol{extractable \, fat} = [(\boldsymbol{M2 - M1 \div M0}] \times \mathbf{100}$$

Where:

M0 = sample weight (g)

M1 = weight of flask + boiling stones (g)

M3 = weight of flask= fat + boiling stones (g)

2.1.6.5 Gross Energy Analysis

Gross energy of the feed was measured using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA) (Rutherfurd, Chung and Moughan 2007 Woyengo, Kiarie and Nyachoti, 2010). Pellets of feed sample, weighing approximately 1 g, were made by adding a small amount of water to the sample before pelleting it with a pellet press (Parr Instruments, USA). The pellets were dried overnight in a drying oven at 105°C, before being weighed into tin crucibles (Sartorius CP1245) and placed in the bomb. The bucket in the bomb jacket was filled with 2 l of water. 10 cm of fuse wire was threaded through the hole, ensuring the wire touched the pellet. The bomb was then assembled, ensuring the top was tightly screwed on,

and then filled with oxygen. Once filled, the bomb was put into the bucket of water, the electrodes were pushed into the bomb, and the lid of the bomb jacket was shut. Sample weight was entered, and the process was started; the calorimeter measures the energy produced (in MJ/kg) when the pellet is exploded.

Sample weight was entered, and the process was started; the 73 calorimeter measures the energy produced (in MJ/kg) when the pellet is exploded. Apparent metabolizable energy (AME) was calculated using the following formula:

$$\boldsymbol{GEdiet - (GEexcreta \times (TiO2\ in\ diet \div TiO2\ in\ excreta))}$$

Where:

$GEdiet$=Gross energy in diet

$GEexcreta$ =Gross energy in excreta

$TiO2$ = Titanium dioxide results

Then, apparent metabolizable energy corrected for nitrogen and nitrogen retention using the formula**:** $\boldsymbol{AME - ((34.4 \times Nret.) \div 1000)}$

Where:

AME =Apparent metabolizable energy

34.4=correction factor

$Nret.$ = Nitrogen retention/g diet

2.1.7. Procedures for analysis of tissue samples

2.1.7.1 Histology

Tissue sampling and fixing for performance trials (T5, and T6)

Jejunum sections

Jejunum cross sections were excised from the distal 5cm part of the jejunum preceding Meckel's diverticulum. All the cross sections were washed with distilled water and fixed in Bouin's fixative (Fisher Scientific, UK) for 6 hours then stored in 70% ethanol before further processing.

Tissue wax embedding.

Samples were taken out of the 70% Industrial Methylated Spirit (IMS) pots with tweezers and a 5mm piece was excised with a scalpel carefully. Damaged areas were removed and the 5mm sections placed into disposable labelled histology cassettes (Fisher Scientific, UK) with identification numbers in pencil. Care was taken that tissue samples did not dry out. For this purpose, all the prepared cassettes were placed in 70% IMS in an air-tight container, as soon as possible. Samples were then moved to NTU Clifton campus histology laboratory where the prepared cassettes with tissue samples were placed in a tissue processor (Leica ASP300S, Leica Microsystems, Milton Keynes, UK), the processor takes the tissue through a series of alcohols to dehydrate then xylene to prepare the samples for impregnation with wax. Once removed from the processor, the samples were embedded in paraffin wax using an embedding machine (Leica EG1150 Modular Tissue Embedding Center, Leica Microsystems, Milton Keynes, UK). The samples were placed on a cold plate to set and stored in the fridge until sectioning and mounting. All the steps followed are given below (Table 2.4).

Table 2.4- Embedding steps of the tissue samples

Chemical	Duration
70% IMS	overnight
70% IMS	1 hour
90% IMS	1 hour
90% IMS	1 hour
100% IMS	1 hour
100% IMS	1 hour
Histoclear	1 hour
Histoclear	1 hour
Paraffin wax 60oC	1 hour
Paraffin wax 60oC	1 hour

IMS: Industrial methylated spirit.

Tissue sectioning and mounting

The waxed tissue sample blocks were trimmed from the sides with a single-edge razor blade. The blocks were cut using a rotary manual microtome (Leitz 1512, Germany) to make a 10-micron thick ribbon of tissue sections. The cut tissue sections were then placed to float on warm water at 40°C to flatten. Then they were then lifted from the water onto the slides. Once on the slides, the samples were placed on a hot plate (Cole-Parmer™ Stuart™ Hot Plate with Stirrer, US152) at 40°C until they dried and then stored in the slide boxes. Four sections of the same sample were added to each slide.

Tissue slides staining.

Once all the sections were mounted onto slides, the following staining procedure was followed using the standard operating procedure (SOP) of the Poultry Research Unit (PRU) of Nottingham Trent University (NTU) (Table 2.5).

Table 2.5- Staining steps of the tissue samples

Chemical	Duration
Xylene	5 minutes
Histoclear	1 minutes
100% IMS	2 minutes
95% IMS	1 minutes
70 % IMS	1 minutes
Running water until runs clear	Until runs clear
Haematoxylin	2 minutes
Running water	until runs clear
1% scott's tap water	1 time for 2-4 seconds
Running water	until runs clear
Observe slide under microscope if nuclei	----------
a. If too blue	A quick dip in acid alcohol then tap water rinse
b. Not blue enough	Either return to scotts or haematoxylin
Eosin	2 minutes
Running water	Quick rinse
70% IMS	Dip
95% IMS	1 minute
100% IMS	2 minute
Histoclear	2 minutes
Xylene	2 minutes
DPX mountant using a small plastic pipette	2-4 drops in fume-hood
Coverslips	In fume-hood
Drying DPX mountant	Few minutes in fume-hood, then on a working bench covered with a protective sheet

Tissue microscopy

Slides were analysed using an Olympus BX51 microscope fitted with an Olympus DP71 camera (Olympus, Pennsylvania, USA). Olympus Cell F software was used to identify and

measure villus height, villus width and crypt depth. For each of the 15 sections cut from each sample, two villi were measured for height and width and two crypts were measured for depth. Where possible the measurements were taken from opposite quarters and, if that was not possible, from different halves. Villi width was measured from either edge of the epithelial cells halfway down the length of the villi, and height was measured from the highest epithelial cells across the top of the villi, down the centre, to the point where the crypt began. Crypt depth was measured from the centre of the opening down to the centre of the innermost epithelial cells (Figure 2.1).

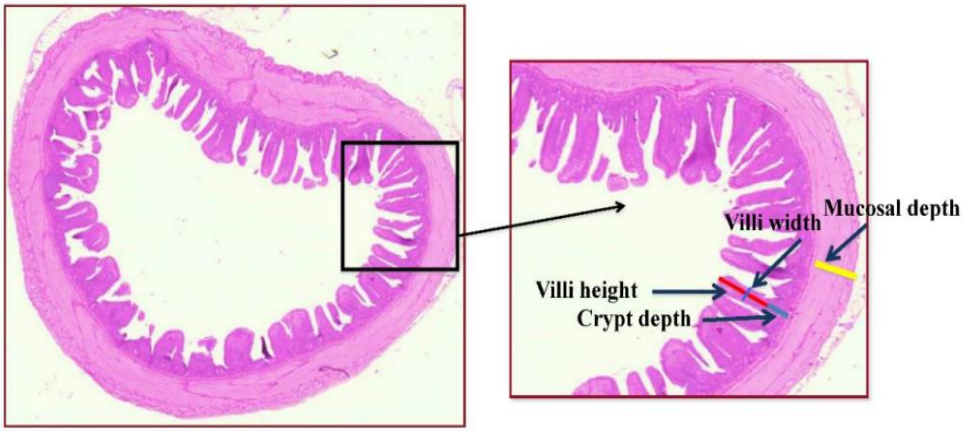

Figure 2.1- Histological parameters of jejunum or ileum sections (Kulshreshtha et al., 2020)

From each sample, villi and crypt were measured (eight villi and eight crypts per pen). An average of the eight measurements was calculated and used for statistical analysis.

2.1.7.2 Immunoglobulin A and Interleukin-6 analysis

Reagent Preparation and Storage

20 minutes before use, all samples and reagents were brought to room temperature.

1- Wash Buffer: crystals have been formed in the concentrate, then warmed with 40°C water bath (Heating temperature should not exceed 50°C) and mixed gently until the crystals have

completely been dissolved. The solution was then cooled to room temperature before use. 30ml of the solution was diluted into 750ml concentrated wash buffer with distilled water.

2-Standards: 1 ml Sample Dilution Buffer is added into one Standard tube (labelled as zero tube), then the tube kept at room temperature for 10 minutes then mixed thoroughly.

3- Preparation of Biotin-labelled Antibody Working Solution: it is prepared within 1 hour before experiment. Required total volume of the working solution is calculated: 0.1ml/well × quantity of wells. The Biotin-detection antibody is diluted with Antibody Dilution Buffer at 1:100 and mixed thoroughly. (i.e. Add 1ul Biotin-labelled antibody into 99ul Antibody Dilution Buffer.). Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution which should be prepared within 30 minutes before experiment, by calculating required total volume of the working solution: 0.1ml/well × quantity of wells. (Allow 0.1-0.2ml more than the total volume.) then diluting the SABC with SABC Dilution Buffer at 1:100 and mixing them thoroughly. (i.e. Add 1ul of SABC into 99ul of SABC Dilution Buffer.)

Assay Procedure

When diluting samples and reagents, they must be mixed completely and evenly. Before adding TMB into wells, equilibrate TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test. 1. Set standard, test samples (diluted at least 1/2 with Sample Dilution Buffer), control (blank) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate. Wash plate 2 times before adding standard, sample and control (blank) wells! 2. Prepare Standards: Aliquot 100ul of zero tube, 1sttube, 2ndtube, 3rdtube, 4thtube, 5thtube, 6thtube and Sample Dilution Buffer (blank) into the standard wells.

Steps

Step1	Wash plate 2 times before adding Standard, Sample (diluted at least 1/2 with Sample Dilution Buffer) and Control (blank) wells!
Step2:	Add 100ul standard or sample to each well and incubate for 90 minutes at 37°C.
Wash step:	Aspirate and wash plates 2 times.
Step3:	Add 100ul Biotin-labeled antibody working solution to each well and incubate for 60 minutes at 37°C
Wash step:	Aspirate and wash plates 3 times.
Step4:	Add 100ul SABC Working Solution into each well and incubate for 30 minutes at 37°C.
Wash step:	Aspirate and wash plates 5 times.
Step5:	Add 90ul TMB Substrate Solution. Incubate 10-20 minutes at 37°C.
Step6:	Add 50ul Stop Solution. Read at 450nm immediately and calculation.

2.1.8. Data analysis

All the collected data were analysed using the statistical software package SPSS v.28 (IBM SPSS statistics, 2021). After KS testing to confirm normality, statistical analysis was carried out by a one-way ANOVA to determine the equality of treatment means, and univariate analysis as appropriate Statistical difference was declared significant at P-value ≤0. 05. Duncan post hoc tests were used where appropriate to elucidate differences between experimental groups.

2.1.8.1 Digestibility related measures

Digesta samples were frozen immediately upon collection and then freeze dried to a constant weight in a Lyotrap freeze drier (LTE Scientific, Oldham, UK). Samples were then finely ground. using a pestle and mortar and stored in sealed pots until analysis. Digesta samples were analysed for titanium dioxide and amino acid content for the amino acid trials NA04, and NA07.

Excreta samples were collected as previously described and freeze dried 7 days. Samples were then ground to pass through a 1mm sieve using a coffee grinder (Whittards, Chelsea, UK) and stored in sealed pots until analysis. Excreta samples were analysed for dry matter, titanium dioxide and gross energy content to calculate the apparent metabolizable energy.

Titanium Dioxide Determination

Titanium dioxide was incorporated into all diets as an inert marker at an inclusion rate of 5g/kg. Titanium dioxide (TiO2) was quantified in diets, excreta and digesta by the method of Short *et al.,* (1996). Samples of 0.3-0.4g of digesta were accurately weighed into ceramic crucibles and ashed at 650° C for 13 hours and allowed to cool. The crucibles were placed on a hotplate and 15ml of 7.4M sulphuric acid was pipetted into each (Figure 2.2). The crucibles were then heated to simmering until the sample was completely dissolved (approximately 2-3 hours). Crucibles were allowed to cool before the contents were qualitatively transferred using distilled water into 100ml volumetric flasks, via a Whatman 541, hardened, ashless filter paper (Fisher Scientific). 10ml of hydrogen peroxide (30 volumes) was added to each flask, before making to volume with distilled water. Flasks were stoppered and mixed and absorbance measured on a UV spectrophotometer (Cecil CE3410, Cecil instruments, Cambridge, UK) at a wavelength of 410nm. A series of standards were prepared from 5ug/ml titanium dioxide and these were used to produce a standard curve. All samples and standards were read against a zero standard as a blank. The amount of titanium in the sample was calculated using the following equation:

$$\boldsymbol{mg\ Ti \div mg\ sample = \left((absorbance \times 100) \div ceofficient\right) \times mg\ Sample\ mass}$$

where the coefficient is obtained from the regression analysis of the standard curve.

Figure 2.2- Crucibles placed on a hotplate and 15ml of 7.4M sulphuric acid was pipetted into each.

2.1.8.2 Amino acid analysis

Amino acid analysis was carried out at 30 ◦C using a Thermo Scientific™ Acclaim™ Trinity P1 mixed mode column (150 mm × 2.1 mm, 3 μM) on a Thermo-Fisher Vanquish (uHPLC) coupled to an Altis Triple Quadrupole Mass spectrometer (MS/MS) with heated electrospray ionization (H-ESI) system. The source conditions were as follows: spray voltage 3500 V, spray current 63.4 μA, ion transfer tube temperature 325 ◦C, vaporizer temperature 370 ◦C, sheath gas 5.58 L/min, auxiliary gas 7.97 L/min, ion transfer tube DC 15 V, RF Lens Amplitude 47 V. Nitrogen gas was produced using a nitrogen generator (Genius NM32LA, Peak Scientific Instruments Ltd). For each amino acid transition of interest, the collision energies (CE) were optimised, and one transition was used as the quantifier. Quantification for all targeted amino acids was achieved using the concentration vs peak area ratio (the integrated peak area of the analyte relative to that of the internal standard). Data acquisition was performed using Thermo

Xcalibur™ mass spectrometry data system and data was processed using Thermo Tracefinder™ 4.1 application. Due to the loss of asparagine and glutamine in the acid hydrolysis process, 17 amino acids could be analysed, including both indispensable amino acids (IAA): Cystine (Cys), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr) and Valine (Val) and dispensable amino acids: Alanine (Ala), Arginine (Arg), Aspartic acid (Asp), Glutamic acid (Glu), Glycine (Gly), Proline (Pro) and Serine (Ser). Recovery of amino acids was calculated using the soy SRM yielding a total amino acid recovery of 93%. All the data was normalized in relation to the expected recovery of the SRM and gross amino acid compositions of the substrates were expressed as g/kg (Muleya *et al*., 2023).

Sample preparation.

Samples were weighed into 100ml glass bottles, 500mg for raw materials and 1g for digesta. The bottles were cooled to 4° C prior to oxidation. Oxidation solution was prepared fresh by mixing 1ml hydrogen peroxide (30 volumes) with 9ml formic acid (73.5%) with 0.05M phenol. The oxidation solution was then incubated at 25° C for an hour and then cooled to 4° C for an hour prior to use. 10ml of cooled oxidation solution was added to each sample bottle to oxidise the cysteine and methionine to cysteic acid and methionine sulphate respectively, in order to prevent their destruction during hydrolysis. Bottles containing the samples and oxidation solution were incubated for 16 to 18 hours at 4° C. Excess oxidation reagent was decomposed by addition of 0.84g of sodium metabisulphate to each sample bottle. 50ml of hydrolysis reagent (6N hydrochloric acid with 0.01M phenol) was then added to each bottle and the samples incubated at 110° C for 24 hours. Bottles were cooled in the freezer before quantitatively transferring the contents into wide neck conical flasks, rinsing with tri-sodium citrate buffer (pH 2.2). The flasks were then partly neutralised by addition of 35ml of 7.5N sodium hydroxide, with the flasks kept on ice to reduce overheating. The pH of each flask was

adjusted to 2.2 using 7.5N sodium hydroxide, 1N sodium hydroxide and hydrolysis reagent as required. Flask contents were transferred to a series of 200ml volumetric flasks, containing 4ml of 10µmol/ml norleucine as an internal standard. The volumetric flasks were then made to volume with tri-sodium citrate buffer (pH 2.2), stoppered and mixed. 10ml from each flask was centrifuged at 3000rpm for 5 minutes and the supernatant filtered through a 0.2µm filter into an analytical glass vial and stored at -80° C until analysis.

Calculation of amino acid content Standards were prepared containing 200 nmol/ml of each of 18 amino acids, 100 nmol/ml each of cysteic acid and methionine sulfone and 200 nmol/ml norleucine. These were used to prepare calibration graphs of peak area against amino acid concentration for each amino acid. After correction of peak areas by comparison with the internal standard, the calibration graphs were used to quantify the amino acid content of the samples, and this result was corrected for sample weight. Determination of coefficient of amino acid digestibility Using the titanium dioxide measurements the amino acid results for the chick digesta samples were used to calculate apparent amino acid digestibility using the following equation:

$$\mathbf{1} - (\boldsymbol{AA_{digesta}} \times \boldsymbol{Marker_{feed}}) \div (\boldsymbol{AA_{feed}} \times \boldsymbol{Marker_{digesta}})$$

Where:

$AA_{digesta}$ = amino acid concentration in the digesta

$Marker_{feed}$ = titanium dioxide concentration in the diet

AA_{feed} = amino acid concentration in the diet

$Marker_{digesta}$ = titanium dioxide concentration in the digesta

Apparent digestible amino acid content of insect larvae meal, and soya protein

The determined apparent digestible amino acid content of the diets was regressed against rate of inclusion of the protein source. The linear regression was then extrapolated to a rate of inclusion of 100% (or 1000g/kg) protein. This method gives a figure for apparent digestible content of the protein source for each amino acid measured. Dividing this figure by the total content of the specific amino acid in the protein gave a coefficient of apparent amino acid digestibility.

2.2.1 Insect trials (T1 and T2)

2.1.2.1 Mealworm (*T. molitor*)

Live mealworm for the two trials (T1, and T2) were brought from Peregrine Livefoods Ltd., UK. Mealworm used were medium sized (20mm length for T1) and aged around 7 weeks, (22 mm length for T2) and aged around 8 weeks.

2.1.2.2 Diet formulations of raising substrates.

For T1 trial, two types of substrates were used (Banana peel (BP), and Distillers grains with solubles (DDGS)). Raw banana peel was brought and blended using a Moulinex food processor to be added to the boxes (100 gm in each box) at the NTU Poultry unit. DDGS was bought from a local mill in Nottingham, UK, and about 700g dry weight were left to soak in 1L of water for 30 minutes and smashed manually with a spatula to reach a homogenous consistency. For T2 trial, three types of waste substrates were bought from bio-waste processing company Green Eco Technologies, Ltd., UK (Food waste (FW), and Sausage waste (SuW), and Sea waste (SEW)), only viable materials were selected to take forward into the T2 trial.

2.2.1.3 Mealworm rearing conditions, and trial design.

T1: was a single factor study comparing two substrates; T2: was a single factor study comparing three substrates. Both studies were completely randomised.

Before larvae placement

The insect vented boxes used in T1, and T2 studies were plastic rectangular vented 1L boxes measuring 19x13x7.5 cm (L x W x H), with a ventilation opening on the lid. The boxes were sourced from Bugzarre.co.uk.

For T1 trial, the insect vented boxes were prepared prior to insect placement by adding 32g of wood shavings each, to a depth of 4 cm as bedding and then approximately 50 g of fresh feeding substrate was placed over the bedding (Figure 2.3).

For T2 trial, approximately 95 g of feeding substrate was added to the corresponding insect box without addition of wood shavings because it was decided from the results of the T1 trial, that adding wood shavings was affecting the precision of calculations of substrate use (Figure 2.4).

All boxes used in the T1, and T2 trials were placed during the trials over Removable Under Tank Heat Pads (27.94 x 15.24 cm) sourced from amazon.co.uk to adjust average box temperature to 27℃, temperature was recorded using a digital thermometer, and small glass beakers with sponges immersed in water were placed between the boxes to raise the humidity to ±37% RH (Figure 2.3, and 2.4). Insect boxes were labelled with treatment, diet number and type of insect.

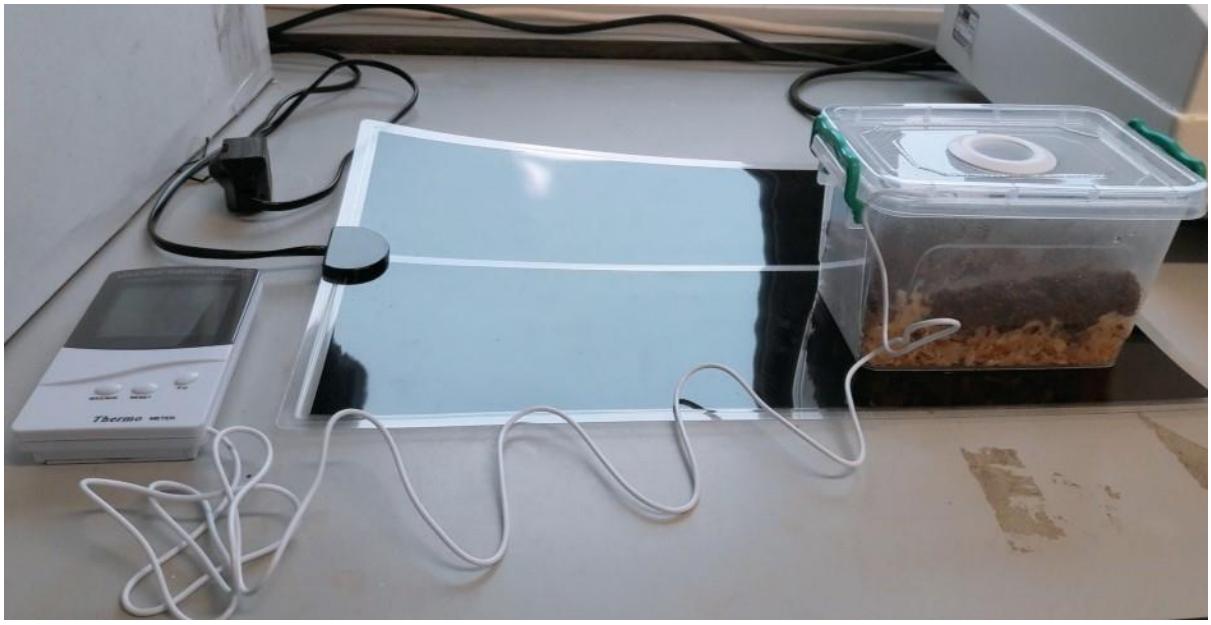

Figure 2.3- T1 trial setup, insect rearing boxes prepared with wood shavings and substrates, then placed over heating pad to moderate the temperature.

Figure 2.4- T2 trial setup, insect rearing boxes prepared without wood shavings only the substrate, then placed over heating pads to moderate the temperature.

2.2.1.4 Experimental procedures for T1, and T2 trials

Larvae feeding trials (T1, and T2) were mainly two pilot studies that depended partly on mealworm breeding guide (www.breedinginsects.com /2020) and partly based on trial and error. For example, adding the wet sponges over the lids of the insect boxes in T1 and then

changing the position of the sponges to be in water beakers around the insect boxes in T2 was mainly to increase humidity over 30% RH, the heating mats were part of temperature control procedure. amount of larvae added in each box was based on personal estimation of the suitable amount of larvae with respect to the volume of the box.

In trial T1, mealworms were weighed, 10 grams per box into 24 boxes, littered with wood shavings. Feeding substrates were added on the top of wood shavings. Beakers with wet sponges were placed on top of the boxes to achieve ±37% Humidity. Larvae were left to feed for 3 weeks and boxes were checked at the end of each week for post feeding larvae. Temperature and humidity were checked day by day to ensure uniformity of environmental conditions. Based on previous experiments, feeding of larvae ended when the prepupal stage was achieved by 40% of the larvae per box. Ethical approval to undertake this study was sought from the Nottingham Trent University School of ARES ethical review group, and granted approval logged as project ARE192045.

In trial T2, Mealworm larvae were weighed, with approximately 18 grams in each box and placed over the feeding substrates (wood shavings were removed in this trial to facilitate weighing the substrate at the end of each week for more precise calculation of substrate use). A total of 24 boxes were used in the trial, with each diet group being offered to 8 replicates. Beakers with wet sponges were placed between boxes over the heating mats to achieve ±37% Humidity. Larvae were left to feed for 2 weeks and then boxes were checked at the end of each week for post feeding larvae, temperature and humidity were checked day by day to ensure uniformity of environmental conditions. based on previous experiments feeding of larvae was ended when prepupal stage was achieved by 40% of the larvae per box. Ethical approval to undertake this study was sought from the Nottingham Trent University School of ARES ethical review group, and granted approval logged as project ARE202148.

2.2.1.5 Study observations

Observations of larvae were performed daily to ensure environmental conditions of temperature ±27° and humidity ±37 % were maintained. At the end of each week, dead larvae were removed and weighed (however, some larvae which reached the pupal stage were discarded without weighing which had a negative effect on the larvae weight gain). Larvae were weighed weekly per box on days 0, 7, 14, and 21 for T1 trial, and 0, 7, and 14 for T2 trial.

Mealworms were collected on day 21 for T1, and on day 14 for T2. Post feeding larvae were collected, weighed, and stored at -20° for further analysis. Post feeding substrates in each box, and dead larvae were also collected for weight determination. Larvae were frozen at -20^{o}C before being freeze dried and ground with a pestle and mortar.

2.2.1.6 Environment

Temperature, Humidity and Lighting settings within the boxes and around the room were appropriate based upon the literature (Kim *et al*., 2015; Kotsou *et al*., 2021). The temperature and humidity were monitored day by day and heat pad temperature was adjusted accordingly. The amount of water in the beakers with soaked sponges around the insect boxes was checked and topped up when needed to maintain humidity.

2.2.1.7 Analytical procedures for larvae and substrate analysis

Feed conversion efficiency was expressed on a fresh matter base as the feed conversion ratio (FCR) according to (Waldbauer, 1968).

Feed conversion ratio (FCR) was calculated as:

$$(\boldsymbol{Weight\ gained} \div \boldsymbol{Weight\ of\ ingested\ feed}) \times \mathbf{100}$$

Mealworm weekly biomass gain including mortality weight was calculated by subtracting initial larvae weight from end of week weight added to mortality weight, and weekly substrate

use was calculated by subtracting end of week substrate weight from initial substrate weight. T1 trial, banana peel and DDGS substrates were analysed for dry matter, ash, nitrogen content (to calculate amount of protein), fats, calcium and phosphorus (ICP), larvae were analysed for dry matter and nitrogen content (analysis procedures were explained in the corresponding sections).

T2 trial, substrates (food waste, sausage waste, and sea waste) were analysed for dry matter, ash, nitrogen content, calcium, phosphorus, copper, iron, and magnesium. Larvae were analysed for dry matter and nitrogen content (analysis procedures were explained in the corresponding sections).

Chapter 3– Effect of different feeding substrate on mealworm (*Tenebrio molitor*) growth performance, survival rate, and nutritional profile.

3.1 Introduction

Literature reports that insect breeding and production for feed have a lower environmental effect, in comparison with other animal production sectors (Oonincx *et al*., 2015). Mealworm (*Tenebrio molitor*) is among the edible insects that can convert a range of feeding substrates from conventional grain-based substrates (Rumbos *et al*., 2021), to many dry wastes or by-products (Harsanyi *et al*., 2020; *Morales-Ramos et al.*, 2020), and even a range of polymer products as polyethylene (Peng *et al*., 2023; Brandon *et al*., 2018) and polystyrene (Tsochatzis *et al*., 2021). Previous studies of insects raised on various feeding substrates, typically showed an acceptable feed conversion rate, survival rate and nutritional profile but meal worm is rarely evaluated, despite its suitability for farming in cooler climates, such as the UK (Bordiean *et al*., 2020). One meal worm feeding study reporting on the effect of substrate choice (through self-selection) on larvae growth and conversion efficiency, showed that substrates with higher concentrations of carbohydrate positively influenced larval growth (*Morales-Ramos et al*., 2020). However, this study focused on evaluating primary food materials rather than waste or by products and did not determine whether substrate nutrient profile impacted on larvae nutrient profile, as shown in other insect species such as black soldier fly larvae in the study for Hopkins *et al*, 2021.

When feeding insects, selecting a rearing substrate that is a low-cost by-product yet able to support a high yield of insect biomass with high nutritional quality protein is essential for

largescale commercial viability of insect farming. In addition, using low value by-products as insect larvae substrate, not only aids in getting rid of waste products, but also transforms them into high quality protein. This leads to direct benefits on the environment through reduced waste, and indirect benefits through increased environmental sustainability of animal feed.

This chapter investigates the efficacy of a range of by-products and waste products as feeding substrate for mealworm (*Tenebrio molitor*) larvae in terms of larval development rate, feed intake, biomass gain and nutritional profile. Test substrates included some conventional substrates that are currently available as registered feed materials (distillers dried grains with solubles (DDGS) and banana peels (BP), alongside three previously unconsidered materials from a company specialising in processing bio-waste for use in anaerobic digestors: food restaurant waste (FW), sausage waste (SuW) and sea water inlet pipe filter waste (SeW). The investigation was conducted under controlled conditions as two meal worm larvae rearing trials: study T1 evaluated dried distiller's grains with solubles and banana peel, and study T2 evaluated food restaurant waste, sausage waste and sea water inlet pipe filter waste.

The main aims of the trials were as follows:

- To investigate the effect of different feeding substrates on mealworm biomass gain, substrate use, and survival rate.
- To determine the effect of feeding substrate on nutritional profile of mealworm.

The hypothesised outcome of this investigation was that meal worm would bio-convert all tested substrates into larval mass, but that substrates with higher level of carbohydrate would support more efficient conversion of substrate into larval mass, while different levels of proteins in different substrates should affect larvae protein content, and therefore that substrate nutrient profile would influence larvae nutrient profile.

3.2 Materials and methods

3.2.1 Mealworm (*T. molitor*)

Live mealworm for the two trials (T1, and T2) were brought from Peregrine Livefoods Ltd., UK. Mealworm used for T1 were medium sized (20mm length) and aged around 7 weeks. To compact the trial to fit external time constraints, older mealworms (aged around 8 weeks) were purchased for T2 (22 mm length) so that larvae end at the end of T2 matched larvae at the end of T1.

3.2.2 Diet formulations of raising substrates.

Diets for T1 and T2 trials, were prepared and formulated according to the methods detailed in chapter 2. In T1 trial, two types of substrates were used (Banana peel (BP), and Distillers grains with solubles (DDGS)) (table 3.1).

In T2, three types of waste substrates were supplied by a bio-waste processing company (Green Eco Technologies, Ltd, UK) who wanted to explore alternative recycling routes to their current use of anaerobic digesters. Three high volume, consistently available, organic materials were proposed by Green Eco Technologies. The Food Waste (FW) stream is collected from facilities like leftover food from hospitals, hotels, restaurants. The Sausage Waste (SuW) is from a meat processing plant, and Sea Waste (SEW) is debris removed from coastal water inlet pipe protection grills (table 3.1). All three streams are collected for organic recycling to diverted them from being incinerated, land fill or entering the sewer system. The Green Eco Technologies waste master system is based on recycling technology that converts organic

waste, without the aid of any bacteria or additives or water to a high density composite, reusable material that retains both the calorific and nutrient value of the inputted waste stream (www.greenecotec.com / 2022). Proximate analysis was performed to confirm composition of T1, and T2 mealworm raising substrate and are shown in table 3.2, and table 3.3, respectively.

Table 3.1- Study codes, study durations, and substrates used in the two studies.

Trial number	Substrate	Abbreviations	Study duration
T1	Distillers dried grains with solubles	DDGS	3 weeks
	Banana peels	BP	
T2	Food restaurant waste	FW	2 weeks
	Sausage waste	SuW	
	Sea water inlet pipe filter waste	SeW	

Table 3.2- Proximate analysis of T1 mealworm substrates based on dry matter basis.

Substrates	Dry matter (g/kg)	Ash(g/kg)	Protein(g/kg)	Fat (g/kg)	Calcium (g/kg)	Phosphorus (g/kg)
Banana peel	104.3	95.5	89.09	35.21	23.48	14.69
DDGS	841.6	54.7	348.61	63.98	20.80	148.80

Table 3.3- Proximate analysis of T1 mealworm substrates based on dry matter basis.

Substrates	Dry matter (g/kg)	Ash(g/kg)	Protein(g/kg)	Calcium (g/kg)	Phosphorus (g/kg)
Food waste	859.7	49.7	176	49.4	164.9
Sausage waste	899.7	45.7	220	98.4	4.3
Sea waste	850.0	138.0	148	206.6	168.5

3.2.3 Mealworm rearing conditions, and trial design.

Trial T1: was a single factor study comparing two substrates; Trial T2: was a single factor study comparing three substrates. Both studies were completely randomised.

Before larvae placement

Preparation of insects vented boxes for T1, and T2 trials for placement of substrate and larvae are detailed in chapter2.

3.2.4 Experimental procedures for T1, and T2 trials

Procedures for trials T1, and T2 regarding feeding, weighing of larvae and environmental conditions are detailed in chapter2.

Ethical approval to undertake trials T1 and T2 was sought from the Nottingham Trent University School of ARES ethical review group, and granted approval logged as project ARE192045, and ARE202148 respectively.

3.2.5 Study observations

Observations of larvae were performed daily to ensure environmental conditions of temperature ±27° and humidity ±37 % were maintained. At the end of each week, dead larvae were removed and weighed (however, some larvae which reached the pupal stage were discarded without weighing which had a negative effect on the larvae weight gain). Mealworm biomass gain, and substrate use were calculated as per the method detailed in chapter 2. Larvae were weighed weekly per box on days 0, 7, 14, and 21 for T1 trial, and 0, 7, and 14 for T2 trial (In the second mealworm feeding trial it was decided to bring larger larvae to shorten the time taken for the larvae to reach the prepupae. Or in other words to perform the second trial in less weeks than the previous trial in order to complete the study prior to a planned poultry study with externally fixed time constraints).

Mealworms were collected on day 21 for T1, and on day 14 for T2 larvae (when larvae were collected at the end of each trial they were nearly the same age because the experimental procedures from literature states that the trial ends when 40 to 80% of larvae reach the prepupae

so the weeks of the in T1 and T2 trials did not affect the larvae final age, and as stated in chapter of insect section that larvae from T1 trial were nearly week younger than larvae brought for T2 trial). Post feeding larvae were collected, weighed, and stored at -20° for further analysis. Post feeding substrates in each box, and dead larvae were also collected for weight determination. Larvae were frozen at -20^{o}C before being freeze dried and ground with a pestle and mortar. Ground mealworm larvae were analysed for Protein via Dumatherm Nitrogen Analyser (Gerhardt, UK) as described in chapter 2. Ash and dry matter were analysed as described in chapter 2. Efficiency of Conversion of Ingested food (ECI) as a measure for feed conversion efficiency on fresh feed basis, was calculated as the formula described in chapter 2.

3.2.5.1 Environment

Temperature, Humidity and Lighting settings within the boxes and around the room were appropriate based upon the literature (Kim *et al*., 2015; Kotsou *et al*., 2021). The temperature and humidity were monitored day by day and the temperature of the heat pads were adjusted accordingly. The amount of water in the beakers with soaked sponges around the insect boxes was checked and topped up when needed to maintain humidity.

3.2.5.2 Statistical analysis of data

Outliers were removed from data if they fell either two standard deviations above or below the mean**.** Statistical analysis was performed using SPSS v.28 (IBM SPSS statistics, 2021). KS testing was used to determine data normality, followed by one-way ANOVA, Duncan post hoc tests were used where appropriate to elucidate differences between experimental groups, and Univariate analysis as appropriate. Statistical significance was declared at $p<0.05$.

3.3 Results

3.3.1 Mortality and substrate use results for T1, and T2 trials.

Weekly larvae mortality, and substrate use data for T1, and T2 trials are shown in table 3.4., and 3.5 respectively. For T1 trial, D0-7, and D7-14 showed significantly (P<0.05) lower larvae mortality rate compared to week 3 (D14-21). On the other hand, larvae raised on Banana peel substrate showed a 100% mortality value from the first week (Figure 3.3). Due to this, banana peels were removed from the analysis as it was apparent that they were not a compatible substrate for mealworm rearing. On day 0-7 larvae showed a significant increase (P<0.05) in substrate use compared to D7-14, however there was no significant difference in the rate of substrate use between D0-7, and D7-14 compared to D14-21.

Table 3.4- Weekly larvae mortality and substrate use for T1 trial (g/week).

Parameters	**Weeks of study T1**			**P value**	**S.E.M²**
	D0-7	**D7-14**	**D14-21**		
Mortality(g)	0.271^{a}	0.2611^{a}	0.733^{b}	<0.001	0.148
Substrate use(g)	39.404^{b}	31.259^{a}	33.739^{ab}	0.045	3.2515

Means within the same raw that do not share a common superscript are significantly different by one-way ANOVA (p<0.05), ²Standard error of means (n=24).

For T2 trial, on D0-7 FW substrate showed significantly (P<0.05) the highest larvae mortality compared to either SuW or SeW (Table 3.5). From D7-14, MW SuW showed significantly (P<0.05) higher mortality rate compared to MW SeW group, however, neither MW SuW nor MW SEW have shown a significant difference in mortality rate compared to MW FW group. 7 From day 0-7, MW SuW group showed a significant increase (P<0.001) in substrate use compared to both MW FW and MW SeW groups. However, from D7-14, MW FW group

showed significantly higher (P<0.001) substrate use compared to both SuW and SeW treatment groups (Table 3.5).

Table 3.5- Weekly larvae mortality and substrate use for NA02 trial (g/week).

Parameters	Treatment			P value	S.E.M[2]
D0-D7	**MW FW**	**MW SuW**	**MW SeW**		
Mortality	11.111[b]	0.8651[a]	0.2609[a]	<0.001	0.329
Substrate use	2.4228[a]	4.4409[b]	1.4717[a]	<0.001	0.336
D7-14					
Mortality	1.7183[ab]	2.0909[b]	1.4067[a]	0.041	0.191
Substrate use	7.7416[b]	5.7138[a]	6.4582[a]	<0.001	0.308

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p<0.05), [2]Standard error of means (n=24).

Figure 3.3- The effect of Banana peel substrate on mealworm from week one of study (Trial T1).

3.3.2 Mealworm larvae weekly biomass and size development for T1 and T2 trials

Results of T1, and T2 trials for weekly larvae biomass are shown in table 3.6 and 3.8 respectively, and larvae size development are shown in table 3.7, and 3.9 respectively. For the

T1 trial, where the mealworm larvae were fed for 3 weeks on DDGS, there was not any significant increase (P=0.975) in larvae biomass (LBM). In addition, the third week of this study (D14-21) resulted in slight but not significant decline in larvae biomass from 13.775g on D7-14 to 13.327g on D14-21 (Table 3.6). On the other hand, larvae size development seemed to have expected normal trend (table 3.7). For the T2 trial, by D7 and D14, larvae had decreased in biomass in all treatment groups (Table 3.8). However, the MW FW group witnessed the most substantial decrease compared to the other substrate groups with a decrease to 3.005g compared to MW SuW (16.806g) and MW SeW (14.599g) on D7. On D14 in the MW FW group, larvae biomass decreased to 0.221g compared to 9.869g, and 8.459g for MW SuW and MW SeW substrates respectively (Table 3.8). Larvae size development showed no significant difference between the larvae groups and overall development showed a normal trend (Table 3.9).

Table 3.6- Weekly larvae weekly biomass(g) for T1 trial.

Parameter	D0-7	D7-14	D14-21	P value	S.E.M[2]
Larvae biomass(g)	10.930	13.775	13.327	0.975	2.075

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$), [2]Standard error of means (n=24).

Table 3.7- Larvae size development (mm) for T1 trial.

Treatment	D0	D0-7	D7-14	D14-21
MW DDGS	20	22	24	25
S.E.M[2]	0.00	0.522	0.669	0.452
P value	----	----	-----	-----

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$), [2]Standard error of means (n=24).

Table 3.8- Effect of substrates on Mealworm larvae weekly Biomass(g) for trial T2

Treatment	D0 (g)	D7 (g)	D14 (g)
MW FW	18.306	3.005[a]	0.221[a]
MW SuW	18.401	16.806[b]	9.869[b]
MW SeW	18.496	14.599[c]	8.459[b]
P value	0.069	<0.001	<0.001
S.E.M²	0.055	0.313	0.455

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$) ²Standard error of means (n=24).

Table 3.9- Larvae size development (mm) with different feeding substrates for T2 trial.

Treatment	D0	D7	D14
MW food waste	22.5	24	26
MW sausage waste	22.6	24	26
MW sea waste	22.1	24	26
P value	0.116	--	---
S.E.M²	0.103	0.00	0.00

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$), ²Standard error of means (n=24).

3.3.3 Mealworm larvae weekly biomass gain and feed conversion ratio for T1.

On D7-14, larvae biomass gain was significantly higher ($P<0.05$) than both D0-7 and D14-21. By day 21, a significant reduction ($P<0.05$) in biomass gain was reported compared to D7-14 (Table 3.10), and it is worth pointing however out that this reduction in biomass gain on D14-21 could be due to the miscalculation of the real biomass weight, as the larvae that developed to pupae were unfortunately discarded without measuring their weight. We can notice a significant difference in FCR of mealworm larvae feeding on DDGS substrates in different feeding stages. On day 7-14, mealworm larvae showed significantly ($P<0.001$) the highest FCR compared to D0-7 and D14-21. On day 14-21, mealworm group showed the lowest FCR compared to D0-7 and D7-14 (Table 3.7).

Table 3.10- Larvae biomass gain including mortality weight with DDGS substrate group.

Treatment	D0-7	D7-14	D14-21	P value	S.E.M[2]
Biomass gain(g)	1.201[ab]	3.106[b]	0.285[a]	0.021	0.9977
FCR on fresh food basis (%)	9.082[b]	13.862[c]	3.874[a]	<0.001	1.4569

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05), [2]Standard error of means (n=24).

3.3.4 Mealworm larvae weekly biomass gain for T2 trial.

According to the previous larvae weekly biomass results (Table 3.11), we can see that there was a weekly reduction in larval biomass in this study. That resulted in negative results regarding biomass gain. On D0-7, FW and SEW groups showed significantly (P<0.01) the least biomass gain results (-4.189, -3.635) respectively, compared to SW group (-0.7304). On D7-14, SW and SEW groups showed significantly (P<0.01) the least biomass gain results (-4.845, -4.733) respectively, compared to FW group. On D0-14, the SW and SEW groups showed significant (P<0.01) increase in larval biomass gain (11.2297g, and 6.2309g respectively) compared to the FW group (-2.249) (table 3.11).

Table 3.11- Mealworm larvae biomass gain including death weight (g).

Treatment	D0-7	D7-14	D0-14
MW food waste	-4.189[a]	-1.0656[b]	-2.249[a]
MW sausage waste	-0.7304[c]	-4.845[a]	11.2297[c]
MW sea waste	-3.635[b]	-4.733[a]	6.2309[b]
P value	<0.001	<0.001	<0.001
S.E.M	0.154	0.282	0.394

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

3.3.5 Dry matter and protein content of post-feeding larvae for T1, and T2 trials.

Sea waste substrate significantly increased the protein content (P<0.001) of mealworm larvae compared to the rest of the study substrates, however, both MW food waste and MW DDGS substrates showed significantly higher (P<0.001) larvae dry matter compared to MW SuW but showed no significant difference in protein content between either MW food waste, MW DDGS, or MW SuW (table 3.12).

Table 3.12- Dry matter and protein content of post-feeding larvae for T1, and T2 trials.

Treatment	Dry matter (g/kg)	Protein (g/kg)
MW food waste	558.5^{c}	572.2^{b}
MW sausage waste	442.5^{b}	554.5^{a}
MW sea waste	405.7^{ab}	635.2^{c}
MW DDGS	303.2^{a}	566.2^{ab}
P value	<0.001	<0.001
S.E.M[2]	2.855	0.371

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05), [2]Standard error of means (n=8).

3.4 Discussion

The main aim of this study was to determine whether the type of feeding substrate had any effect on the mealworm development and mealworm protein content. A second aim was to see whether unconventional substrates could work as feeding substrates for mealworm and produce more larvae mass yield compared to a conventional feeding substrate as DDGS. So, the rationale behind the mealworm feeding studies was to investigate the ability of mealworm to valorise different types of wastes or byproducts. For example, in T1 trial, banana peels, were chosen to test its viability as feeding substrate for mealworm and thus reduce wastes from banana peel, however, after the banana peel failed as a mealworm substrate it became very

intriguing to look for reasons behind mealworm mortality. Two theories have developed one of which is that the substrate was too moist for mealworm to feed and the larvae drowned therefore it is recommended that in future research banana peel could be dried first and ground instead of freshly blended and added to the boxes, the other theory was probably the nature of banana peels that contains high levels of ethylene (Zhang *et al*., 2012) which might have been deterrent to the larvae. The second substrate choice was wheat DDGS which is a valuable byproduct and according to literature (Kluth and Rodehutscord, 2010; Abdel-Raheem *et al*., 2011), the nutrients value of DDGS as a direct poultry feed can't be fully utilized, therefore, it was thought to introduce DDGS as feed through mealworm by converting DDGS to an insect protein mass and investigate the viability of DDGS as an insect feeding substrate.

Proximate analysis of test substrates showed the significant difference in mineral content, where food waste, sausage waste, and sea waste showed 4.94, 9.84 and 20.66 Ca (g/kg) content and 16.49, 0.43, and 16.85 P (g/kg) content respectively. SuW substrate typically showed the highest protein content (22%) compared to SeW, and FW (14.8%, and 17.6% respectively). Although the substrates used in trial T2 are from different sources of wastes especially the seawaste substrate that is mainly organic wastes from sea inlet pipes, however, the substrates were all subjected to the same processing method that does not involve any bacterial activity. That technique uses oxygen molecules to interact with the organic wastes and breaks them to a cellular level eliminating any pathogens or extra water into a composite form (www.greenecotec.com/benefits).

The performance data from the first trial (T1) indicates that, from the parameters measured, there appears to be no deleterious effect of feeding DDGS to mealworm larvae. On the other hand, mealworm fed banana peel substrate showed 100% mortality after the first week which was under fixed conditions of the study of temperature and humidity, so seems a clear indication of effect of this feeding substrate on larvae survival rate. Banana peel substrate was

blended fresh and was not submitted to any drying process, so this could be one reason behind the larvae mortality since mealworm mainly feed on spent grains, cereals, leftover cake mix, and maize (Riaz *et al*., 2023), however, the DDGS substrate was also soaked in water prior to feeding but the results showed less than 15% mortality of these larvae. Another reason behind the failure of banana peel as a substrate for mealworm might be that it could have contained a high amount of ethylene, as researchers have reported the use of ethylene to quicken the process of banana ripening (Ge *et al*., 2017; Vu *et al*., 2019), and this could be toxic to the mealworm. Another theory could be that banana peel is a high adsorbent of mycotoxins such as aflatoxin (Shar *et al*., 2016; Akpomie and Conradi, 2020), which may have led to the failure of the mealworms fed this substrate. The results of the current study could show that although mealworm can efficiently valorise a range of by-products, there are limitations for some products that deleteriously effect mealworm mortality and growth.

Substrate use data for the DDGS group showed significantly increased substrate (39.404 g) use for the first week of the trial compared to the second week (31.259g), however the cumulative data showed overall high substrate use. On the other hand, weekly biomass gain for the DDGS group showed the highest increase in week 2 (3.1061g) and a significant decline by the third week (end of trial). This could be due to the failure to record the weight of the pupae, as the pupae were simply picked and discarded. If the weight of discarded pupae had been taken in account, larvae mass yield would have been higher in all weeks of the study, but especially the last week. This study could indicate that DDGS could work as an efficient raising substrate for mealworm. This hypothesis was confirmed by a study for Van Broekhoven *et al*., 2015, where different mealworm species were reared on 20% maize DDGS diet as a high protein- low starch diet, and this 20% maize DDGS diet significantly increased larvae protein content and enhanced shorter development time compared to a low protein- high starch diet.

In the 2nd trial (T2), mealworm larvae aged approximately 8 weeks were fed on three different waste substrates for two weeks. Some of the substrates used were unconventional such as sea wastes substrates which are from sea inlet pipe filter wastes, sausage wastes and a food waste substrate that can be included as a conventional substrate for insect rearing. Proximate analysis showed that sausage waste contained the highest protein content compared to the other tested substrates. The results analysed in this chapter indicate that, there appears to be significant effects of the feeding substrates on the mealworm. The food waste substrate negatively affected the larvae, as the FW substrate group showed a 11.111g mortality mass on D0-7, 1.7183 g on D7-14, with the sum of 12.829 g through the whole trial (73.5% mortality) compared to the Sausage waste and the Seawaste substrate groups which showed 16.9%, and 9.6% mortality biomass respectively. This indicates the significant effect of substrate on larvae survival rate in this trial. All the conditions were constant across all the experimental larvae groups (temperature and humidity), and so this clearly shows that the negative effect on the larvae was due to the nature of the substrate. The survival rate was based on the weight of dead larvae for each substrate group. However, none of the substrates witnessed any biomass gain, and all the biomasses gain data recorded gave negative values. The negative results reflect the impact of the pupal weight which were discarded and not taken into consideration; however, the overall biomass gain of SW and SEW group compared to FW, showed how the type of substrate could affect the larvae survival rate and biomass gain.

There were no obvious reasons behind the negative value of biomass gain of the larvae fed on the waste substrates, but there are certain factors that influence these results. During the two weeks of the trial, any post feeding larvae which have reached the pupal stage would be discarded without recording the weight, and that could have affected the biomass gain values, particularly if a specific substrate encouraged the mealworm to move to the pupal stage. Another reason is that it was observed in this trial that mealworm tend to show a cannibalistic

behaviour if the feeding medium is too crowded or if either the conditions or the feeding substrates are suboptimal or have the presence of pathogenic bacteria (Maciel-Vergara *et al*., 2018). Although the environmental conditions were monitored daily, and adjusted accordingly, this could still have been an issue. In Van Broekhoven *at al*., 2015, and Kotsou *et al*., 2021, only 50 larvae were added to each experimental box unlike in the current study where 18g of larvae was added to each box (about 300 larvae), which that might have elicited cannibalistic behaviour.

Substrate use data for the MW SuW group showed significantly increased substrate use (4.4409 g) on D0-7 compared to either the MW FW or the MW SeW substrate group, however on the second and final week of the trial, the FW group showed significantly higher substrate use (7.7416 g) compared to week 1.This helps explain the lower mortality rate on week 2 compared to week 1 for the FW group, however, the high mortality rate in week 1 does indicate that the mealworm were negatively affected and that the food waste substrate failed be as a mealworm raising substrate. Larvae weekly biomass results for the three substrates confirms the previously mentioned conclusion. The MW FW group showed a net biomass of 3, and 0.22 g on Day7 and day 14 respectively from a starting weight of 18.3 g on day 0. This shows how the food waste substrate negatively affected the larvae survival rate and total biomass compared to both Sausage waste and sea waste substrates, which showed net biomass of 16.8g and 14.6 g respectively on day7, and 9.87g, and 8.46g respectively on day 14. Therefore, it is clear from the results that sea waste, and sausage waste substrates were favourable for mealworm and could be recommended as mealworm raising substrates.

Results of the dry matter and protein content of mealworms after feeding on the different substrates in trials T1, and T2 are shown in table 3.12. Feeding different substrates seems to have significantly affected the dry matter as well as the protein content of post feeding mealworm. Surprisingly the sea waste substrate had significantly increased protein content of

mealworm, more than the DDGS substrate which is considered a conventional substrate for mealworm. The food waste substrate and sausage waste did not deleteriously affect the protein content of mealworm, although from the proximate analysis data, the sausage waste substrate did have the highest protein content before feeding, however, that did not result in the MW SuW group having the highest protein content. This also was clear in Broekhoven *et al*., 2015, where there was no significant difference in *Tenebrio molitor* larvae protein content (which is the same mealworm species used in the current study), even with the mealworm fed the high protein substrates, and it is worth noting that none of the post- feeding *T. molitor* exceeded 50% protein content. According to the literature, the average protein content of mealworm can reach 45% (Ravzanaadii *et al*., 2012), therefore, in the current study, waste substrates have succeeded to raise the average *T. molitor* protein content above this. However, it must be noted that insect feeding and performance trials in a research setting are much different from rearing insects in insect farms, as the first involves precision in calculating the starting and final weight of larvae to monitor the performance and to investigate to what extent was the feeding substrates utilised by the larvae on the other hand, insect rearing farms for protein yield overlooks lab procedures and focuses more on getting the largest amount of larvae. The conditions of the T1 and T2 trials were not the most optimum conditions especially the humidity conditions couldn't exceed 37% RH when 60% is the most optimum for mealworm. Also, larvae numbers inside each box might have exceeded the ecological density limits for meal worm larvae growth in the experimental box, and the excessive crowding may have induced abnormal behaviour within the larvae, such as cannibalism. Another error leading to inaccuracy in the investigation was discarding the pupae without taking their weight in account, unfortunately, this was not based on any rationale except that it was a mis- handling of a typical performance trial probably due to lack of experience, therefore, it is definitely recommended

for future research in mealworm feeding trials to take into accounts all the previously mentioned limitations.

3.5 Conclusion

Mealworm could convert low value by-products, and useless waste products into a high-quality protein mass, however there are certain limitations for the type of raising substrates for mealworm, thus, banana peel, and fruit waste can't be recommended due to the high insect mortality witnessed from the results of the current study.

Feeding substrate can have a significant effect on the protein content of mealworm. And sea waste substrate has significantly increased the mealworm protein content.

Mealworm mass production requires certain conditions of temperature and humidity although it can acclimatize to a range of temperature and humidity if required. However, this acclimatization can be at the expense of larvae development in the form of a prolonged larval stage or the appearance of cannibalism activity. This would be detrimental for yield and mortality so cannot be recommended.

Chapter 4- Effect of rearing substrates on the nutrient digestibility profiles of insect larvae for broiler chickens compared to soyabean meal.

4.1 Introduction

Mealworm and BSF are among the insect species that are suitable for feed for poultry, due to the ability of these insects to feed on a variety of substrates including waste materials and manure, making use of the residual AA from the substrate by incorporating them into their own biomass (Makkar *et al*., 2014). To make insect farming for poultry feed economically and environmentally sustainable, it is likely to be waste streams rather than agricultural crops which will be used as a substrate for insects. In addition to that, some studies have shown the role of black soldierfly larvae meal in enhancing the chicken growth rate (Hale, 1973; Oluokun, 2000).

The previous chapter investigated the effect of raising substrate (DDGS, banana peel, sea waste, food waste, sausage waste) on the nutritional profile of mealworm (*Tenebrio molitor* larvae) and accordingly, protein content of mealworm was affected by the different raising substrates. The substrate had a significant effect on the protein content with some substrates resulting in a high protein content reaching 65% on dry matter basis. Whilst the nutrient profile is important for chicken nutrition it is vital to consider the digestibility of these nutrients for poultry. In a study by *DeMarco et al*., 2015, investigating the effect of 25% BSFL or mealworm replacement on the amino acid digestibility of broilers compared to a control diet, both insects were raised on a cereal by-product substrate. This study showed the 25% addition of insect meal clearly enhanced the amino acid profiles of both dispensable and indispensable amino acids, especially the levels of two crucial amino acids (Methionine and Lysine), compared to the amino acid profile of control soyabean meal diet (Table 4.1).

Table 4.1- Amino acids concentration (g/kg DM) of 25% mealworm, and 25% BSFL meal inclusions against soyabean meal control diet (data taken from *De Marco et al*., 2015).

amino acids	Control soyabean meal diet	25% Mealworm raised on cereal by-products	25% BSFL raise on cereal by-products
Indispensable amino acids			
Arginine	16.6	19.3	18.2
Histidine	7.69	9.92	9.09
Iso-Leucine	9.74	12.4	10.4
Leucine	17.8	20.4	19.4
Lysine	9.84	15.5	11.2
Methionine	4.86	6.24	5.48
Phenylalanine	14.0	15.3	14.1
Threonine	9.54	11.6	11.3
Valine	9.80	13.7	12.0
Dispensable amino acids			
Alanine	7.01	14.8	13.0
Aspartic	17.2	23.2	19.7
Cystine	4.56	6.83	6.98
Glutamic	30.7	37.4	34.4
Glycine	10.8	13.8	12.9
Proline	13.4	18.1	20.0
Serine	11.9	14.7	14.1
Tyrosine	9.71	15.0	10.8
Total	409.36	454.53	299.59

Therefore, the main aims of this study are.

- To quantify the amino acid content and availability of the mealworm and black soldierfly larvae from different sources (fed on different waste substrates)
- To investigate the effect of these different feeding substrates on the amino acid profile of these insects compared to soyabean meal as well as their effect on the amino acid digestibility of the broilers.

The first hypothesis is that the insects raising substrate will significantly affect the amino acids profile of the studied insects as well as the protein content (as already seen in Chapter 3). The second hypothesis would be that the insect source will not have a significant effect on the coefficient of digestibility of broilers, but that insect meal will show a positive effect on digestibility of broilers fed insect meal compared to soyabean meal.

4.2 Materials and methods

Amino acid determination was conducted across 2 trials due to the large number of replicates needed to conduct amino acid digestibility trials. T3 was intended to assess BSFL, and T4 meal worm larvae, both with soya as a control material, but the final BSFL test material (BFSL fed dried distillers grains with solubles) was not supplied in time for T3, and was therefore added later into T4 to ensure amino acid digestibility values were captured for this material. Amino acid digestibility of BSFL and MW reared on different substrates for broiler nutrition was determined using the regression method of Short *et al*. (1999) over 2 experiments (T3, and T4). This approach has the advantage that the estimated digestibility automatically considers the basal endogenous losses and is regarded as the gold standard approach for determining amino acid digestibility in poultry (Akinde, 2016) as the approach does make a generic assumption over the endogenous losses which are needed to correct the apparent ileal digestibility values. On the other hand, since the model uses three ingredient inclusion levels, the regression method is laborious and costly compared with other methods. Although it is time intensive and expensive, the insect material is a new one and therefore it probably was important to be thorough and to have a measured value for endogenous losses in case the insect meal affected these.

4.2.1 Birds husbandry conditions

For each of T3 and T4 trials, 144 one-day old male Ross 308 broilers were sourced from PD Hook Cote hatchery from a flock aged 34 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. All birds from each trial were fed a commercial chick starter crumb (GLW Feeds, Shepshed, UK) from day 1 to day 20 for T3 and to day 19 for T4, with water provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible.

For the test feeding period, chicks were individually weighed, then randomly assigned to mesh sided pens littered with wood shavings substrate and fed the corresponding trial diets assigned to each pen for a further 3 days. Each protein source was fed to the corresponding experimental group at 3 inclusion levels to 3 pens per inclusion level. For T3 trial, each pen had colour marked and unmarked chicks, so it was practically two replicates in each pen by sum of 6 replicates per inclusion. For T4 trial each pen was treated as one replicate, so each diet inclusion had 3 replicates. Specifics for each trial are detailed in table 4.3.

Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was granted by the University ARES Ethics Committee and was logged as project ARE212203.

Table 4.3- Demonstrating number of replicates and pens per trial.

Trial identifier	Protein sources	Drying	No. of reps per inclusion level
T3	Soyabean meal/Insect meal	Freeze dried	6
T4	Soyabean meal/Insect meal	Freeze dried	3

4.2.1 Insect larvae meals

In T3 trial, live black soldierfly larvae from three different feeding substrates (Fruit waste (FW), Bran and Brewery wastes (Brew)), were brought from AgriGrub Ltd., UK, then were freeze dried and ground to pass through a 1mm sieve. In addition to this, a commercial BSFL fed DDGS was evaluated from Hexafly Ltd, Ireland. The BSFL were bought in the form of ground powder. In T4 trial, live mealworm from two different substrate sources; Bran (conventional mealworm feeding substrate), and DDGS (non-conventional mealworm feeding substrate) were purchased from Live Foods Direct Ltd., UK. The MW were freeze dried and ground to pass through a 1mm sieve. Both trials included soyabean meal as a protein control at the three different inclusion levels. The two trials in this chapter, and the insect larvae meal used in each trial are detailed in Table 4.2.

Table 4.2- Amino acid digestibility studies on Insect larvae meal

Trial number	Insect source	Abbreviations	Drying process
T3	Black soldierfly larvae fed fruit waste	BSFL FW	Freeze dried (FD)
	Black soldierfly larvae fed bran	BSFL Bran	Freeze dried (FD)
	Black soldierfly larvae fed brewery waste	BSFL Brew	Freeze dried (FD)
T4	Mealworm fed bran	MW Bran	Freeze dried (FD)
	Mealworm fed distillers grains	MW DDGS	Freeze dried (FD)
	Black soldierfly larvae fed distillers grains	BSFL DDGS	Freeze dried (FD)

4.2.2 Diets formulation

Diets were mixed onsite for both trials. The BSFL and MW as per table 4.2 plus soyabean meal as control were incorporated into mash diets at 200, 400, and 600g/kg inclusion, as a sole protein source with added vitamins, minerals and oil. An inert marker was added at 5g/kg (Titanium dioxide) and the remainder of the diet made up with an equal mix of dextrose and

wheat starch. Diet formulation is shown in table 4.3. Diets were mixed in house using a ribbon mixer, with the titanium dioxide incorporated into a portion of the dextrose prior to adding to the mixer to ensure homogenous mixing. Dry ingredients were mixed for 5 minutes before addition of oil and then mixed for a further 5 minutes.

Table 4.3- Diet formulation for amino acid digestibility studies (g/kg).

Ingredient	200g/kg inclusion	400g/kg inclusion	600g/kg inclusion
Soyabean meal/test protein	200	400	600
Soyabean meal oil	50	50	50
Vitamin/mineral premix*	50	50	50
Titanium dioxide	5	5	5
Dextrose	347.5	247.5	147.5
Wheat starch	347.5	247.5	147.5

* 1Premix (per kg of diet): Calcium; 10g, Phosphorus; 4.5g, Sodium; 1.5g, Chloride; 1.5g, Magnesium; 0.6g, Manganese; 60mg, Zinc; 50mg, Iron; 80mg, Copper; 6mg, Iodine; 0.5mg, Molybdenum; 0.2mg, Selenium; 0.15mg, Retinol; 2.25mg, Cholecalciferol; 37.5µg, Tocopherol; 10mg, Menadione; 3.0mg, Thiamine; 3.0mg, Riboflavin; 5.0mg, Pantothenic acid; 10mg, Pyridoxine; 4.0mg, Niacin; 30mg, Cobalamin; 10µg, Folic acid; 1.5mg, Biotin; 0.15mg, Choline; 1.3mg, Amprolium; 125mg, Antioxidant; 125mg.

4.2.3 Study observations

After three days of feeding the test diets, the birds were culled by cervical dislocation and digesta collected from the distal end of the small intestine (ileum) as identified as the portion between Meckel's diverticulum and the ileal-caecal-colonic junction. Care was taken to use gentle digital pressure when removing digesta to minimise disruption of the mucosal lining of the intestine. For T3 trial digesta samples were pooled into two pots per pen (one pot for colour marked replicate and one for unmarked), for T4 trial, digesta samples were pooled into one pot per pen. Pots of digesta were frozen at -20^{o}C, prior to freeze drying and grinding.

4.3 Results

Proximate analysis for each of the protein sources studied in this chapter is detailed in table 4.4. It is notable that the protein content of the mealworm from different sources is almost equal or higher than that of soyabean meal, while the BSFL from fruit waste substrate had less protein content than either BSFL from other substrates or mealworm or soyabean meal.

Table 4.4- Proximate analysis of protein sources for amino acid digestibility trials (T1, and T2).

Trial	Protein source	Dry matter g/kg	Protein g/kg DM	Fat g/kg DM	Ash g/kg DM	Gross energy MJ/kg DM
T3	Soyabean meal	875.503	488.4	20.922	91.15	16.99515
	BSFL Fruit waste	826.95	313.4	371.49	72.09	---------
	BSFL Bran	750.41	399.4	167.20	74.92	20.283
	BSFL Brewery waste	927.29	485.2	105.80	110.82	--------
T4	MW DDGS	919.85	499.8	340.60	33.19	25.8314
	MW Bran	974.69	461.9	222.76	32.57	26.8265
	BSFL DDGS	858.25	409.04	228.05	106.33	19.036

4.3.1 Total amino acid content of soyabean meal, black soldierfly larvae, and mealworm raised on different feeding substrates.

The total amino acid content of soyabean meal and insect meal from different sources used in the amino acid digestibility trials are shown in table 4.5. Mealworm raised on bran substrate (MW Bran) showed numerically the highest amino acid content compared to the rest. Also, lysine and methionine content were slightly higher in MW Bran when compared with soyabean meal. On the other hand, BSFL from DDGS and Bran substrates had less total amino acid content (320.9, and 299.6 g per kg DM respectively) compared to soyabean meal (409.4mg/kg DM).

Table 4.5- Amino acid concentration (g/kg DM) of insect larvae meals and soyabean meal.

Amino acids	Soya	BSFLFW	BSFL Bran	BSFL Brew	MW Bran	MW DDGS	BSFL DDGS
Indispensable amino acids							
Arginine	26.8	12.06	14.56	20.23	21.32	24.16	17.65
Histidine	9.88	7.78	10.46	11.57	12.73	12.89	10.83
Iso-Leucine	6.34	27.87	6.51	11.33	8.19	9.25	8.11
Leucine	30.05	120.52	20.80	33.68	32.49	33.58	23.62
Lysine	25.13	14.11	19.84	24.27	23.08	25.29	22.19
Methionine	5.51	3.21	4.98	7.06	5.56	5.90	5.77
Phenylalanine	18.88	8.71	12.57	18.45	14.99	16.21	14.79
Threonine	16.08	10.44	12.74	20.70	18.01	19.24	14.99
Valine	16.87	17.89	20.47	20.48	28.59	18.40	16.11
Dispensable amino acids							
Alanine	21.2	24.43	26.51	34.49	41.13	39.09	25.28
Aspartic	47.1	25.97	32.76	41.45	38.41	40.47	32.07
Cystine	8.54	23.09	3.80	3.94	5.57	5.67	4.24
Glutamic	95.7	66.45	42.08	71.49	100.18	131.38	46.91
Glycine	23.8	37.39	22.56	20.69	51.22	60.66	25.26
Proline	21.81	25.44	17.16	24.59	46.55	53.28	18.83
Serine	29.11	17.32	18.4	25.09	28.41	29.16	18.92
Tyrosine	6.56	11.87	13.39	15.94	28.27	23.49	15.38
Total	409.36	454.53	299.59	405.44	504.71	548.11	320.96

4.3.2 Digestible amino acid content of insect larvae meals and soyabean meal (T3, and T4).

The digestible amino acid content of two different types of insects (BSFL and MW) from different substrate sources, and soyabean meal from the two current studies are shown in table 4.6 and 4.7 (T3 and T4 respectively). For the T3 trial, BSFL Brew showed a significantly

highest digestible amino acid content ($p<0.001$) in all but 7 amino acids. BSFL Bran was equal to or higher than soyabean meal for most of the amino acids apart from cysteine, glutamic, leucine, lysine, phenylalanine, serine, and threonine. BSFL FW showed significantly ($P<0.005$) the lowest digestible amino acids content in 8 amino acids while Glutamic acid was significantly lower ($P<0.005$) than that in soyabean meal but comparable to that in BSFL Brew and significantly ($P<0.005$) higher to that in BSFL Bran group. However, the digestible AA content of cystine, glycine, Isoleucine, leucine, and proline were significantly ($P<0.005$) the highest in BSFL FW group compared to the other treatment groups as well as soyabean meal (Table 4.6).

For T4 trial, MW Bran group showed the highest digestible AA content except for Cystic acid, and Aspartic acid. MW DDGS had digestible AA content equal or even higher than soyabean meal in most of the amino acids apart from Arginine, Aspartic acid, Cystic acid, and phenylalanine. On the other hand, BSFL DDGS showed the least digestible AA content compared to the other insect protein sources and soyabean meal apart from Alanine which was significantly higher ($P<0.005$) than soyabean meal but less than the other insect protein sources, and Methionine which was significantly ($P<0.005$) the highest in digestible AA compared to that of the other insect larvae meals as well as soyabean meal (Table 4.7).

Table 4.6- Digestible amino acid content of soyabean meal and insect larvae meals from different substrate sources (T3 trial).

Amino acid	Soyabean meal	BSFL FW	BSFL Bran	BSFL Brew	P value
Alanine	16.91 (0.238)^{a}	23.6 (0.376)^{b}	23.70 (0.210)^{b}	31.99 (0.388)^{c}	<0.001
Arginine	23.13 (0.386)^{d}	11.38 (0.373)^{a}	13.02 (0.324)^{b}	18.94 (0.176)^{c}	<0.001
Aspartic	34.18 (0.981)^{c}	25.43 (0.708)^{a}	30.28 (0.589)^{b}	36.87 (0.739)^{c}	<0.001
Cystine	7.32 (0.151)^{b}	23.11 (0.204)^{c}	3.60 (0.173)^{a}	3.49 (0.066)^{a}	<0.001
Glutamic	76.11 (1.627)^{c}	65.56 (1.278)^{b}	36.78 (0.439)^{a}	66.21 (1.369)^{b}	<0.001

Glycine	18.11 (0.301)[a]	36.28 (0.423)[c]	20.22 (0.524)[b]	18.66 (0.616)[ab]	<0.001
Histidine	7.90 (0.535)[b]	7.12 (0.178)[a]	9.51 (0.177)[c]	10.16 (0.104) [d]	<0.001
Iso-Leucine	1.78(0.675)[a]	26.94(0.272) [d]	5.49(0.143)[b]	9.15(0.194)[c]	<0.001
Leucine	21.26(0.286)[b]	119.72(0.561) [d]	18.11(0.269)[a]	30.93(0.251)[c]	<0.001
Lysine	20.71(0.322)[c]	13.72(0.441)[a]	17.99(0.318)[b]	22.004(0.288)[c]	<0.001
Methionine	4.32(0.123)[b]	2.47(0.096)[a]	4.39(0.209)[b]	5.815(0.145) [d]	<0.001
Phenylalanine	14.81(0.411)[c]	8.07(0.293)[a]	11.13(0.374)[b]	16.44(0.192)[d]	<0.001
Proline	15.71(0.448)[a]	24.54(0.365) [c]	14.89(0.191)[a]	22.16(0.337)[b]	<0.001
Serine	23.62(0.353)[b]	16.87(0.457)[a]	17.35(0.269)[a]	23.74(0.524)[b]	<0.001
Threonine	12.42 (0.434)[b]	9.57(0.364)[a]	10.37(0.208)[a]	18.87(0.112)[c]	<0.001
Tyrosine	5.082 (0.147)[a]	10.84(0.214)[b]	12.58(0.308)[c]	13.65(0.282) [d]	<0.001
Valine	11.97(0.190)[a]	16.84(0.421)[b]	18.15(0.188)[c]	18.005(0.226)[c]	<0.001
				highest statistically	
				lowest statistically	

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

Table 4.7- Digestible amino acid content of soyabean meal and insect larvae meals from different substrate sources (T4 trial).

Amino acid	Soyabean meal	MW Bran	MW DDGS	BSFL DDGS	Pvalue
Alanine	18.88(0.435)^{a}	37.19(0.567)^{c}	39.21(1.138)^{c}	23.54(0.599)^{b}	<0.001
Arginine	24.63(0.397)^{c}	23.74(0.319)^{c}	20.94(0.478)^{b}	17.01(0.288)^{a}	<0.001
Aspartic	41.14(0.929)^{c}	38.23(0.845)^{bc}	36.83(0.944)^{b}	30.44(1.069)^{a}	<0.001
Cystine	6.85(0.453)^{b}	4.11(0.269)^{a}	4.64(0.487)^{a}	3.237(0.377)^{a}	0.001
Glutamic	86.48(1.631)^{b}	129.1(1.411)^{d}	99.07(2.398)^{c}	41.89(2.769)^{a}	<0.001
Glycine	20.03(0.839)^{a}	56.93(0.659)^{c}	47.59(1.975)^{b}	20.19(1.929)^{a}	<0.001
Histidine	8.8(0.245)^{a}	12.25(0.137)^{c}	11.76(0.463)^{c}	10.205(0.204)^{b}	<0.001
Iso-Leucine	5.04(0.181)^{a}	9.12(0.121)^{c}	8.07(0.332)^{b}	7.927(0.036)^{b}	<0.001
Leucine	26.81(0.267)^{b}	32.53(0.673)^{c}	31.9(0.484)^{c}	22.39(0.937)^{a}	<0.001
Lysine	22.91(0.434)^{ab}	24.71(0.489)^{b}	22.64(0.678)^{ab}	21.37(0.395)^{a}	0.011
Methionine	5.02(0.109)^{a}	5.74(0.147)^{b}	5.51(0.126)^{ab}	5.59(0.107)^{b}	0.016
Phenylalanine	16.48(0.399)^{c}	15.63(0.289)^{bc}	14.49(0.463)^{ab}	13.98(0.729)^{a}	0.003
Proline	18.75(0.584)^{a}	51.33(0.388)^{c}	44.21(1.194)^{b}	16.104(0.943)^{a}	<0.001
Serine	25.84(0.788)^{b}	26.36(0.717)^{b}	25.66(0.722)^{b}	16.71(0.917)^{a}	<0.001
Threonine	13.6(0.504)^{a}	17.39(0.299)^{b}	16.36(0.696)^{b}	13.596(0.444)^{a}	0.001
Tyrosine	5.65(0.188)^{a}	22.77(0.125)^{b}	26.89(0.304)^{d}	14.41(0.265)^{b}	<0.001
Valine	14.89(0.275)^{a}	17.52(0.253)^{b}	27.33(0.759)^{c}	13.77(0.634)^{a}	<0.001
				highest statistically	
				lowest statistically	

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

4.3.3 Coefficients of digestibility of two types of Insects (BSFL, and Mealworm) raised on different substrates against soyabean meal.

Coefficients of digestibility (COD) of the two different types of insects (BSFL, and MW) from different substrate sources, compared with soyabean meal from the two current studies are shown in table 4.8 and 4.9. There were no significant differences in COD for methionine. BSFL FW had the highest COD for most of the amino acids, followed by BSFL Bran, and BSFL Brew, which were higher than soyabean meal for 16 out of 17 amino acids with exception to methionine being the excluded amino acid. Accordingly, Soyabean meal showed significantly ($p<0.05$) the least COD compared to insect protein sources (BSFL FW, BSFL Bran, and BSFL Brew) for 15 out of 17 amino acids (Table 4.8).

For T4 trial, there were no significant differences in COD between either the insect protein sources (MW Bran, MW DDGS, and BSFL DDGS) or compared to soyabean meal except for only iso-leucine, and tyrosine. Insect protein sources did not show any significant difference in COD for all the 17 amino acids, however all insect sources showed significantly higher ($P<0.05$) COD for iso-leucine, and tyrosine than soyabean meal (Table 4.9).

Table 4.8-Coefficients of digestibility for insect larvae meals and soyabean meal (T3 trial)

Amino acid	Soyabean meal	BSFL FW	BSFL Bran	BSFL Brew	P value
Alanine	0.79(0.011)^{a}	0.97(0.015)^{c}	0.89(0.008)^{b}	0.93(0.011)^{bc}	<0.001
Arginine	0.86(0.014)^{a}	0.94 (0.03)^{b}	0.89(0.022)^{ab}	0.94(0.01)^{b}	0.042
Aspartic	0.73(0.021)^{a}	0.98(0.027)^{c}	0.92(0.018)^{bc}	0.89(0.018)^{b}	<0.001
Cystine	0.86(0.018)^{a}	----------	0.95(0.046)^{ab}	0.89(0.017)^{a}	0.004
Glutamic	0.79(0.017)^{a}	0.99(0.019)^{c}	0.88(0.01)^{b}	0.93 (0.019)^{bc}	<0.001
Glycine	0.76(0.013)^{a}	0.97(0.011)^{b}	0.89(0.023)^{b}	0.901(0.029)^{b}	<0.001
Histidine	0.79(0.022)^{a}	0.92(0.023)^{b}	0.91(0.017)^{b}	0.88(0.009)^{b}	<0.001
Iso-Leucine	0.28(0.043)^{a}	0.97(0.01)^{c}	0.84(0.022)^{b}	0.81(0.017)^{b}	<0.001
Leucine	0.71(0.009)^{a}	0.99(0.005) ^{d}	0.87 (0.013)^{b}	0.92(0.008)^{c}	<0.001
Lysine	0.82(0.013)^{a}	0.97(0.031)^{b}	0.91(0.016)^{b}	0.91(0.012)^{b}	<0.001
Methionine	0.79(0.022)	0.77(0.03)	0.88(0.042)	0.82(0.021)	0.063
Phenylalanine	0.78(0.022)^{a}	0.93(0.034)^{b}	0.89(0.01)^{ab}	0.89 (0.01)^{b}	0.005
Proline	0.72(0.021)^{a}	0.96 (0.014)^{c}	0.87 (0.011)^{b}	0.901(0.014)^{b}	<0.001
Serine	0.81(0.012)^{a}	0.97(0.026)^{b}	0.94(0.015)^{b}	0.95(0.021)^{b}	<0.001
Threonine	0.77(0.027)^{a}	0.92(0.035)^{b}	0.81(0.016)^{a}	0.91(0.006)^{b}	<0.001
Tyrosine	0.77(0.022)^{a}	0.91(0.018)^{bc}	0.94(0.023)^{c}	0.86(0.018)^{b}	<0.001
Valine	0.71(0.011)^{a}	0.94(0.024)^{c}	0.89 (0.009)^{bc}	0.88(0.011)^{b}	<0.001

highest statistically	
lowest statistically	

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

Table 4.9- Coefficients of digestibility for insect larvae meals and soyabean meal (T4 trial)

Amino acid	Soyabean meal	MW Bran	MW DDGS	BSFL DDGS	P value
Alanine	0.89(0.021)	0.95(0.015)	0.95(0.028)	0.93(0.024)	0.237
Arginine	0.92(0.015)	0.98(0.013)	0.98(0.022)	0.96(0.016)	0.089
Aspartic	0.87(0.02)	0.95(0.021)	0.96(0.025)	0.95(0.033)	0.142
Cystine	0.80(0.053)	0.73(0.047)	0.83(0.087)	0.76(0.089)	0.742
Glutamic	0.90(0.017)	0.98(0.011)	0.99(0.024)	0.89(0.059)	0.153
Glycine	0.84(0.035)	0.94(0.011)	0.93(0.038)	0.80(0.076)	0.176
Histidine	0.89(0.025)	0.95 (0.01)	0.92(0.036)	0.94(0.019)	0.385
Iso-Leucine	0.80 (0.028)^{a}	0.98(0.013)^{b}	0.99 (0.04)^{b}	0.98(0.005)^{b}	0.002
Leucine	0.89(0.009)	0.97(0.02)	0.98(0.015)	0.95(0.039)	0.112
Lysine	0.91(0.017)	0.98(0.019)	0.98(0.029)	0.96(0.017)	0.167
Methionine	0.91(0.02)	0.97(0.025)	0.99(0.023)	0.97(0.019)	0.129
Phenylalanine	0.87(0.021)	0.96(0.018)	0.97(0.031)	0.95(0.005)	0.041
Proline	0.86(0.027)	0.96(0.007)	0.95(0.026)	0.86(0.05)	0.075
Serine	0.89(0.027)	0.90(0.025)	0.90(0.025)	0.88(0.0484)	0.954
Threonine	0.85(0.031)	0.90(0.016)	0.91(0.039)	0.91(0.029)	0.437
Tyrosine	0.86 (0.029)^{a}	0.97(0.005)^{b}	0.95(0.011)^{b}	0.94(0.017)^{ab}	0.012
Valine	0.88 (0.016)	0.95(0.024)	0.96(0.027)	0.86(0.039)	0.059

lowest statistically

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

4.3.4 Bodyweight gain measures from amino acid study (T3, and T4 trials)

For both amino acid trial, the total bodyweight gain of the birds fed the different test diets can be seen in tables 4.10, and 4.11. In T3, bodyweight gain was significantly higher for the 40% soyabean meal, and 60% BSFL Bran inclusion diets (p=0.014), and was significantly lower in 20% BSFL Brew inclusion diet (p=0.014), but there was no significant diffences in the bodyweight gain of the other diets (table.4.10).

Table 4.10- Effect of graded levels of black solder fly and mealworm fed on different substrates on D17-D20 bird weight gain (T3 trial).

Treatment	Bodyweight gain per bird (g) (S.E.)
20% Soyabean meal	65.5(20.51)^{ab}
40% Soyabean meal	118.8(29.49)^{b}
60% Soyabean meal	95.4(16.39)^{ab}
20% BSFL FW	47.4(12.94)^{ab}
40% BSFL FW	25.9(8.63)^{ab}
60% BSFL FW	55.1(22.32)^{ab}
20% BSFL Bran	13.0(16.4)^{ab}
40% BSFL Bran	76.2(17.92)^{ab}
60% BSFL Bran	115.5(32.15)^{b}
20% BSFL Brew	1.0(35.23)^{a}
40% BSFL Brew	78.3(19.55)^{ab}
60% BSFL Brew	62.7(14.26)^{ab}
P value	0.014

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

For T4 trial, bodyweight gain was significantly higher (p<0.001) for the 60% inclusions of insects meals (MW Bran, MW DDGS, and BSFL DDGS) compared to the lower inclusions, and although 60% insect meals were slightly higher than 60% soyabean meal inclusionthey were not significantly different. The semisynthetic diets deleteriously effect feed intake as they

are less palatable to the broilers so, it would be expected that the birds eat less of the 20% diets. Therefore, as expected 20% inclusion diets of all studied groups, the broilers had less BWG compared to the rest of diets and inclusions (table.4.11).

Table 4.11- Effect of graded levels of mealworm black solder fly and fed on different substrates on D17-D20 bird weight gain (T4 trial).

Treatment	Bodyweight gain per bird (g) (S.E.)
20% Soyabean meal	220.4(9.44)^{ab}
40% Soyabean meal	252.02(5.47)^{cd}
60% Soyabean meal	259(8.45)^{cde}
20% MW Bran	225.9(7.17)^{ab}
40% MW Bran	274.2(5.64)^{de}
60% MW Bran	283.3(6.39)^{e}
20% MW DDGS	238.5(5.89)^{bc}
40% MW DDGS	270.2(3.72)^{de}
60% MW DDGS	276.2(9.59)^{e}
20% BSFL DDGS	209 (3.79)^{a}
40% BSFL DDGS	273.2(9.6)^{de}
60% BSFL DDGS	280.2(8.85)^{e}
P value	<0.001

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

4.3.4 Apparent metabolizable energy

Apparent metabolizable energy values for T3 and T4 trials are shown in Table 4.12, and 4.13 respectively. For T3, chicken groups fed 20% inclusion level of BSFL from all the substrates, showed no significant difference AME, even when AME was corrected for nitrogen retention, results of AMEn of broilers treated 20% BSFL from different raising substrates were still not significant (table 4.12). for the broilers fed 40% BSFL inclusions the difference in AME between groups was not significant (P=0.091), however when the results were corrected for N retention, BSFL Bran group had significantly higher AMEn than BSFL Brew (P=0.051), all broiler groups fed 60% BSFL from different substrates on the other hand, showed a significant

difference for both AME, and AMEn, compared to broilers fed 60% soya inclusion, however there was no significant difference in either AME or AMEn between the different BSFL groups (Table 4.12).

Table 4.12- Apparent metabolizable energy and AME corrected for nitrogen for T3 amino acids trial.

Treatment	% inclusion		
	20	**40**	**60**
		AME	
Soyabean	11.04(0.469)	10.803(0.401)	10.69^{a}(0.709)
BSFL FW	11.81(0.256)	11.92(0.259)	14.22^{b}(1.049)
BSFL Bran	11.97(0.342)	12.21(0.735)	13.31^{b}(0.434)
BSFL Brew	11.06(0.661)	10.35(0.709)	13.08^{b}(0.368)
Pvalue	0.354	0.091	0.012
		AMEn	
Soyabean	10.63(0.436)	10.053^{ab}(0.399)	9.501^{a}(0.653)
BSFL FW	11.68(0.232)	11.49^{ab}(0.248)	13.39^{b}(1.007)
BSFL Bran	11.69(0.321)	11.59^{b} (0.692)	12.24^{b}(0.4103)
BSFL Brew	10.79(0.617)	9.82^{a}(0.652)	11.857^{b}(0.342)
Pvalue	0.185	0.051	0.04

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

For T4, both 20%, and 40% insect meal inclusion groups didn't show any significant difference either between each other or compared to soya inclusions (table 4.13). On the other hand, for broilers fed 60% BSFL inclusions, MW DDGS group had significantly the highest AME (P=0.007) and AMEn (P=0.011) compared to the other groups fed 60% insect meal as well as compared to control (table 4.13).

Table 4.13- Apparent metabolizable energy and AME corrected for nitrogen for T4 amino acids trial.

Treatment	% inclusion		
	20	**40**	**60**
		AME	
Soyabean	11.354(0.128)	12.079(0.243)	12.453^{a}(0.179)

MW Bran	11.323(0.737)	13.451(0.743)	13.813[a](1.227)
MW DDGS	10.566(0.847)	12.931(0.576)	16.138[b](0.451)
BSFL DDGS	10.927(0.342)	12.376(0.607)	11.639[a](0.246)
Pvalue	0.758	0.395	0.007
		AMEn	
Soyabean	10.9004(0.118)	11.164(0.234)	10.983[a](0.152)
MW Bran	11.0109(0.7404)	12.573(0.7233)	12.519[a](1.202)
MW DDGS	10.214(0.818)	12.024(0.557)	14.738[b](0.482)
BSFL DDGS	10.625(0.318)	11.8102(0.575)	10.908[a](0.246)
Pvalue	0.771	0.399	0.011

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

4.3.5 Nitrogen retention

Tables 4.14, and 4.15, shows the nitrogen retention values for T3, and T4 amino acids trials respectively. BSFL FW showed significantly ($p<0.001$) the lowest nitrogen retention in all the inclusion levels compared to the rest of treatments as well as soyabean meal. Soyabean meal 20% and 40% inclusion levels showed significantly increased nitrogen retention compared to other treatments of the corresponding inclusion levels, however neither BSFL Bran nor BSFL Brew showed a significant difference in the nitrogen retention value compared to soyabean meal in the 60% inclusion level (Table 4.14). For T4, all insect meal of 20% inclusion showed significantly low nitrogen retention ($p=0.003$) compared to soyabean meal. Significantly lower nitrogen retention can be seen with BSFL DDGS 40% ($p=0.003$), and 60% ($p<0.001$) inclusions compared to the rest of diets in the corresponding inclusion level as well as soyabean meal, MW Bran 60% also showed significantly lower nitrogen retention ($p<0.001$) compared to soyabean meal, nevertheless, there was no other significant differences between the rest of diets or as compared to soyabean meal (Table 4.15).

Table 4.14- Nitrogen retention (g/kg) for T3 amino acids trial

Ni ret (g/kg)	Treatment Level

	20%	40%	60%
Soyabean meal	11.85 (0.998)[c]	21.8 (0.880)[c]	34.42 (1.668)[b]
BSFL FW	3.64 (0.771)[a]	12.42 (0.648)[a]	24.24 (1.314)[a]
BSFL Bran	8.06 (0.671)[bc]	18.03 (1.272)[bc]	30.99 (0.791)[b]
BSFL Brew	7.64 (1.324)[b]	15.38 (1.745)[ab]	35.54 (0.827)[b]
Pvalue	<0.001	<0.001	<0.001

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

Table 4.15- Nitrogen retention for T4 amino acids trial.

Ni ret (g/kg)	**Treatment Level**		
	20%	40%	60%
Soyabean meal	13.19 (0.309)[b]	26.60 (0.329)[b]	42.73 (0.811)[c]
MW Bran	9.08 (0.152)[a]	25.52 (1.178)[b]	37.6 (0.812)[b]
MW DDGS	10.21 (0.876)[a]	26.37 (0.685)[b]	40.71 (0.974)[bc]
BSFL DDGS	8.80 (0.740)[a]	21.26 (0.370)[a]	16.46 (0.953)[a]
Pvalue	0.003	0.003	<0.001

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

Table 4.16 shows the variation between test proteins in total Lys and Total Met is reduced when the amino acids are expressed as a proportion of crude protein rather than as a proportion of the whole material.

Table 4.16- Lysine and Methionine in Insect larvae meals and soyabean meal as a proportion of crude protein

Trial	Protein source	Crude protein	Lysine %	Methionine %	Lysine g/100g CP	Methionine g/100g CP
NA04	Soyabean meal	488.4	2.513	0.551	5.145	1.128
	BSFL Fruit waste	313.4	1.411	0.321	4.502	1.025
	BSFL Bran	399.4	1.984	0.498	4.967	1.247
	BSFL Brewery waste	485.2	2.427	0.706	5.001	1.455
NA07	MW DDGS	499.8	2.308	0.556	4.618	1.112
	MW Bran	461.9	2.529	0.59	5.476	1.277
	BSFL DDGS	409.04	2.219	0.577	5.426	1.411

Table 4.17- Mean coefficients of digestibility and digestible amino acid content of two different insect larvae meals raised on different substrates compared to soyabean meal in the two amino acids trials (T3, and T4).

Trial	Protein source	Coefficients of Digestibility	Digestible amino acid content
NA04	**Soyabean meal**	0.751	18.551
	BSFL Fruit waste	0.947	26.003
	BSFL Bran	0.893	15.74
	BSFL Brew	0.893	21.593
NA07	**Soyabean meal**	0.873	21.283
	MW Bran	0.943	30.863
	MW DDGS	0.949	28.418
	BSFL DDGS	0.911	17.198

4.4 Discussion

The aim of the two studies in this chapter was to evaluate the nutritional profile of two type of insect species, each were raised on various waste products compared to soya, which is considered a standard poultry feed, particularly with respect to the amino acid profile, the digestible amino acids and the coefficient of digestibility. A second aim was to evaluate the energy quality of graded insect meal inclusions in comparison to graded levels of soya.

Performance data over 3 days recorded while birds are adapting to diets not designed to fully meet their long term need for structural form and fibre in the diet has limited value. The data was recorded to confirm birds grew while on the test diets. The data for both studies confirm birds on all treatments gains weight but it is difficult to draw deeper meaning from the findings as the main effect was from the extreme differences in dietary protein supply that are unavoidable in linear regression studies to determine the digestible amino acid content of a protein source, especially for the 20% protein source diet inclusions. The optimal inclusion levels and linearity of response to graded levels of protein source have been extensively evaluated (Clarke, 2001, Ravindran and Bryden, 19993; Rodehutscord *et al*., 2004) and indicated that 3 levels of inclusion at 20%, 40%, and 60% are sufficient for regression analysis. Amino acid contents of insect larvae meals were equivalent or even higher for both dispensable and indispensable amino acids compared with soya, apart from the BSFL larvae groups raised on either fruit waste or bran, which leads to speculation that these raw materials were contaminated with frass, thereby reducing the total content of digestible of AA.

The proximate analysis data of insect proteins showed that insects are a good source of protein and fat. Mealworm from different sources showed protein content equal or higher than soyabean meal, although the fat content of insect larvae meals was very high, these results are similar to those reported by *De Marco et al.,* (2015). This latter study showed high protein content of insect meal raised on cereal grains substrate especially mealworm, and also showed high fat content for both mealworm and BSFL fed cereal grains substrate (*De Marco et al*., 2015). However, BSFL protein sources especially BSFL FW, and BSFL Bran showed poor protein content, less than soyabean meal and that could rather indicate that either the insects feeding substrates (Fruit wastes and Bran) had poor protein content or that these insect larvae meals had high amount of frass mixed in with the insects which rendered the nutritional composition poorer. Nevertheless, the total amino acid content of insect larvae meal compared to soyabean meal was high overall, and it is worth noting that most of the insects used in these trials were fed waste materials. Utilising waste materials in this manner reduces waste as they are converted into larval mass and improves the sustainability and economic viability of insect production. Insect frass could be considered a waste product from insect production, but it can be utilised in sustainable agriculture as agrochemical substitute and as a viable fertilizer as well as its role in improvement of plant soil (Poveda, 2021).

4.4.1 Digestible amino acid content and coefficients of digestibility

Mealworm proved to contain high quality protein and a substantial amino acid concentration, amino acids digestibility results confirmed the hypothesis as well as the BSFL amino acids digestibility results showing high digestible amino acids on both dispensable and indispensable amino acids). Lysine is considered to be the first limiting amino acid, and therefore, lysine is frequently incorporated with the basal soyabean meal diet for poultry, even where the rest of amino acids are abundant (Evans and Patterson, 2007). Methionine is considered the second limiting amino acid (Remus *et al*., 2015). Focusing on two crucial amino acids (Lysine, and Methionine) therefore, applying these amino acids in synthetic form as additives to the basal diets compensates for the environmental effect of losing a large percentage through nitrogen excretion, and aiming for a better muscle deposition (Remus *et al*., 2015). The lysine and methionine percentage and content in g/100g crude protein for T3, and T4 trials are represented in table 4.16. The content of both lysine and methionine for insect larvae meal was comparable to soyabean meal, except for BSFL raised on Fruit waste or raised on Bran substrates which has lower protein content as previously described.

Bran as a substrate is considered the highest in Methionine and lysine content for example rice bran contains 6.53 g/100g and 1.67 g/100g crude protein for Lysine, and methionine respectively (Zaky *et al*., 2020) compared to DDGS which contains 1.93 g/100g, and 1.55 g/100g crude protein for Lysine and methionine respectively (Youssef *et al*., 2008), however, that didn't show in the amino acids profile of BSFL, on contrary, BSFL fed on DDGS showed higher concentrations of Methionine and lysine compared to BSFL fed on bran. That probably could be the result of mis utilization of bran as a substrate although it is considered standard mealworm substrate, that also shows the crucial difference in substate utilisation between different insects and how the effect of substrate could differently affect each type of insects' nutrient profile.

The digestible amino acid contents of the protein sources investigated in this chapter are shown in figure 4.2, and 4.3 and the coefficients of digestibility for the protein sources have been detailed in table 4.8 and 4.9; and for simplicity, the average COD and digestible amino acid content are shown in table 4.16 to show a comparative ranking and a feed stock comparison. Ravindran *et al*., (2005) recorded the COD of soyabean meal to 0.82, while Scholey, (2012) reported the COD of soyabean meal as 0.78. the same difference in COD of soyabean meal from two different amino acids trials that were performed in this chapter was noticed, as in T3 the COD of soyabean meal was 0.75 while in T4 was reported as 0.87, which could be due to a difference in source of soyabean meal. It is unlikely to be due to either bird age or a difference in methodology as both T3 and T4 were performed in under same conditions with the same age and strain of bird. This assumption that the soya batches were variable is supported by the results of the COD of Insect larvae meal of different types and from different raising substrates which was very consistent in both trials (table 4.17). No digestible amino acid content for insect larvae meal in broilers have been reported in previous literature, however in these trials the values of the digestible AA from chickens fed BSFL from different raising substrates varied substantially however, the overall values were still significantly higher than that of soyabean meal. This indicates that insect larvae protein from BSFL has high digestible amino acids in broilers. Similar results were noted in T4 with the overall high concentration digestible amino acids of chickens fed MW or BSFL raised on DDGS substrate. The BSFL protein sources from Agrigrub which were raised on fruit wastes and bran did have reduced the protein yield (313.4, and 399.4 g/kg on dry matter basis respectively), however, this did not negatively affect either the digestible amino acid content or coefficients of digestibility (table 4.17). That could highlight the value of insect larvae frass and its nutritious value and could be an important point for future research to evaluate the insects which have lower protein content but good AA digestibility on these sources.

Frass is a common term for insects post feeding substrates and frass resulting from BSFL post feeding activity is called residue or BSFL digestate (Green and Poppa, 2012). Frass is a substance in the form of composit and the properties of immature composit. Insects' frass is basically the combination of microbes that is responsible for fermentation of substrate, these microbes resulted for the insect feeding activity, in addition to both insect defecation and shedding of exoskeleton through the larval stages (Schmitt, and de Vries, 2020).

BSFL prefer the high moisture content substrate which affects the later post feeding treatment and separation from frass. The more efficient separation the better the quality of larvae protein product. Reducing moisture content of the frass to facilitate the separation of larvae by temperature, tends to slow larval development. It was suggested by Dortmans *et al.*, 2017, that the management of frass at larvae post feeding stage using compositing method which is mainly mixing the frass with any other wastes or earlier dry frass from insect rearing facility is considered the best method to obtain free of frass high quality insect protein (Surendera *et al*., 2020)

4.4.2 Apparent metabolizable energy of insect larvae meals and soyabean meal

Metabolizable energy (AME) is a method used to determine the available energy in feed with proven viability in the poultry sector., Energy is not a nutrition indicator, it is rather a measure for the nutrients that are the source of energy in the diet such as lipids, carbohydrates, and proteins. AME can be corrected for zero Nitrogen retention and the value corrected is referred to as AMEn (Abdollahi *et al.*, 2021). The correction of AME for zero N retention tends to re-adjust and highlight the true energy values especially for high quality protein sources (Abdollahi *et al*., 2021). The apparent metabolizable energy corrected for zero nitrogen retention of the Insect larvae protein sources with different protein levels (20%, 40%, and 60%) as well as soyabean meal investigated in this chapter are summarised in figure 4.6. experimental diets with higher levels of insect larvae protein (40%, and 60%) seems to have overall higher values for AMEn, with MW being slightly higher than BSFL protein source. On the other hand, values of different grades of soyabean meal were very low compared to insect larvae protein sources. It was also noticed that the contamination with frass in BSFL protein sources where larvae were fed either fruit wastes or bran did not reduce energy the values as expected (Fig. 4.6). That result indicates the effect of high fat content of insect larvae meal on the levels of AMEn, however, that cannot be confirmed unless further analysis of the AMEn of defatted insect larvae meal from both mealworm and Black soldierfly larvae is performed.

4.5 Conclusion

In conclusion, amino acid profile of insect larvae meal has proven to be rich and of high-quality protein compared to soyabean meal which is the main source of protein used in poultry feed even though the feed sources for the larvae were waste substrates, underlining the ability of insects to convert waste into high quality protein mass. The insect raising substrate have also proven to affect the protein and the amino acid content, where DDGS substrate improved the protein content of BSFL, and mealworm compared to the rest of substrates, but didn't affect the digestibility coefficient of broilers fed the insects larvae meal. Insect processing and effective separation of frass could improve insect protein content. Larvae suppliers need to pay attention to the separation techniques of insect larvae from frass to maximise the value of the insect protein. Last but not least, the source of soyabean meal has been shown to affect the coefficient of digestibility of broilers that was well evidenced when the COD of broilers fed soyabean meal from T4 was significantly higher than that of T3 trial. Nevertheless, the COD of Insect larvae meal from both trials was consistent.

Chapter 5– Effect of mealworm inclusion in broiler diets on performance, nutrient digestibility, and intestinal morphology.

5.1 Introduction

This study was to investigate the effect of replacing soya in diets with graded levels of insect meal (Mealworm larvae). There is an ongoing drive to find a protein alternative for poultry production to ensure the industry becomes less dependent on soya. This is due in part to environmental reasons where soya production and transportation participates in greenhouse gas emissions and that leads to a high carbon footprint. Soya also has sustainability problems as the overcultivation of soya comes at the expense of wild land. Insects could work as a perfect alternative for soya meal due to the rich amino acid profile that is comparable to soya (*De Marco et al*., 2015; Khan *et al*., 2018). However, the total replacement of soya by insect meal would be economically costly. A crucial factor that is affecting the decision to utilise insects in the UK is the price of insect meal in the market. In 2020, the price of insect meal reached $4,250, and $6,066 per metric tonne, which is significantly higher than both soya and fishmeal (www.feednavigator.com, 2021).

Previous studies have shown the value of using insect meal as animal feed (Sanchez Muros *et al*., 2014; Iaconisi *et al.,* 2017). However, the financial cost of replacing the total soyabean inclusion with insect meal could lead to disregarding the benefits. This raises questions over whether a dose response study using low doses could have positive effect on chicken performance, gut histology and immunity markers. Previous studies have investigated the potential use of varying inclusions of partially defatted BSFL (Dumas *et al*., 2018) or full fat BSFL (Mohan *et al*., 2023), as a soya replacement for poultry feed. Other studies have provided

varied results in response to inclusions of insect meal gaining some more insight on the potential use of Mealworm and BSFL as potential prebiotics or gut health enhancers.

Józefiak *et al*., (2018) fed a soya-based diet with BSFL and mealworm at 0.05 and 0.2% and reported that at days 15-35, feed intake significantly increased ($P \leq 0.05$) in broilers. Moreover, the supplementation with BSFL in the previously mentioned doses resulted in a significant change in the broiler's gut microbiota (Józefiak *et al.,* 2018). Furthermore, a study by Borrelli *et al.* (2017) reports a similar hypothesis with significant increases in the diversity of microbiota, where experimental groups of layer hens provided an insect-based diet showed a higher count of Firmicutes (57.69±2.37%) and Proteobacteria (8.38±0.47%). The mechanism behind these modulations to intestinal flora in broilers fed BSFL is currently uncertain, but emerging research indicates multiple mechanisms may be at play. In a study that examined the potential of black soldier flies as a source for antimicrobial peptides (AMPs), researchers injected black soldier fly larvae with Escherichia coli bacteria to induce an immune response (Alvarez *et al.,* 2019). They found over 90 peptides in their analysis, four of which they determined to have pronounced anti-*H. pylori* activity. Only larvae injected with *E. coli* were found to have anti-*H. pylori* antimicrobial peptides, indicating that these peptides were produced in response to bacterial exposure. Following this research, others have speculated that the AMPs in BSFL are responsible for reduced energy loss via unnecessary gastro-intestinal immune response (de Souza Vilela *et al.,* 2021). The authors reported a 4-fold decrease in CD3+ T lymphocytes and a 9.7-fold decrease of CD3+CD8+ intestinal cytotoxic T lymphocytes occurred in broilers fed 20% BSFL compared to the control group. Mucin is a protein produced by the goblet cells in the epithelial layer lining the gastrointestinal tract and the source of mucus formation which are the secretions in turn responsible for management of gut microbiota and gut health (Kang *et al.,* 2022). A study by Colombino *et al*., (2021) showed that low doses as low as 5% of insect meal has not affected the mucin formation, which is

considered a positive sign however, it has affected the composition of caecal microbiota by making minor modulations in the caecal microbial fauna and slightly enhancing the presence of beneficial fatty acids making bacteria, these fatty acids are considered a very essential energy source for enterocytes in the gut. This is among other research that proved how low levels of insect meal supplementation can be more useful to gut health than higher insect meal inclusions (Biasato *et al*., 2018; Biasato *et al*., 2019).

In chapter 4, semisynthetic diets were used to determine the amino acid digestibility, however these diets gave no indication of how the products are utilised in realistic poultry rations. Additionally, based on the above-mentioned evidence from previous literature, the beneficial impact of insect meal inclusion at low doses could be that of a prebiotic. Higher doses of insect meal act as a protein replacement and could also beneficially impact bird performance and body immunity markers.

The aim of the current chapter is: Investigate the effect of differing dietary inclusion levels of yellow meal worm on broiler performance, health, immune status and nutrient utilisation. Therefore, a 35-day bird trial was designed using nutritionally balanced diets to compare broiler health and performance when fed increasing doses of MW against a control diet. Mealworm was included at 0.3% to investigate its use as an additive or at a higher level of 5% to be included as a protein replacement. An intermediate level of 1% was also included.

The first hypothesis is that the MW inclusion will have enhance the broiler performance compared to the control diet, and the second hypothesis is that the MW supplementation will improve nutrient digestibility as well as the jejunum morphology of broilers.

5.2 Materials and methods

5.2.1 Husbandry Conditions

320 one-day-old male Ross 308 broilers were sourced from PD Hook Cote hatchery from a flock aged 34 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. Chicks were individually weighed on arrival, then randomly assigned to 50 mesh sided pens (8 birds per pen), littered with a wood shavings substrate. Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible. Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was granted by the University ARES Ethics Committee and was logged as project ARE212203.

5.2.2 Diet formulation and condition of animals

Mealworm was externally sourced from a commercial company, in dried and ground form and all the treatment diets were manufactured by NTU, and another product from a different company (COMP) was also sourced for the study. The trial used four treatments (table 5.1) with three phases (starter, grower, and finisher). Three basal diets (one per phase) were commercially manufactured by Target Feeds (Shropshire, UK). Diets were formulated by a commercial nutritionist to meet the age and strain of the bird (table 5.2). Each basal diet was divided into six treatments in house. The externally sourced mealworm was added to treatments EXT0.3, EXT1, and EXT5 at either 3g/kg (EXT0.3) 10g/kg (EXT1) or 50g/kg (EXT5), and a competitor mealworm product was added to COMP5 treatment at 50g/kg. Proximate analysis was performed to confirm composition (table 5.3)

Table 5.1- Dietary treatments, soya replacement with insect meal (g/kg)

Diet code	Diet Treatments	Soya inclusion	Insect inclusion
Control	Control	324.3	0
EXT0.3	0.3% Mealworm	321.3	3
EXT1	1% Mealworm	314.3	10
EXT5	5% Mealworm	274.3	50
COMP5	5% Mealworm	274.3	50

Table 5.2- Dietary Treatments: basal diets (g/kg)

Ingredient	Starter	Grower	Finisher
Wheat	615	635	670
HiPro soya	324.3	291.3	252.9
Soya oil	15.8	29.4	38.9
Salt	3.8	5	3.8
Limestone	1.6	3.8	1
Dicalcium Phos, 18%P	21.7	19.1	17.4
Lysine HCl	2.8	2.2	2.3
DL-Methionine	3.2	2.7	2.6
Threonine	1.6	1.2	1.2
Vitamin & Mineral premix	5	5	5
Phytase (QB)	0.1	0.1	0.1
Titanium dioxide	5	5	5

Table 5.3- Proximate analysis of basal diets for dose response trial

Starter	% DM	% Ash	% Fat	% Protein

Control	87.51	5.88	2.66	24.75
EXT0.3	87.59	5.77	2.81	23.05
EXT1	87.68	5.91	2.93	22.70
EXT5	87.93	5.54	3.85	19.46
COMP5	87.96	6.09	3.19	23.14
Grower				
Control	87.81	5.63	3.88	19.89
EXT0.3	87.77	5.87	4.05	21.45
EXT1	88.01	5.63	4.31	21.21
EXT5	88.40	5.37	5.52	19.63
COMP5	88.31	5.54	4.27	20.37
Finisher				
Control	87.78	5.05	4.79	19.29
EXT0.3	88.12	4.84	5.03	19.99
EXT1	88.58	5.14	5.23	18.19
EXT5	88.27	4.87	6.22	18.80
COMP5	88.71	5.00	5.32	19.29

5.2.3 Treatment Schedule / randomisation plan / condition of animals

Each treatment diet was fed to 10 replicate pens containing 8 birds each and only birds weighing between 40g and 50g were placed on trial. The combined weight of each pen was recorded on day 0. Treatments were randomly allocated via an online randomisation tool (random.org) by block (one block of 5 pens to include all treatments) to reduce any effect of ventilation and temperature differences within the room. Pen allocation is shown in table 5.4 and a plan of the experimental room is shown in appendix B.

Appendix B: Pen allocation

pen number	block	Treatment		pen number	block	Treatment	
1	1	B	T2	28	6	B	T2
2	1	E	T5	29	6	A	T1
3	1	A	T1	30	6	D	T4
4	1	D	T4	31	7	C	T3
5	1	C	T3	32	7	D	T4
6	2	B	T2	33	7	A	T1
7	2	C	T3	34	7	E	T5
8	2	A	T1	35	7	B	T2
9	2	E	T5	36	8	C	T3
10	2	D	T4	37	8	B	T2
11	3	C	T3	38	8	E	T5
12	3	E	T5	39	8	A	T1
13	3	A	T1	40	8	D	T4
14	3	D	T4	41	9	B	T2
15	3	B	T2	42	9	D	T4
16	4	B	T2	43	9	C	T3
17	4	E	T5	44	9	E	T5
18	4	C	T3	45	9	A	T1
19	4	A	T1	46	10	A	T1
20	4	D	T4	47	10	B	T2
21	5	E	T5	48	10	D	T4
22	5	B	T2	49	10	E	T5
23	5	C	T3	50	10	C	T3
24	5	A	T1				
25	5	D	T4				
26	6	C	T3				
27	6	E	T5				

Randomized by block using random.org.

5.2.4 Study observations

Bird observations were performed a minimum of twice daily to ensure bird welfare and environmental conditions were maintained. Temperature and/or ventilation were adjusted depending on bird behaviour. Dead birds were removed and weighed, and any unhealthy birds (defined as any bird displaying discomfort or distress) were culled and recorded. Bird feed intake was calculated as per the method detailed in section 2.4.1. Birds were weighed weekly by pen on days 0, 10, 21, 28 and 35, as per section 2.4.1. Additionally, feed intake and bird weight were used to calculate weekly feed conversion ratio (FCR).

On day 35, birds were sequentially fed fresh diet for a minimum of 30 minutes to ensure adequate gut fill prior to euthanasia. Two average sized birds per pen were humanely euthanized by cervical dislocation. Post-mortem blood samples were collected and split into tubes containing EDTA as an anti-coagulant for post-trial immunoglobulin analysis (as per section 2.4.2) or into serum tubes for antioxidant analysis (section 2.5.10). The blood was centrifuged at 3000RPM for 10 minutes (Thermo Scientific, Megafuge 8, Fisher, UK) and plasma/serum removed and stored at -20°C.

The ileum and jejunum were excised from the end of the duodenal loop to the ileal-caecal-colonic junction then split at Meckel's diverticulum. Ileal digesta was collected from each bird by gentle digital pressure into one pot per pen. 5 cm of jejunum was fixed in Bouin's solution for a minimum of four hours prior to transfer into 70% IMS solution for histology analysis.

Ileal digesta was pooled into one pot per pen and frozen at -20^{o}C before being freeze dried and ground with a pestle and mortar. Diets and ileal digesta were analysed for gross energy by bomb calorimetry at an external lab (PAS, Shropshire, UK). Protein content was analysed via Dumatherm (Gerhardt, UK) as described in section 2.5.2. Titanium dioxide content of the diet

and digesta was measured using the method of Short *et al*., (1996; section 2.5.5). Dry matter was analysed as per sections 2.5.4, and 2.5.1. Left and right breast, thigh, and drumstick were collected for estimating carcass yield from 1 bird per pen.

5.2.5 Statistical analysis of data

Outliers were removed from data if they fell either two standard deviations above or below the mean**.** Statistical analysis for performance data was performed using SPSS v.28 (IBM spss statistics, 2021). KS testing was used to determine data normality, followed by one-way ANOVA and Univariate analysis as appropriate. Duncan post hoc tests were used to elucidate differences in treatments. Statistical significance was declared at $p<0.05$.

5.3 Results

5.3.1 Environment

Temperature and ventilation settings within the room were maintained based upon the age of the bird as stipulated in the breed guide. The ammonia level within the room was monitored through husbandry observations and any suspected increase in level was reported so that the ventilation could be adjusted as necessary.

5.3.2 Health and Conditions

Mortality data is shown in table 5.4. During the whole 35d study, mortality was 2% which is considered standard for trials conducted at the NTU unit and lower than would be expected in a commercial setting which would typically be more than 4%. There was no apparent effect of treatment on mortality as can be seen in table 5.4 which splits the mortality by week and dietary treatment.

Table 5.4- Bird mortality for dose response trial

Treatment	d0-10	D10-21	D21-28	D28-35	Total
Control	0	1	0	0	1
EXT0.3	0	2	0	0	2
EXT1	2	1	0	0	3
EXT5	2	0	0	0	2
COMP5	0	0	0	0	0

5.3.3 Bird Uniformity

Upon arrival at the research unit, all chicks were individually weighed and only birds weighing between 38 and 46g were placed. The mean start weights for each treatment are shown in table 5.5. There was no statistical difference in the start weight of the chicks between treatments.

Table 5.5- Average start weight for chicks for dose response trial

Treatment	d0 BW/bird (g)
Control	39.9
EXT0.3	40.4
EXT1	40.3
EXT5	40.5
COMP5	40.4
S.E.M	0.24
P value	0.353

5.3.4 Weekly average bird weight

By day 10, there was a significant increase ($P< 0.05$) in bodyweight for the birds fed the higher dose of mealworm treatment (5%) compared to the control, with birds weighing on average over 25g more. By day 21 the EXT5 treatment group had significantly higher ($P<0.05$) bird

body weight compared to Control fed birds, (table 5.6). For D28 and D35 there was no significant difference between any of the treatment groups for bird weight compared to control (Table 5.6)

Table 5.6- Weekly Average Bird Weight for dose response trial (g ±SEM)

Diet	D0 BW	D10 BW	D21 BW	D28 BW	D35 BW
Control	39.9	191 [b]	765 [b]	1330	2059
EXT0.3	40.4	201 [ab]	774 [ab]	1370	2108
EXT1	40.3	201 [ab]	780 [ab]	1362	2105
EXT5	40.5	216 [a]	830 [a]	1412	2139
COMP5	40.4	217 [a]	799 [ab]	1392	2126
SEM	**0.235**	**8.09**	**21**	**33.8**	**44.6**
P value	**0.353**	**0.026**	**0.03**	**0.512**	**0.758**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

5.3.5 Weekly Performance

Body weight gain

Cumulatively there was a significant increase in body weight gain during the period of day 0-10 for EXT5 and COMP5 (higher mealworm inclusion) compared to the control (p=0.02). However, there was no significant difference in body weight gain during the periods of day 10-20, 21-28, and 28-35 between the treatments compared to control (table 5.7).

Table 5.7- Body weight gain for dose response trial (g +/- S.E.M)

Treatment	D0-10 BWG	D10-20BWG	D21-28 BWG	D28-35 BWG

Control	151 [a]	573	566	729
EXT0.3	161[ab]	573	596	738
EXT1	161[ab]	579	582	743
EXT5	175[b]	614	582	727
COMP5	177 [b]	582	593	735
S.E.M	**8.0**	**16.7**	**23.3**	**16.3**
P Value	**0.020**	**0.402**	**0.901**	**0.959**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$).

Feed intake.

Results of Feed intake (FI) of the current study is represented in table 5.7. On D0-10, EXT 5 birds showed significantly higher FI ($p=0.016$) compared to EXT1, COMP5, and control, however, the rest of treatment groups EXT0.3, EXT1, and COMP5 did not show any significant difference in FI compared to the control. On D10-20, EXT5 group had significantly higher FI ($p=0.02$) compared to COMP5, and control, while there was no significant difference in FI between EXT5 and either EXT0.3 or EXT1 and no significant difference in FI among EXT0.3, EXT1, and COMP5 treatment groups or compared to control. On D21-28, EXT0.3, and EXT5 showed significantly higher FI compared to COMP5 treatment group as well as control, while EXT1 did not have any significant difference in FI compared to any of the treatments as well as control. On D28-35, EXT0.3 showed higher FI compared to COMP5 and control however, EXT0.3 didn't show any significant difference with either EXT1 or EXT5 treatment groups (table 5.8). EXT5 also recorded higher FI than COMP5 for this week (Fig.5.1).

Table 5.8- Feed intake for dose response trial (+/- S.E.M)

Treatment	D0-10 FI	D10-20 FI	D21-28 FI	D28-35 FI
Control	287[a]	830 [a]	921[a]	1155[ab]
EXT0.3	314[ab]	862[ab]	1036[b]	1264[c]
EXT1	298[a]	854[ab]	977[ab]	1203[abc]
EXT5	347[b]	897[b]	1047[b]	1248[bc]
COMP5	285[a]	832[a]	922[a]	1120[a]
S.E.M	**13.6**	**20.1**	**28.6**	**33.9**
P Value	**0.016**	**0.020**	**0.001**	**0.023**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

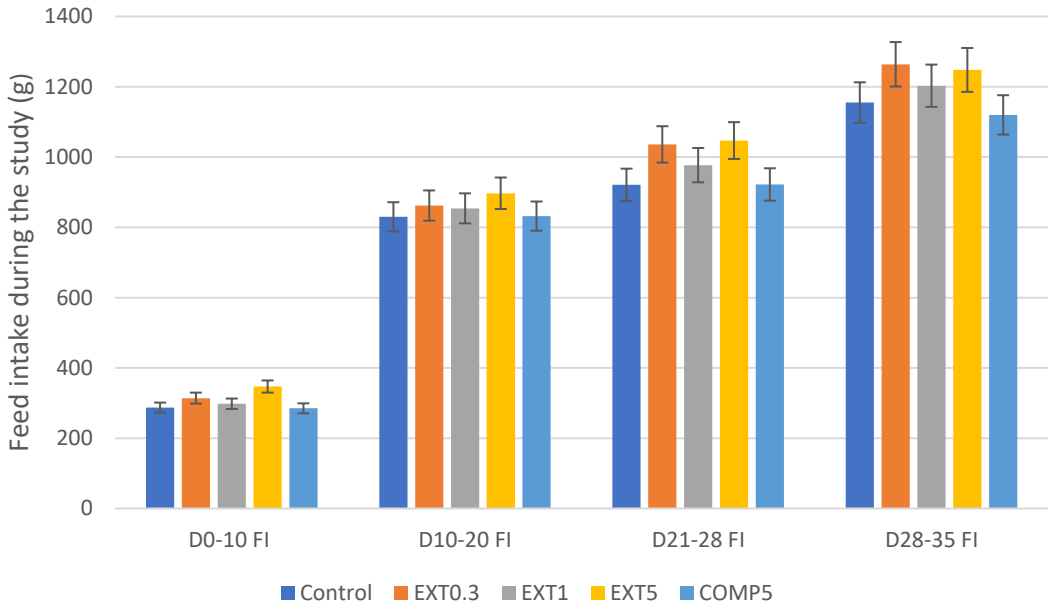

Figure 5.1- Feed intake values of mealworm graded doses against control.

Feed conversion ratio.

Results for feed conversion ratio of the current study are shown in table 5.9. During the periods of day 0-10 and 10-20 there was no significant difference in feed conversion ratio between treatments. However, between day 21-28 and 28-35 there was a significant increase in FCR for

EXT0.3 and EXT5 ($p<0.05$) compared to COMP5, however there was no significant difference between any of the treatments compared to control (table 5.9).

Table 5.9- Feed conversion ratio for dose response trial +/- SEM

Treatment	D0-10 FCR	D10-20 FCR	D21-28 FCR	D28-35 FCR
Control	1.96	1.45	1.64^{ab}	1.59^{ab}
EXT0.3	1.98	1.54	1.77^{b}	1.72^{b}
EXT1	1.93	1.48	1.71^{ab}	1.62^{ab}
EXT5	2	1.46	1.8^{b}	1.72^{b}
COMP5	1.65	1.43	1.56^{a}	1.53^{a}
S.E.	**0.127**	**0.052**	**0.073**	**0.047**
P Value	**0.308**	**0.661**	**0.005**	**0.023**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p=<0.05$)

5.3.6 Cumulative Bird body weight gain

By day 21, there was a significant increase in bodyweight gain for EXT5 compared to control ($p=0.043$) but there were no other significant differences with other treatment groups. For D0—D28, and D0-D35 there was no significant differences in cumulative BWG between any of the treatment groups or compared to control (Table 5.10).

Table 5.10- Cumulative Body Weight Gain (BWG) for dose response trial (g) (+/- S.E.M)

BWG (g)	Control	EXT0.3	EXT1	EXT5	COMP5	S.E.M	p value
D0-D21	725^{a}	733^{ab}	740^{ab}	789^{b}	759^{ab}	21.0	0.043
D0-D28	1291	1330	1322	1371	1351	33.8	0.519
D0-D35	2019	2067	2065	2099	2086	44.6	0.763

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA ($p=<0.05$)

5.3.7 Carcass yield

There was no significant difference in breast yield and total carcass yield between treatment groups, except for COMP5, which showed significantly higher breast yield and total carcass yield compared to the control. On the other hand, there was no significant difference in thigh yield, drumstick yield, and dressing % between any diets (table 5.11) (Fig.5.2).

Table 5.11- Carcass yield for dose response trial (±SEM)

Carcass yield	Breast yield(g)	Thigh yield(g)	Drumstick yield (g)	Total carcass yield (g)	Dressing(%)
Control	438[a]	214	185	837[a]	38.9
EXT0.3	503[ab]	232	192	927[ab]	40
EXT1	483[ab]	228	188	900[ab]	40.1
EXT5	461[ab]	215	181	857[ab]	39.4
COMP5	518[b]	232	196	946[b]	40.2
S.E.M	26.5	7.1	6.0	38.0	0.52
P value	0.040	0.168	0.457	0.045	0.392

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

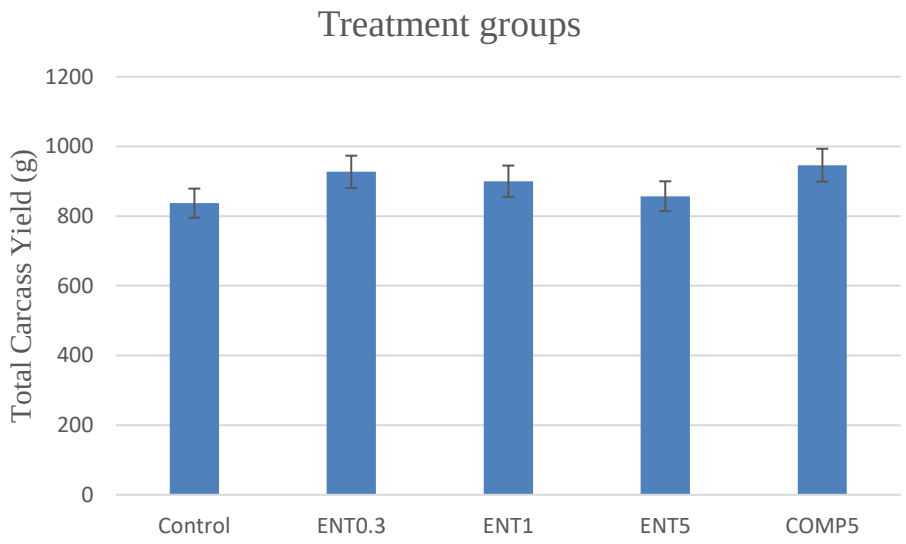

Figure 5.2- Total Carcass yield of the mealworm doses compared to control.

5.3.8 Nitrogen digestibility

Results of Nitrogen Digestibility Coefficient (NDC) for dose response trial for day 21, and day 35 of the study are represented in table 5.12. On day 21, NDC of the lower MW dose (EXT0.3) was significantly higher than EXT5, and COMP5 but was not significantly different to the control. Additionally, all MW treatment groups had no significant difference in NDC at D21 compared to the control group. On day 35, NDC of EXT0.3 was significantly lower (p=0.012) than EXT1, EXT5, but showed no significant difference compared to COMP5 or the control. On the other hand, EXT1, and EXT5 treatment groups were significantly higher in NDC (p=0.012) compared to the control. (Table 5.12).

Table 5.12- Nitrogen digestibility coefficient (NDC) for dose response trial on day 21, and 35(±SE)

Treatment	Nitrogen Digestibility Coefficient	
	D21	**D35**
Control	0.825 (0.009)^{ab}	0.687 (0.009)^{a}
EXT0.3	0.852 (0.009)^{b}	0.689 (0.019)^{a}
EXT1	0.824 (0.012)^{ab}	0.729 (0.010)^{bc}
EXT5	0.817 (0.010)^{a}	0.739 (0.008)^{c}
COMP5	0.804 (0.010)^{a}	0.701 (0.013)^{ab}
P value	0.035	0.012

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

5.3.9 Apparent metabolizable energy

Apparent metabolizable energy (AME), apparent metabolizable energy corrected for nitrogen retention (AMEn), and nitrogen retention (Ni ret) values on days 21 and 35 of treatments in the dose response trial are shown in table 5.13, and 5.14 respectively. On day 21, there was a significant increase (p=0.019) in AME for COMP5 compared to EXT5 as well as control, however no significant difference compared to either EXT0.3 or EXT1. When AME was

corrected for nitrogen retention, AMEn values showed that COMP5 was significantly higher than EXT0.3, EXT5 and control (p=0.006), but not significantly different to EXT1. On the other hand, EXT0.3 showed significantly the highest Ni ret (p<0.001) compared to the rest of the treatments as well as control, while COMP5 was significantly lower compared to EXT0.3, EXT1, and EXT5 as well as control, and EXT5 showed lower Ni ret compared to EXT0.3 and to control but was not significantly different from EXT1 (Table 5.13).

Table 5.13- Day 21 apparent metabolizable energy for dose response trial(±SE)

Treatment	AME (MJ/kg)	AMEn (MJ/kg)	Ni ret (g/kg)
Control	11.74 (0.244)^{a}	10.76(0.235)^{a}	28.44(0.313)^{c}
EXT0.3	12.08 (0.109)^{ab}	11.01(0.099)^{a}	31.17(0.336)^{d}
EXT1	12.18(0.205)^{ab}	11.22(0.191)^{ab}	27.97(0.422)^{bc}
EXT5	11.69(0.171)^{a}	10.75(0.161)^{a}	27.25(0.323)^{b}
COMP5	12.54 (0.201)^{b}	11.64 (0.191)^{b}	26.202 (0.383)^{a}
P Value	**0.019**	**0.006**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

On day 35, there was a significant increase in AME of COMP5 (p<0.001) compared to EXT0.3, EXT1 and control but was not significantly different to EXT5. However, although EXT5 was significantly higher than EXT0.3 (p<0.001), it was not significantly different to EXT1, COMP5 or control. Even when AME was corrected for nitrogen retention, AMEn of COMP5 showed the same distinction, the only difference between AME and AMEn recorded was that EXT5 was also significantly higher (p<0.001) than control for AMEn. On the other hand, the Ni ret of EXT0.3 was the highest (p<0.001) compared to the rest of the treatments, whereas COMP5 was not significantly different to either EXT5 or control but was significantly higher (p<0.001) than EXT1 and significantly lower (p<0.001) than EXT0.3 (table 5.14).

Table 5.14- Day 35 apparent metabolizable energy for dose response trial(±SE)

Treatment	AME (MJ/kg)	AMEn (MJ/kg)	Ni ret (g/kg)
Control	13.09 (0.111)^{ab}	12.2 (0.103)^{a}	25.756 (0.271)^{c}
EXT0.3	13.03(0.132)^{a}	12.09(0.126)^{a}	27.322(0.227)^{d}
EXT1	13.08(0.972)^{ab}	12.26(0.089)^{ab}	23.98(0.271)^{a}
EXT5	13.37(0.098)^{bc}	12.52(0.092)^{bc}	24.89(0.227)^{b}
COMP5	13.68(0.113)^{c}	12.81(0.108)^{c}	25.44(0.189)^{bc}
P Value	**<0.001**	**<0.001**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

5.3.10 Other measures

Table 5.15 represents the effect of the treatment for dry matter digestibility (DMD) at D21 and D35, and D35 litter DM at D10. There was no significant effect of the dietary treatment on D21 DMD or D35 litter DM at D10. EXT5 had significantly higher D35 DMD compared to the other dietary treatments. All birds had normal scores for lesions at D21 and D35 (gizzard, proventriculus and small intestine).

Table 5.15- Litter dry matter content and vent score

Bird weight gain (g)	Control	EXT0.3	EXT1	EXT5	COMP5	SEM	p value
D21 DMD	94.9	94.6	94.8	94.8	94.8	0.25	0.947
D35 DMD	95.6^{b}	95.6^{b}	95.4^{b}	96.2^{a}	95.6^{b}	0.18	0.043
D35 litter DM (%)	81.4	84.5	84.0	83.4	82.1	1.11	0.274
proportion of birds with vent score on D10	0.15	0.20	0.11	0.15	0.24	0.051	0.456

Means within the same raw that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

5.3.11 Immunoglobulin A, and Interleukin-6 (immunity markers)

From understanding the connection between the different physiological parameters and their effect on the overall health status of poultry, it was important to investigate the effect of the mealworm supplementation at 5% dose (from both sources) on the presence of two crucial immunity markers, Immuno-globulin A (Ig A), and interleukin-6 (IL-6). IL-6 is a cytokine that is usually released in large amounts upon infection or stress. IL-6 mediates the production of antibodies, or on other occasions it stimulates or inhibits cell growth, while IgA production within the normal levels promotes cell immunity (Huang *et al*., 2007).

Interleukin-6 was investigated on day 21 for the EXT5 and COMP5 treatment groups to compare to control. There was no significant difference (P> 0.05) in the quantity of immunoglobulin A present in the serum between the dietary treatments and compared to control (table 5.17). On the other hand, there was a significant reduction (P<0.05) of IL-6 in the 5% competitor mealworm treatment group on day 21 compared to the control and numerically compared to EXT5 (Table 5.16) (Fig. 5.4).

Table 5.16- Levels of immunoglobulin A present in the blood on day 21 (±SE)

Treatment	Ig A (ug/ml)	IL-6 (ug/ml)
Control	9.856 (0.355)	140.487^{b} (23.458)
EXT5	10.588 (0.839)	92.348^{ab} (6.767)
Comp5	9.232 (0.557)	74.255^{a} (4.813)
P Value	**0.304**	**0.006**

Means within the same raw that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

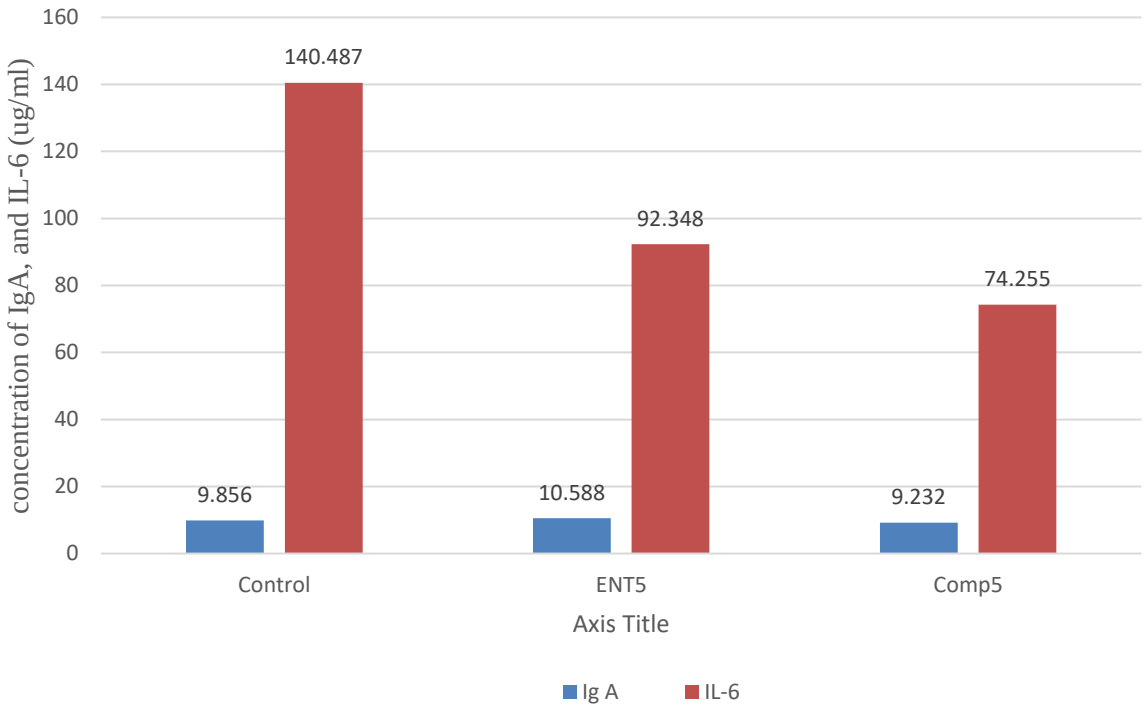

Figure 5.4- IgA, and IL-6 concentrations for day 21 of the 5% MW treatments from two different sources against the control.

5.3.12 Jejunum histology

Histology results showed that by day 21, there was a significant difference between treatment groups in terms of villus height, villus width, villus area and villus to crypt ratio. EXT1 increased villus height significantly ($P<0.05$) compared to other treatments as well as the control, while the rest of the treatment groups showed no significant differences. EXT1 also increased villus width significantly ($P<0.05$) compared to all other diets, while COMP5 decreased villus width significantly compared to other treatments as well as control. EXT1 also increased villus area significantly ($P<0.05$) compared to other treatment groups as well as control, while COMP5 decreased villus area significantly ($P<0.05$) compared to other treatments as well as control. In terms of villus to crypt ratio, EXT1 showed a significant increase in villus to crypt ratio compared to other treatment groups except for EXT5. Other

treatment groups showed no significant difference in villus to crypt ratio compared to control. At Day 21, there was no significant difference in crypt depth between groups (table 5.17).

Table 5.17- Effect of insect meal on jejunal histology of broilers at D21 (Mean±SEM)

Jejunum histology	Treatments						
D21	**Control**	**EXT0.3**	**EXT1**	**EXT5**	**COMP5**	**P value**	**S.E.M**
Villus height (µm)	727.37[a]	728.74[a]	823.19[b]	713.81[a]	703.59[a]	<0.001	7.720
Villus width (µm)	124.09[b]	122.43[b]	139.03[c]	121.53[b]	111.56[a]	<0.001	1.561
Crypt depth(µm)	109.80	111.93	113.17	104.86	110.02	0.102	1.023
Villus area (mm)	0.091[b]	0.092[b]	0.116[c]	0.088[ab]	0.08[a]	<0.001	0.0018
Villus/crypt ratio	6.649[a]	6.646[a]	7.406[b]	7.018[ab]	6.453[a]	<0.001	0.0779

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05).

By day 35, there was a significant difference between treatment groups in terms of villus height, villus width, and crypt depth. EXT1 increased villus height significantly (P<0.05) compared to other treatments except EXT0.3. COMP5 significantly (P<0.05) decreased villus height compared to EXT0.3 and EXT1, while the rest of the treatment groups showed no significant difference compared to the control. EXT1 and EXT0.3 decreased villus width significantly (P<0.05) compared to the control, and EXT1 was also significantly narrower than EXT5 and COMP5. EXT5 had significant increase in crypts depth (P<0.05) compared to COMP5. By Day 35, there was no significant difference in either villus area or villus to crypt ratio between treatment groups compared to control (table 5.18).

Table 5.18- Effect of insect meal on jejunal histology of broilers D35 (Mean±SEM).

Jejunum Histology	**Treatments**						
D35	**Control**	**EXT0.3**	**EXT1**	**EXT5**	**COMP5**	**Pvalue**	**S.E.M**
Villus height (µm)	937.13[ab]	969.76[bc]	1002.17[c]	935.86[ab]	900.36[a]	<0.001	6.969

Villus width (µm)	122.84^{c}	112.84^{ab}	106.23^{a}	119.02^{bc}	117.34^{bc}	<0.001	1.207
Crypt depth (µm)	104.045^{ab}	106.26^{ab}	106.88^{ab}	112.32^{b}	101.308^{a}	0.015	1.053
Villus area (mm)	0.11607	0.1094	0.10737	0.11047	0.10829	0.447	0.002
Villus/crypt ratio	9.01813	9.24069	9.45779	8.82654	8.92758	0.104	0.081

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

5.4 Discussion

The main aim of this study was to determine if lower doses of mealworm (0.3%, 1%, and 5%) can be beneficial in supporting the performance of broilers whilst also comparing one of these doses (5%) of MW from two different sources (EXT5 and COMP5). A second aim was to try to investigate the effect of low MW doses on nutrient digestibility and intestinal morphology at day 21 and day 35 and whether there is a significant difference in immunity markers between the same doses of the MW from the two different sources on day 21.

Performance

The performance data from this trial indicates that, from the parameters measured, there seems to be no detrimental effects of feeding mealworm (MW) at either 0.3%, 1% or 5% as mortality was 2% which is considered standard for trials conducted at NTU unit and lower than what would be expected in commercial trials. As birds were fed identical basal diets, it can be assumed that any beneficial effects seen are a result of the MW supplement and not due to inconsistencies in nutritional provision. All cumulative measuring points up to day 35 show normal values and no significant difference in bird weight gain compared to the control, except for the first 10 days where the higher doses of MW from two different sources (EXT5 and COMP5) showed significant increases in weight gain compared to the control while for the lower levels (EXT0.3, and EXT1) showed equal or relatively better performance in term of

BWG (table 5.6). Feed intake results of the current study are represented in Table 5.11, and for clarification the results are also shown in figure 5.1. Feed intake differed from BWG results, it clearly showed a substantial fluctuation in FI values between treatment groups and some of the treatments had higher values of FI compared to BWG. For example, during the first four weeks there was significant increase in feed intake for the higher dose of MW from the externally source MW compared to both the same dose of MW from another company source (COMP5) as well as control. This resulted in higher feed conversion ratio for EXT5 and EXT0.3 compared with the rest of treatments.

From D0-10, the higher dose of MW (EXT5, COMP5) produced a significant increase in body weight gain by 24g, and 26g on average respectively compared to the control and a slight increase for the lower dose treatments (EXT0.3, and EXT1) by 10g higher than control though this difference was not significant. A proportionate improve in BWG continues for the higher MW doses reaching for EXT5 to 41, and 16g, and for COMP5 by 9, and 27g better than the control on the second and third week of the trial respectively. Probably the insect meal is driving the palatability of the diets up as the results shows higher feed intake for EXT5 compared to control (figure 5.1).

Feed intake results on the other hand were very interesting, where EXT5 had significantly higher feed intake in all weeks of study compared to COMP5, and control, except for the final study week. Since both EXT5 and COMP5 had the same mealworm percentage inclusions, the significant difference might be due to how mealworm was raised and probably the mealworm substrate had effect but since we do not have information about the type of substrate the externally sourced MW were raised on. While COMP5 showed no difference in feed intake compared to control although COMP5 showed better BWG by 34g more than control. Normally when increase in body weight is met with low feed intake or normal feed intake it's a commercially a good indication that the supplement has positive effect on the animal

performance, and this showed in the group of broilers given COMP5 dose. While the increase in BWG noticed in the broilers with the EXT5 dose was met by significantly higher feed intake than control which means the broilers are increasing in weight due to getting to much feed and that is not an economically positive trait; however, sometimes improving palatability can be positive as this could encourage the birds eat more of a cheaper diet and therefore that can lead to use of local feed ingredients and could have good consequences on improving carbon footprint. It has been reported that avians have taste buds as much as these found in mice (Mistretta *et al*., 1999), however, taste buds in poultry are not normally found in the dorsal surface of the tongue but mainly at the end of the tongue near the pharynx with the pharynx and by the salivary glands (Kurosawa *et al*., 1983). The response of chicken to umami taste is wider ranged compared to humans. While humans translate glutamate and aspartic acid as umami, chicken palatability towards an amino acids balanced diet is more adequate than a diet lacking essential amino acids such as lysine or tryptophan or methionine (Picard *et al*., 1993). Therefore, increased feed intake for EXT5 diet might be due to increased palatability of chicken towards this particular diet and the mealworm coming from externally sourced MW source might have been subjected to supplementation with amino acids. Results of FI against BWG are in agreement with Sedgh-Gooya *et al*., (2022), who found that when broilers were supplemented with 2.5%, or 5% of MW, the performance showed a significant increase in BWG of in broilers compared to a control diet with no significant difference in FI. This contrasts with the findings of the current trial where FI was increased. This may be due to differences in the origin of insect meal in terms of nutritional quality like the sort of raising substrate, or amino acids supplementation which may have made the EXT mealworm material palatable to the chicks.

Another important performance criterion is the feed conversion ratio (FCR) which is a measure of the amount of feed needed to produce a kilogram of poultry meat, with the quality of feed

being interpreted by a low FCR value. In the current study, the weekly FCR results are demonstrated in table 5.8. There was no significant difference in FCR in the first two weeks of the study for any of the treatments compared to control. In the final two weeks of the study, FCR of COMP5 was significantly less than the lowest MW dose (EXT0.3), and the other high MW dose (EXT5), This significance in FCR between COMP5 and EXT5 with respect to the BWG, and FI results suggests that the origin of the mealworm (probably the substrate type, or environmental conditions) reflects on the insect meal quality and therefore reflects on birds performance. That results were consistent with several studies. In a study by Khosravi *et al*., (2018), graded doses of MW (up to 16%) showed improved performance of juvenile rock fish in terms of increased BWG and improved growth rate compared to fishmeal, however, with doses above 16% the performance declined compared to fishmeal, worth mentioning that, fish meal is a very expensive high quality protein source (about 1000 to 1700 €/ton), and the production volume from fish meal was estimated by about 2.359 million metric tonnes in 2020, compared to insect protein production of about 10 thousand tonnes in 2020 (Fletcher, 2021) which is priced around 3500 to 5500 €/ ton, however, there is a direction towards replacing fishmeal with other more sustainable protein sources like insect meal, towards what is called blue strategy, and although insect meal is more expensive that is probably due to low annual production (www.fao.org/2021). Nevertheless, low production is considered normal at the beginning from an emerging product, moreover, producers in multinational aquafeed companies are so optimistic about an estimate of increase in insect meal production to about 500 thousand tonnes 2030 which might force prices of insect protein to drop (Rabobank, 2021). Another study by Panini *et al.*, (2017) showed no negative effect on BWG, FI, and FCR of pacific white shrimp with a partial MW diet (30%) with results comparable to total fishmeal. Other studies on the effect of increasing doses of full fat MW (5%, 10%, and 15%) showed improvements in chicken performance in terms of BWG, and FI (Biasato *et al*., 2017; Biasato

et al., 2018) which confirmed the results of broilers performance supplemented with EXT5 in terms of higher BWG and FI , while a study by Ballitoc and Sun, (2013), showed that 10% replacement of SBM with MW improved FI, and FCR of broilers, similar to COMP5 treatment group in the current study which also improved broilers FCR; however, the study by Ballitoc and Sun, (2013) did not provide details about the mealworm feeding substrate.

Carcass yield

Carcass yield results for this study are represented in Table 5.11. Total carcass yield of the higher dose of MW (COMP5) showed a significant increase compared to control, with the significance resulting from an increase in the breast yield of COMP5.The total carcass yield of the rest of the treatments were comparable with the control with slight improvement compared to control showing EXT0.3, EXT1, and EXT5 with more 90g, 63g, and 20g carcass yield compared to control respectively (Figure 5.2). These results show a potential positive effect of supplementing MW to broilers with an increase in meat yield for the higher dose competitor source. These results are in agreement with Ballitoc and Sun, (2013) which showed that 1% MW dietary inclusion significantly increased carcass yield in broilers compared to total SBM diet. However, worth mentioning that perhaps the significantly high carcass yield of MW COMP5 group is related to the FCR results where in D21-D28 and D28-D35 of the trial MW COMP5 group showed significantly less FCR (1.56^{a}, and 1.53^{a} respectively) compared to EXT0.3 (1.77^{b}, and 1.72^{b} respectively), and EXT5 (1.8^{b}, and 1.72^{b} respectively) group and also relatively less FCR than EXT1 (1.71^{ab}, and 1.62^{ab} respectively) as well as control (1.64^{ab}, and 1.59^{ab} respectively) which is a very interesting relation because EXT diets have increased chicken palatability and made them eat more which showed in high FI, however the high FCR of the broilers fed MW EXT diet probably have affected the carcass yield while the FI in the COMP5 group was not met by high FCR which in turn showed significant increase in carcass yield of the COMP5 broilers.

Gizzard and ileal lesions score

There were no statistically significant results on gizzard or ileal lesions score either on day 21 or day 35 of the trial. That is a positive indication of no deterrent effect resulting for MW supplementation.

Apparent metabolizable energy

The results for apparent metabolizable energy (AME), apparent metabolizable energy corrected for nitrogen (AMEn) and nitrogen retention (Ni ret) for D21, and D35 of the study are shown in Table 5.13, and 5.14. The results appear to suggest that the higher dose of MW from the competitor source has improved both AME and AMEn compared to the EXT5 treatment as well as control. The results of day 35 of study for AME and AMEn showed the same significance of COMP5. The AME, and AMEn results showed that the higher dose of MW from both sources were comparable to one another at the end of the study but were significantly higher than control. The increase in AMEn of higher doses of MW indicates that by day 21both 5% MW diets had improved energy utilisation so broilers were using the diets more effectively. Also, generally supplementation of MW even in low doses did not negatively affect feed quality. That results highlight the importance of AME as a parameter to measure the energy quality of feed, and MW supplementation in 5% doses has significantly positive effect on enhancing the energy of nutrients and adding value to the feed even in small dose. That results were not consistent with those of Zadeh *et al*., (2019), where Japanese quails supplemented with increasing doses of MW (7.5, 15, and 22.5 %) showed no difference in AMEn probably because the doses used in the study are higher than the current study. It also contrasts with the results of Biasato *et al.*, 2017 that showed no significant difference between increasing MW doses (5%, 10%, and 15%) up to day 25. Another observation from the current study was that both AME and AMEn in the current study did not reflect in the Ni ret as EXT5 was significantly lower than the rest of the treatments except for EXT1, and COMP5 interestingly had

significantly lower Ni ret Compared to control on day 21, the lower N retention might be the probability of presence of less digestible protein. What was interesting that the lower dose of MW (EXT0.3) showed significantly the highest Ni ret control on both day 21, and day 35 of the study, that may indicate that low MW doses improve protein digestibility better than higher doses, suggesting the probability of lower MW levels having a prebiotic effect on broilers.

Jejunum histology

Jejunum histology results for increasing doses of MW compared to control for day 21, and 35 are demonstrated in table 6.18, and 6.19. The increasing doses of MW did not have adverse effect on jejunum histology compared to control group in terms of either villus height, villus width, villus area or villus/crypts ratio on day 21 and 35. Additionally, EXT1 showed positive results in terms of Villus height on day 35, and both villus height, and villus width on day 21 the increase in Villus height and villus width indicates that EXT1 affected the Jejunum villi by improving absorptive area. Investigation of the effect of MW dose as low as 1% on the gut histology of broilers has not been investigated to date in the literature, however Biasto *et al*., 2018, showed that a higher dose of MW (15%) significantly decreased intestinal villi and increased crypt depth compared to a control and a lower dose (5%). That suggests that a low MW dose may have a favourable effect on gut health. Additionally, as mentioned earlier that birds in the current study were fed identical basal diets, therefore the positive significant effect of the 1% MW dose on the villus height can be due to the effect of the MW supplementation taking into consideration that the rest of the treatments were consistent with control and didn't demonstrate any negative effect on broilers jejunum histology. However, it still unclear, why the MW higher doses are not effective compared to the lower doses.

Nitrogen digestibility coefficient

The nitrogen digestibility coefficients (NDC) investigated in this chapter are shown in table 5.12, and for more clarification an illustration of the difference in NDC of the treatment groups as well as control between the D21 and D35 of study is shown in Fig. 5.3. All MW doses showed no significant difference in NDC on D21 compared to the control and all had protein digestibility that exceeded 80%, however, the lower dose of MW (0.3%) showed significantly higher NDC compared to 5% MW dose from the two different sources (EXT5, and COMP5), that confirms the results of Nitrogen retention that showed o.3% mealworm supplementation improved nitrogen retention of 21 days old broilers, which probably means lower endogenous, and exogenous nitrogen loss in addition to better hydrolysis of protein. On day 35 of the trial, the protein digestibility of all diets did not exceed 74% nevertheless, EXT1, and EXT5 had significantly the highest NDC compared to the rest of the diets that also reflected on the nitrogen retention where Nitogen retention of broilers on day 21 was higher than that on day 35. The clear decline in NDC, and nitrogen retention between the two trial days (D21, and D35) was interesting (Figure 5.3) and there was no information in literature about the effect of low supplementation of MW on broiler protein digestibility, but only an investigation by Bovera *et al*., 2016 where 30 days old broilers were fed 29.65% MW until Day 62 and results showed a significant decrease in NDC in the 29.65% MW compared to control. Therefore, it seems that probably age of birds might have an effect on their Ni ret, and NDC. Yang *et al*., 2020, have investigated the relation between nitrogen retention and bird age where it was found that nitrogen retention seems to at its peak on day 7 of broilers age and significantly decline by day 35 of age even with supplementations with enzymes. that might be the reason why the broilers nitrogen retention and digestibility declined by day 35 in the current study. Additionally, no record of any unusual change during the study was observed. Nevertheless,

the MW treatment groups still performed better than control especially MW from the externally sourced MW.

Markers of inflammatory and immune response

Infection with diseases triggers a stress response in the infected animal which is translated by production of certain immune markers. Accordingly, immunologically immature chickens can be affected easily by disease, and this can reflect on birds' performance in terms of decline in feed intake (Haung *et al*., 2007). Results of IgA, and IL-6 concentrations for day 21 of the current study are shown in table 5.17 and for clarification, an illustration of the results is represented in figure 5.5. Results of IgA did not show any significant difference either between the two MW sources or compared to control. On the other hand, IL-6 concentration showed that IL-6 release in blood for COMP5 treatment group was significantly lower than the control. The elevation of IL-6 in blood can be the result of a stress response because IL-6 is a cytokine that mediates against pathogen infection, these results highlight the positive effect of low doses of MW supplementation on changing the cytokines-mediated- microenvironment of broilers, that might indicate that the low doses have better immune effect on broilers. According to explanation of Colombino *et al*., (2021) for pro versus anti- inflammatory cytokines where the anti-inflammatory cytokines such as IL-6 regulate the action of the pro-inflammatory cytokines such as IL-2 that is responsible for variety of immune responses within the cells such as apoptosis and necrosis and referring to the role of chitin in promoting the cytokines production. It was also mentioned that mealworm have lower content of chitin compared to for example the black soldierfly larvae and that low chitin content could lead to down regulation of anti-inflammatory cytokines, so one of the interpretations of the results of the current chapter is that the low chitin level in mealworm have probably affected the production of IL-6 cytokines in plasma of the studied broilers. The study of Colombino *et al*., 2021 have mentioned no change

in the gut mucin when broiler supplemented the 5% mealworm diet where the gut mucin is also responsible for the secretion of IgA which might explain the insignificant difference in IgA concentration between the 5% mealworm dose and the control. The results of the current study are contradicted by Haung *et al*., 2007 who investigated the effect of oligochitosan supplementation on IgA in serum and IL-6 mRNA in mononuclear cells in broilers where the supplementation increased both IgA and IL-6 which was interpreted as an improvement in broilers immunity. Further investigation is needed in this area, as analysis of IgA and IL-6 were only performed for day 21 on 3 treatments due to funding limitations, and further analysis may elucidate the effect and provide more clarity as to the benefit of low doses of MW.

5.5 Conclusion

The results of the current study clearly show that MW supplementation had a positive effect on bird performance, that showed clearly in increased BWG of broilers supplemented MW doses compared to control and significantly lower feed intake especially with 5% doses. However, the high feed intake of broilers in the EXT5 group might suggest increased palatability of broilers towards MW diets.

MW 5% dose improved energy utilisation in broilers compared to higher doses, considering AMEn as a crucial parameter to determine the quality of feed, suggesting that MW dietary inclusions can be a good additive to the broilers feed.

Results of jejunum histology, for broilers supplemented with increasing doses of MW were compatible with control. That could mean that MW supplementation did not compromise gut integrity and what was also interesting is that the 1% MW dose showed superior results compared to control in terms of increased villus hight in the two days of the study (D21, and

D35). Since all birds in the study were fed the same basal diet, any improve in any of the parameters could probably be because of MW supplementation.

Generally, 5% MW inclusion performed well in comparison to control although the two 5% mealworm from different sources showed different effects on broilers in some parameters, that could highlight the role of insect feeding substrate in manipulating the nutritional profile of insects and thus could be used to improve insect meal quality prior introduction as feed. Ultimately, the results have proven the viability of using mealworm as supplementation rather than having to use higher inclusions or even a complete MW meal to get effective results. Not due to any negative impact of MW on the health integrity of broilers but rather economically. Making mealworm a suitable feed additive in poultry industry.

Chapter 6– Effect of Black soldier fly larvae on nutrient digestibility, jejunum histology, growth performance, and carcass yield of broilers.

6.1 Introduction

Soybean meal is highly exploited as a vegetable protein source for both broilers and layers, due to the high-quality protein and amino acid profile comparable to animal protein (Veld-kamp *et al*., 2012). However, in addition to the high cost of providing soyabean meal (Van Huis *et al*., 2013), there are also antinutritional and environmental factors surrounding the extensive use of soybean meal as feed for poultry (Ruiz *et al*., 2020).

Black soldierfly larvae (BSFL) has been investigated as a feed for poultry for a few years now (Hartinger *et al*., 2021; Dumas *et al*., 2018) especially at low inclusion levels (Dabbou *et al*., 2018). BSFL is among the seven insect species that are permitted to be used in feed by the European Commission (EU, 2017). This is due to several physiological, behavioural and environmental characteristics the BSFL have. BSFL contain around 40% protein and 30% lipid on a dry matter basis (Lu *et al*., 2022). Chapter 5 discussed the effect of including mealworm in graded doses (0.3%, 1%, and 5%) on the performance, digestibility and gut integrity of broilers, with positive results in terms of digestibility and histology. Although, previous studies in this thesis have shown that mealworm contains more protein on a dry matter basis, BSFL have the ability of converting any waste substrate into high quality protein mass. Mealworm on the other hand, requires specific conditions for the raising substrates so are less flexible. There are additional costs of breeding of mealworms due to their long life cycle, which may last to 12 weeks (www.breedinginsects.com, 2020), while the black soldier fly life cycle may only last for two weeks if at a suitable temperature (27°C) (Tomberlin *et al*., 2002). BSFL

adults have not been reported to transmit any disease or interact with humans and the larval stage is the only feeding stage, and they feed mainly on waste substrates.

There is little information on the nitrogen and amino acid digestibility of BSFL for broilers in literature Previous studies have investigated the potential use of varying doses of partially defatted BSFL (Dumas *et al*., 2018) or full fat BSFL (Kumar *et al*., 2021). This is because testing the viability of lower doses could be economically important as it is currently very expensive to utilise a complete insect meal for broilers. Insect feed market prices create economic limitations compared to soya even considering the lower carbon footprint of insects compared to soya production, and the reduction in wild land use (Fearnside, 2001).

the aim of the current chapter is to investigate the effect of partial replacement of soya in diets by graded levels of black soldierfly larvae on bird performance to d35, and to determine the effect of these inclusions on gut health and nutrient digestibility on day 21 and day 35.

The first hypothesis is that graded doses of black soldierfly larvae will positively affect the broilers performance and gut integrity. The second hypothesis is that graded doses of black soldierfly larvae will enhance nitrogen digestibility of broilers compared to a soyabean meal control.

6.2 Materials and methods

6.2.1 Diets, animals, and husbandry

Husbandry guidelines were followed as described in chapter 2 and adhered to the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). Ethical approval was granted by the University ARES Ethics Committee and was logged as project ARE1628825. A total of 320 one-day-old male Ross 308 broilers were sourced from PD Hook Cote hatchery from a flock aged 34 weeks. Birds were feather sexed on the day of hatch and any poor birds were discarded on arrival. Chicks were individually weighed on arrival, then randomly assigned to 40

mesh sided pens (8 birds per pen), littered with a wood shavings substrate. Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible.

6.2.2 Diet formulation and condition of animals

Black soldierfly larvae used in the study were brought from Hexafly Ltd, Ireland, in the form of a ground powder, and diets were manufactured in house at NTU. The trial used four treatments (table 6.1) with three phases. Starter phase was from study day 0 to day 10 Study, grower phase from study Day10 to Day 21 and the finisher phase was from day 21- 35. Four basal diets (one per phase). Worth mentioning that in BSF1 grower diet preparation, some problems encountered with mistakenly adding less amount of wheat than mentioned in the diet formulations, but the problem was compensated by adding the wheat and remixing the diet. Chicks were individually weighed on arrival, then randomly assigned to 40 mesh sided pens (8 birds per pen), bedded with wood shaving. The pens were allocated to one of the following dietary treatments, Control (standard broiler diet), BSF0.3 (black soldier fly larvae meal substituting 0.3% of soyabean meal of Control), BSF1 (black soldier fly larvae meal substituting 1% of soyabean meal of Control), and BSF5 (black soldier fly larvae meal substituting 5% of soyabean meal of Control). The trial used 3 phases (starter, grower, and finisher) (Table 6.2). Proximate analysis was performed to confirm composition (table 6.3). Food and water were provided *ad libitum* and care taken to ensure chicks were eating and drinking as soon as possible. The ammonia level within the room was monitored through husbandry observations and ventilation adjusted as necessary.

Table 6.1- Dietary treatments, for partial replacement of soyabean meal with black soldierfly larvae graded inclusions (g/kg).

Diet code	Diet Treatments	Soya inclusion	Insect inclusion
Control	Control	324.3	0
BSF0.3	0.3% BSFL	321.3	3
BSF1	1% BSFL	314.3	10
BSF5	5% BSFL	274.3	50

Table 6.2- Dietary Formulations: treatment diets (g/kg)

Starter	**T1**	**T2**	**T3**	**T4**
Wheat	615	615	615	615
Soya oil	15.8	15.8	15.8	15.8
Salt	3.8	3.8	3.8	3.8
Limestone	1.6	1.6	1.6	1.6
Dicalcium Phos, 18%P	21.7	21.7	21.7	21.7
Lysine HCl	2.8	2.8	2.8	2.8
DL-Methionine	3.2	3.2	3.2	3.2
Threonine	1.6	1.6	1.6	1.6
Vitamin & Mineral premix*	5	5	5	5
Phytase (QB)	0.1	0.1	0.1	0.1
Titanium	5	5	5	5
Soya inclusion	324.4	321.4	314.4	274.4
Insect inclusion	0	3	10	50

Grower	**T1**	**T2**	**T3**	**T4**
Wheat	635	635	635	635
Soya oil	29.4	29.4	29.4	29.4
Salt	5	5	5	5
Limestone	3.8	3.8	3.8	3.8
Dicalcium Phos, 18%P	19.1	19.1	19.1	19.1
Lysine HCl	2.2	2.2	2.2	2.2

DL-Methionine	2.7	2.7	2.7	2.7
Threonine	1.2	1.2	1.2	1.2
Vitamin & Mineral premix*	5	5	5	5
Phytase (QB)	0.1	0.1	0.1	0.1
Titanium	5	5	5	5
Soya inclusion	291.5	288.5	281.5	241.5
Insect inclusion	0	3	10	50

Finisher	**T1**	**T2**	**T3**	**T4**
Wheat	670	670	670	670
Soya oil	38.9	38.9	38.9	38.9
Salt	3.8	3.8	3.8	3.8
Limestone	1	1	1	1
Dicalcium Phos, 18%P	17.4	17.4	17.4	17.4
Lysine HCl	2.3	2.3	2.3	2.3
DL-Methionine	2.6	2.6	2.6	2.6
Threonine	1.2	1.2	1.2	1.2
Vitamin & Mineral premix*	5	5	5	5
Phytase (QB)	0.1	0.1	0.1	0.1
Titanium	5	5	5	5
Soya inclusion	252.7	249.7	242.7	202.7
Insect inclusion	0	3	10	50

* 1Premix (per kg of diet): Calcium; 10g, Phosphorus; 4.5g, Sodium; 1.5g, Chloride; 1.5g, Magnesium; 0.6g, Manganese; 60mg, Zinc; 50mg, Iron; 80mg, Copper; 6mg, Iodine; 0.5mg, Molybdenum; 0.2mg, Selenium; 0.15mg, Retinol; 2.25mg, Cholecalciferol; 37.5µg, Tocopherol; 10mg, Menadione; 3.0mg, Thiamine; 3.0mg, Riboflavin; 5.0mg, Pantothenic acid; 10mg, Pyridoxine; 4.0mg, Niacin; 30mg, Cobalamin; 10µg, Folic acid; 1.5mg, Biotin; 0.15mg, Choline; 1.3mg, Amprolium; 125mg, Antioxidant; 125mg.

Table 6.3- Proximate analysis of diets for partial replacement of soyabean meal with black soldierfly larvae.

Starter	**% DM**	**% Ash**	**% Fat**
Control	88.20	6.72	7.014935
BSF0.3	88.16	6.65	3.306977
BSF1	88.44	7.29	4.80996
BSF5	88.65	6.89	4.428392
Grower			
Control	88.62	7.19	5.216196
BSF0.3	88.37	7.34	4.117023
BSF1	88.57	5.93	4.844537
BSF5	88.58	6.63	5.680064
Finisher			
Control	87.91	5.77	6.13751
BSFL0.3	87.94	5.108	5.978665
BSF1	87.09	5.35	5.945934
BSF5	88.49	5.68	7.935349

6.2.3 Treatment Schedule, and randomisation plan

A replicate consisted of 10 pens containing 8 birds each and only birds weighing between 36g and 42g were placed on trial. The combined weight of each pen was recorded on day 0. Treatments were randomly allocated via an online randomisation tool (random.org) by block (one block of 10 pens) to reduce any effect of ventilation and temperature differences within the room.

6.2.4 Study observations

Bird observations were performed per the method detailed in the previous chapter 5 (trial T5) Bird feed intake was calculated as per the method detailed in chapter 2. Birds feeding, weighing, and sampling were performed as per the method detailed in chapter 5.

6.2.5 Statistical analysis of data

Statistics were performed as per method detailed in the previous chapter 5.

6.3 Results

6.3.1 Bird mortality

Mortality data is shown in table 6.4. During the whole 35d study, mortality was 2.8% which is considered standard for trials conducted at the NTU unit and lower than would be expected in a commercial setting which would be typically in excess of 4%. There was no apparent effect of treatment on mortality as can be seen in table 6.4 which splits the mortality by week and dietary treatment.

Table 6.4- Bird mortality for partial replacement of soyabean meal with black soldierfly larvae graded inclusion.

Treatment	d0-10	D10-21	D21-28	D28-35	Total
Control	0	0	0	1	1
BSFL0.3	1	1	0	0	2
BSF1	2	1	0	0	3
BSF5	1	2	0	0	3

6.3.2 Bird Uniformity

Upon arrival at the research unit, all chicks were individually weighed and only birds weighing between 36 and 42g were placed. The mean start weights for each treatment are shown in table 6.5. There was no statistical difference in the start weight of the chicks between treatments.

Table 6.5- Average start weight (g) for chicks for partial replacement of soyabean meal with black soldierfly larvae graded inclusion.

Treatment	d0 BW/bird (g)
Control	38.7
BSF0.3	38.6
BSF1	39.1
BSF5	38.6
S.E.M	0.275
P value	0.504

6.3.3 Bird Performance

Weekly average bird weight

There was no significant difference in bird weight on either day 0 or day 10 between the experimental groups and compared to control. However, by day 21 and until the end of the trial, BSF1 showed significantly less BW (P<0.001) compared to BSF0.3, and BSF5 treatment groups as well as control (table 6.6).

Table 6.6- Weekly Average Bird Weight for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M)

Diet	D0 BW	D10 BW	D21 BW	D28 BW	D35 BW
Control	38.656	160.3775	700.99^{b}	1205.01^{b}	2175.99^{b}
BSF0.3	38.595	160.751	723.42^{b}	1200.98^{b}	2069.37^{b}
BSF1	39.103	162.214	455.12^{a}	807.06^{a}	1627.3^{a}
BSF5	38.596	167.745	689.23^{b}	1143.15^{b}	2056.02^{b}
S.E.M	**0.275**	**4.004**	**15.364**	**25.154**	**66.436**
P value	**0.504**	**0.544**	**<0.001**	**<0.001**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

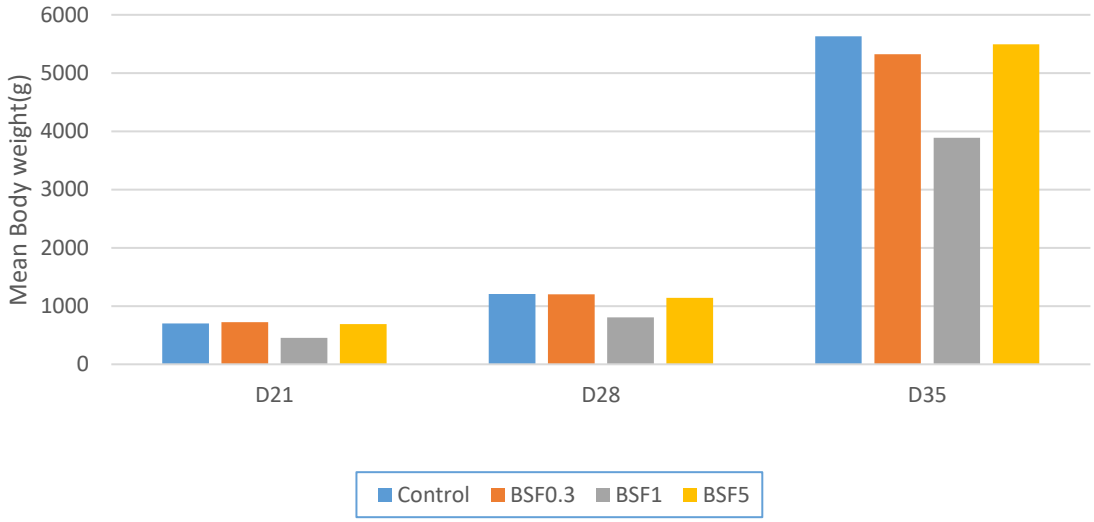

Figure 6.1- Mean body weight on days 21, 28, and 35 of study for BSFL dose response trial

Weekly and cumulative bird weight gain

There was no significant difference in body weight gain during the period of day 0-10 and day 28-35 between treatment groups compared to control. However, there was a significant difference in body weight gain during the periods of day 10-20, and 21-28 where BSF1 showed significant (P<0.001) decrease in BWG compared to other treatments and compared to control. While both BSF0.3, and BSF5 showed no significant difference in BWG compared to control on day 10-28, and day 21-28 (table 6.7). BSF1 treatment group showed significantly the least cumulative BWG, on D0-D21, D0-D28, and D0-D35 of study, compared to the rest of the treatments as well as control (table 6.8).

Table 6.7- Body weight gain for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M).

Treatment	D0-10 BWG	D10-20BWG	D21-28 BWG	D28-35 BWG
Control	121.7	540.6[b]	504.0[b]	970.9
BSF0.3	122.2	562.7[b]	477.6[b]	868.4
BSF1	123.1	292.9[a]	351.9[a]	820.2
BSF5	129.2	521.5[b]	453.9[b]	912.9
S.E.M	3.95	12.73	21.70	54.55
P Value	**0.520**	**<0.001**	**<0.001**	**0.263**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p<0.05$).

Table 6.8- Cumulative Body weight gain for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M).

Cumulative bird weight gain (g)	D0-D21	D0-D28	D0-D35
Control	662.34[b]	1166.36[b]	2137.33[b]
BSF0.3	684.82[b]	1162.38[b]	2030.77[b]
BSF1	416.02[a]	767.95[a]	1588.19[a]
BSF5	650.63[b]	1104.56[b]	2017.43[b]
S.E.M	15.31	25.15	66.41
P Value	**<0.001**	**<0.001**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA ($p=<0.05$).

Weekly feed intake

From day 10-20, there was a significant decrease in feed intake of BSF1 compared to the rest of treatment groups ($P<0.001$) as well as control. On D21-28 BSF1 had significantly less FI than BSF5 treatment group but showed no significant difference in FI compared to either BSF0.3 or to control. On D28-35, BSF5 group showed significant increase ($P<0.05$) in feed intake compared to BSF1 group and to control, however no significant difference in feed intake between BSF0.3 and BSF5 treatment group (table 6.9).

Table 6.9- Weekly feed intake for partial replacement of soyabean meal with black soldierfly larvae graded inclusions (g) (±S.E.M).

Treatment	D0-10	D10-20	D21-28	D28-35
Control	212.9	684.57^{b}	787.9^{ab}	1126.3^{b}
BSF0.3	226.79	727.55^{b}	803.7^{ab}	1066.1^{ab}
BSF1	232.93	563.801^{a}	728.3^{a}	972.8^{a}
BSF5	227.94	737.85^{b}	843.2^{b}	1142.4^{b}
S.E.M	**14.92**	**18.18**	**29.223**	**38.207**
P Value	**0.581**	**<0.001**	**0.049**	**0.015**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

Feed conversion ratio.

On D0-10, and D28-35, there was no significant difference in feed conversion ratio between treatment groups or compared to control. However, on D10-20, there was a significant increase (P<0.001) in FCR of BSF1 compared to BSF0.3 and BSF5 treatment groups as well as control. On D21-28 there was a significant increase in FCR of BSF1 compared to control (P<0.05), however, there was no significant difference in FCR between any of the other treatment groups (table 6.10).

Table 6.10- Weekly feed conversion ratio for partial replacement of soyabean meal with black soldierfly larvae graded inclusions (g) (±S.E.M).

Treatment	D0-10 FCR	D10-20FCR	D21-28 FCR	D28-35 FCR
Control	1.779	1.268^{a}	1.583^{a}	1.185
BSF0.3	1.874	1.297^{a}	1.728^{a}	1.241
BSF1	1.895	1.945^{b}	2.087^{b}	1.203
BSF5	1.786	1.417^{a}	1.906^{ab}	1.276
S.E.M	**0.109**	**0.053**	**0.11**	**0.05**
P Value	**0.826**	**<0.001**	**0.013**	**0.594**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05).

6.3.4 Carcass yield

There was a significant difference in Breast yield and total carcass yield (P<0.05) between treatment groups compared to control, where BSF1 showed the lowest breast, thigh, drumstick yield, total carcass yield, and % dressing compared to BSF0.3, and BSF5 treatment groups as well as control. BSF5 showed significantly (P<0.001) less breast, thigh, drumstick yield, and total carcass yield compared to BSF0.3, and control. On the other hand, there was no significant difference in dressing % between BSF0.3, and BSF5 treatment groups as well as control (Table 6.11)

Table 6.11- Carcass yield for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M).

Carcass yield	Breast yield(g)	Thigh yield (g)	Drumstick yield (g)	Total carcass yield (g)	Dressing %
Control	443.75^{c}	233.1^{c}	183.22^{c}	859.89^{c}	38.87^{b}
BSF0.3	406.31^{bc}	209.03^{bc}	169.09^{bc}	784.43^{bc}	38.02^{b}
BSF1	255.66^{a}	154.28^{a}	125.95^{a}	535.89^{a}	35.46^{a}
BSF5	361.92^{b}	199.71^{b}	160.42^{b}	722.05^{b}	38.18^{b}
S.E.M	20.597	7.003	5.105	30.753	0.656
P value	<0.001	<0.001	<0.001	<0.001	0.004

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

6.3.5 Nitrogen digestibility

Results of Nitrogen Digestibility Coefficient (NDC) for the dose response trial at day 21, and day 35 are represented in table 6.12. On day 21, NDC of all treatment groups was significantly higher than control group (p<0.001), however, there was no significant difference between in NDC between any of the treatment groups. On day 35, BFS1 had significantly the highest NDC (p<0.001) compared to the rest of treatment groups as well as control group, on the other hand, there was no significant difference in NDC between BSF0.3, and BSF5 treatment group compared to control group (Table 6.12).

Table 6.12- Nitrogen digestibility coefficient (NDC) for partial replacement of soyabean meal with black soldierfly larvae graded inclusions on day 21, and 35 (±S.E.M).

Treatment	Nitrogen Digestibility Coefficient	
	D21	**D35**
Control	0.833[a]	0.879[a]
BSF0.3	0.8698[b]	0.865[a]
BSF1	0.8603[b]	0.905[b]
BSF5	0.8599[b]	0.862[a]
S.E.M	0.006	0.007
P value	<0.001	<0.001

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05).

6.3.6 Apparent metabolizable energy

Apparent metabolizable energy (AME), apparent metabolizable energy corrected for nitrogen retention (AMEn), and nitrogen retention (Ni ret) values on days 21, and 35 of treatment in the dose response trial are shown in table 6.13, and 6.14. On day 21, AME of BSF5 was significantly higher than BSF1, and the control group ($p<0.001$). When AME was corrected for nitrogen retention, AMEn values for BSF0.3, BSF1, and BSF5 were significantly higher than that of control group ($p<0.001$). Ni retention for BSF5 was significantly the highest compared to the rest of treatments as well as the control group ($p<0.001$). Also, BSF0.3 showed significantly higher Ni ret than BSF1 treatment group as well as control group($p<0.001$), and BSF1 showed significantly the lowest Ni ret ($p<0.001$) compared to the rest of treatment groups as well as the control group (6.13).

Table 6.13- Apparent metabolizable energy and nitrogen retention on day 21 for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M).

Treatment	AME (MJ/kg)	AMEn (MJ/kg)	Ni ret (g/kg)
Control	13.63[a]	12.73[a]	26.01[b]
BSF0.3	14.87[bc]	13.79[b]	31.19[c]
BSF1	14.39[b]	13.74[b]	18.87[a]
BSF5	15.02[c]	13.9[b]	32.39[d]
S.E.M	0.313	0.289	2.356
P Value	**<0.001**	**<0.001**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05).

On day 35, AME of BSF5 was significantly higher than BSF0.3, and BSF1, treatment groups (p=0.017) but, showed no significant difference with control, on the other hand, both BSF0.3, and BSF1 showed significantly lower AME (p=0.017) compared to control group. When AME was corrected for nitrogen retention, BSF0.3, and BSF1 had significantly low AMEn compared to control but no significant difference compared to BSF5. BSF0.3, and BSF5 treatment groups showed significantly higher Ni ret (p<0.001) compared to BSF1 treatment group but showed no significant difference with control group, on the other hand, the Ni ret of BSF1 was significantly low compared to BSF0.3, and BSF5 treatment groups (p<0.001) as well as control group (Table 6.14).

Table 6.14- Apparent metabolizable energy and nitrogen retention on day 35 for partial replacement of soyabean meal with black soldierfly larvae graded inclusion (g) (±S.E.M).

Treatment	AME (MJ/kg)	AMEn (MJ/kg)	Ni ret (g/kg)
Control	15.06[b]	14.12[b]	27.06[b]
BSF0.3	14.67[a]	13.74[a]	27.004[b]
BSF1	14.68[a]	13.79[a]	25.98[a]
BSF5	14.98[b]	14.02[ab]	26.92[b]
S.E.M	0.102	0.096	0.221
P Value	**0.017**	**0.02**	**<0.001**

Means within the same column that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

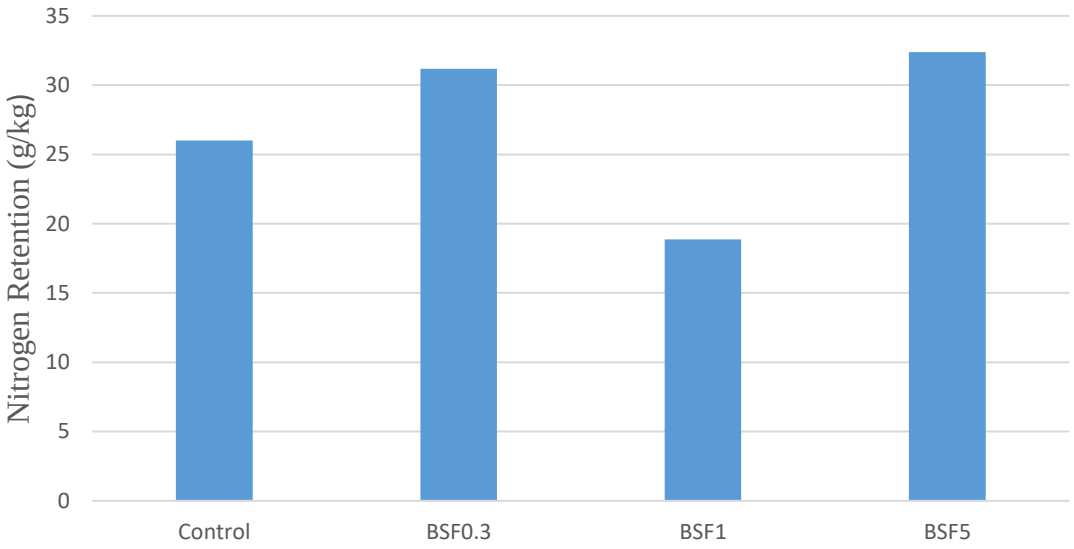

Figure 6.2- Day 21 nitrogen retention values for soyabean meal partial replacement with black soldierfly larvae graded inclusions.

6.3.7. Jejunum histology

Histology results showed that by day 21, there was no significant difference between treatment groups in terms of villus height, villus width, villus area and villus to crypts ratio. However, we can notice a significant increase (p=0.005) in crypts depth of BSF 0.3 treatment group compared to control, but no significant difference compared to either BSF1 or BSF5. but neither BSF1 nor BSF5 treatment groups had any significant difference in crypts depth compared to control (table 6.15).

Table 6.15- Effect soyabean meal partial replacement with black soldierfly larvae graded inclusions on jejunal histology of broilers on day 21(±S.E.M).

Jejunum histology	**Treatments**					
D21	**Control**	**0.3%BSFL**	**1%BSFL**	**5%BSFL**	**P value**	**S.E.M**
Villus height (µm)	727.689	732.65	796.53	805.82	0.050	25.203
Villus width (µm)	141.29	143.35	144.44	147.20	0.679	3.464
Crypts depth(µm)	105.25^{a}	126.12^{b}	107.57^{ab}	123.42^{ab}	0.005	5.075
Villus area (mm)	0.104^{a}	0.115^{b}	0.104^{a}	0.119^{b}	0.038	0.005
Villus/crypts ratio	6.97	6.48	6.96	6.91	0.467	0.354

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05)

By day 35, there was no significant difference between the treatment groups and compared to control in terms of villus width, crypts depth, and villus area. There was a significant difference between treatment groups in terms of villus height, and Villus to crypts ratio. BSF1 decreased villus height significantly ($P<0.05$) compared to other treatments, however, there was no significant difference compared to the control. There was a significant decrease ($p<0.001$) in villus to crypts ratio for BSF1 compared to BSF0.3, and BSF5 experimental group but no significant difference compared to control. BSF0.3, and BSF5 showed no significant difference in any of the villus height or villus to crypts ratio (Table 6.16).

Table 6.16- Effect soyabean meal partial replacement with black soldierfly larvae graded inclusion on jejunal histology of broilers on day 35(±S.E.M).

Jejunum histology	**Treatments**					
D35	**Control**	**0.3%BSFL**	**1%BSFL**	**5%BSFL**	**P value**	**S.E.M**
Villus height (µm)	964.86^{ab}	980.84^{b}	884.42^{a}	980.47^{b}	0.017	24.472
Villus width (µm)	138.42	148.82	151.43	172.69	0.620	26.391
Crypts depth(µm)	107.14	103.66	109.36	98.28	0.096	3.280
Villus area (mm)	0.133	0.148	0.133	0.164	0.460	0.0228
Villus/crypts ratio	9.21^{ab}	9.77^{b}	8.19^{a}	10.16^{b}	<0.001	0.316

Means within the same row that do not share a common superscript are significantly different by one-way ANOVA (p=<0.05).

6.4 Discussion

The main aim of this study was to determine whether BSFL could be used as feed additive (at a level of 0.3%) or as a protein source (1% and 5%) in broiler nutrition.

Performance data

Bird mortality was 2.8% which is considered standard for trials conducted at NTU and lower than what would be expected in commercial trials. The performance data from this trial indicates that, from the parameters measured, there seems to be no detrimental effects of feeding BSFL at either 0.3%, or 5%. There appears to be a detrimental effect in the bird group supplemented with 1% black soldierfly larvae meal, but this may be due to a diet manufacturing problem as mentioned in the diet formulation section in materials and methods.

During the first week, BWG of all treatment groups seemed good compared to control group, with the higher dose of BSFL producing a slight increase in body weight gain by 8g on average compared to control, however D10-20, and D21-28, broiler supplemented 1% BSFL demonstrated significant lower BWG compared to the rest of treatment groups as well as control. The same decline was noticed in the cumulative BWG results as well, although in the first 10 days of the study BWG of BSF1 treatment group was normal compared to control, then on the final study week, the BSF1 broilers group seemed to be compensating for the low BWG in the previous two weeks, and by the end of final week the BSF1 broilers group have gained 468.3g which is relatively equivalent to the BWG of the control group(466.9g) at the same week, which only indicates there was a problem with the grower phase diet for this particular treatment diet, probably during manufacture, probably less nutrients/ vitamins were accidently added to the diet or the amount of missing wheat was added and when the diet was remixed that caused an irregularity to the BSF1 grower diet. Phase, as confirmed by the FI results on D10-20 where BSF1 group FI declined significantly from the previous week compared to the

rest of treatment groups as well as control but was relatively improved in the following week, however, by the final week BSF1 group showed significantly lower FI compared to the BSF5 and control group even with improve in BWG by the final week of study, this only could be explained that BSF1 broilers group haven't eaten too much of the diet to make up for the week that contained the grower phase, however, the BSF1 broilers have utilised the diet in a way that improved the BWG, however, a significant increase in FCR on the second and third week of the trial compared to the other diets and control was interesting. The 0.3%, and 5% BSFL supplemented doses encouraged relatively normal BWG compared to control, and that also reflected on FI results where BSF0.3, and BSF5 groups have shown not much difference in FI compared to control, and the FCR of BSF0.3, and BSF5 was compatible to control through the study. The results of feed intake of Hartinger *et al.*, 2021 which investigated the substitution of SBM by graded levels of BSFL 15%, and 30%, was consistent with this study for 15% BSFL dose, however the 30% (higher dose) showed significant higher feed intake compared to the lower dose as well as the control diet on day 28, and 35. 5% supplement of BSFL showed FI values comparable with the control in Dabbou *et al*., 2018 study, which could confirm the results of the current study.

The significant decrease in average weekly BWG and FI of BSF1 group compared to both higher dose treatment and lower dose treatment is a clear indication that the birds supplemented the 1% BSFL did not perform well, that performance also reflected on the significantly high FCR. The difference in body weight of BSF1 on the later days of the study compared to the other doses (BSF0.3, and BSF5) as well as control was interesting. At day 21 of the current study mean body weight for the BSF1 group is nearly 240g lower (35%) than the other BSFL doses as well as control in line with BSF1 group (figure 6.1). Likewise, at day 28, and 35, this result is more pronounced with the BSF1 reducing the mean body weight by approximately

400g - (30%), and 1740g - (33%) respectively, lower than the other BSFL doses as well as the control. This very poor performance of the BSF1 treatment group couldn't be explained, since all experimental birds were randomly distributed since day1 of the study and were all fed the same basal diet, however, there might have been issues during diet manufacturing especially in the grower phase of 0.3% MW supplemented diets.

Hartinger *et al*., (2021) showed that 30% of BSFL substitution significantly reduced FCR on D14, and D35 compared to control but did not show a significant difference with 15% BSFL. However, Cummins et al., (2017) reported that FCR of pacific white shrimps increased with higher doses of BSFL, as 36% BSFL showed the highest FCR even compared to the fishmeal, but that the 7% BSFL resulted in lower FCR. That results could explain the different results might be due to the difference in the experimental animal, since shrimps need higher fat and protein in the diet and insect meal suited them better, or the environment, the conditions, and the difference in nutrition profile between fish meal and soybean meal. However, the results of the current study regarding FCR showed no significant difference except BSF1 inclusion which is likely due to an issue in the grower phase but cannot be taken as detrimental effect of BSFL at this dose (Table 6.1).

The results of this study seem to indicate that with the exception of the final week of the study, there seems to be no deterioration in performance for 0.3% BSFL supplemented birds.

Carcass yield

The BSF1 treatment group have witnessed significant reduction in meat yield (approximately 38%) compared to control and the rest of treatments, however, as mentioned earlier the BSF1 grower phase diet might have affected the decrease in BWG at the second and third week of study, and although there was improvement by the final week with the finisher diet, it still affected the carcass yield results. Probably, if the BSF1 will be reinvestigated again in a future study the performance results might be different. On the other hand, 0.3% BSFL

supplementation did not improve carcass yield but had no negative effect compared to control, while the BSF5 dietary inclusion have reduced meat yield by 17% compared to control group (Table 6.11).

Gizzard and ileal lesions score

There was no evidence of either gizzard or ileal lesions in birds supplemented with BSFL graded doses and this indicates no detrimental effect of insect meal at the studied BSFL levels on the mucosa in the gut.

Apparent metabolizable energy

The results for apparent metabolizable energy (AME), apparent metabolizable energy corrected for nitrogen (AMEn) and nitrogen retention for day 21, showed that BSFL supplement to diet at any of the dose levels used in the current study (0.3%, 1%, and 5%) have increased AME and AMEn compared to the control group. This suggests that insect meal at low doses could improve the energy quality of feed. That might be due to the high fat content in BSFL, worth noting that apart from the 1% BSFL inclusion, the study showed a dose dependent increase in AMEn with insect inclusions (table 6.13). Additionally, both higher dose (5%), and lower dose (0.3%) significantly improved Nitrogen retention compared to control diet by 20%, and 24.5% respectively. That may suggest that protein hydrolysis in the 0.3%, and 5% BSFL supplemented broilers was better than control. On the other hand, the group supplemented with the 1% BSFL dose showed significant decline in nitrogen retention compared to the rest of supplemented diets as well as control (Figure 6.2), given the fact that there probably were issues with grower phase BSF1 diet which as mentioned previously have reduced broilers performance in the second and third week and that reflected in nitrogen retention (Table 6.13).

By day 35 of the current study (Table 6.14), it seems that both BSFL supplementation (0.3%, and 1%) have decreased the AME, compared to the higher BSFL dose (5%), and the control,

and also decreased AMEn compared to control. The 5% BSFL inclusion showed comparable energy quality to control diet, which suggests that this level of BSFL supplementation is not detrimental to the energy utilisation of broiler diets. Only the nitrogen retention of the 1% BSFL supplemented diet was significantly declined at d35, but this was much less marked than the d21 results, suggesting that the finisher diets were less problematic than those of the grower phase for the BSFL1 diet (table 6.14). The day 35 results of 5% BSFL AMEn in the finisher phase are in agreement with Dabbou *et al*., 2018, that demonstrated no difference in AMEn values either between different BSFL doses (5%, 10%, and 15%) or control. However, this study also showed no difference in AMEn in the grower phase between different BSFL doses (5%, 10%, and 15%) and the control, unlike our findings that the similar 5% BSFL inclusion significantly increased AMEn compared to control.

Jejunum histology

Jejunum histology results for increasing doses of BSFL compared to control for day 21, and 35 are demonstrated in table 6.15, and 6.16. The increasing doses of BSFL did not have an adverse effect on jejunum morphology compared to the control group in terms of either villus height, villus width, villus area or villus/crypt ratio on day 21 or 35. BSF0.3 showed significantly increased crypt depth compared to the control diet at d21 only, which suggests an increased turnover of intestinal cells for this treatment. BSF1 demonstrated significant decrease in villus height, and villus/crypt ratio at d35 compared to the higher BSFL dose (5%), the lower BSFL dose (0.3%) but was compatible with control. This reduction in villus height reduces absorptive area of the small intestine for this diet and this matches with the reduction in N retention and performance seen in this diet. however significant increase in crypts depth on day 21 can be observed in 0.3% BSFL treatment dose significantly higher than control and since according to studies that has been correlated with the presence of bacterial communities that positively affects gut integrity especially in the jejunum region (Dung *et al*., 2021). That all

could confirm no detrimental effect of BSFL doses on contrary with further investigation to jejunum bacteria could prove positive impact of 0.3% BSFL supplementation. That results were consistent with Hartinger *et al.,* 2021 as the increasing levels of BSFL (15%, and 30%) didn't have a significant effect on any of the criteria involving jejunum histology, except for BSFL 15% inclusion level that had a significant increase in jejunum villus width compared to control. And another study by Cutrignelli *et al*., 2018, where SBM was completely replaced by BSFL (100%), showed that total replacement of SBM with BSFL have relatively improved villus height and crypts depth of jejunum villi of laying hens.

Nitrogen digestibility coefficient

The results for nitrogen digestibility coefficient (NDC) investigated in this chapter are shown in table 6.12. All BSFL doses showed higher NDC on D21 of the study compared to control and a protein digestibility that exceeded 86% on both D21 and D35 of study. These are consistent with the results in chapter four where broilers fed 20%, 40%, and 60% total BSFL inclusions from different raising substrates. Broilers of the BSFL group raised on fruit waste or bran showed protein digestibility exceeding 80% and those raised on brewery waste showed 75% protein digestibility similar to the soya protein source. Although, it has been shown in the results of chapter four that insect protein content is highly affected by raising substrate and the potential effectiveness of separation of larvae from frass, although NDC was not affected. Surprisingly, NDC was high even with 1% BSF dose that showed significantly low nitrogen retention and that was unexplainable because NDC and nitrogen retention are considered measures for effectiveness of protein utilisation.

These results were in agreement with results of study performed by Dumas *et al*., 2018 that studied the effect of 20% partially defatted BSFL meal, or 20% BSFL oil on the apparent digestibility coefficients of rainbow trout. The 20% BSFL meal inclusion showed 88% nitrogen

digestibility while the 20% BSFL oil diet showed 90% nitrogen digestibility. This was not the case for the study performed by Hartinger *et al*., 2021, in which the nitrogen digestibility coefficient in broilers fed 30% full fat BSFL meal inclusion was slightly less than that of the lower full fat BSFL inclusion (15%) and significantly lower than control Also in this study, the NDC of both insect inclusions did not exceed 76%. This study also mentioned some factors that could be the cause of that reduction in NDC in high black soldierfly inclusions as the study investigated Janssen *et al*., 2017 who mentioned an issue in the typical nitrogen to protein conversion factor of 6.25, and that a more suitable specific conversion factor for BSFL protein would be 4.76. That is because part of the nitrogen content (3-6.8%) is associated with chitin in the larvae exoskeleton. Nevertheless, studies in this thesis on the full fat BSFL meal have reported digestibility values of Broilers fed BSFL meal either in high inclusions (20%, 40%, and 60%) or in low doses (0.3%, 1%, and 5%), that could encourage further investigation to compare full fat BSFL meal to partially defatted BSFL meal. Additionally, it would be interesting to do further investigation of the digestible amino acids in low dose supplements of black soldierfly larvae meal compared to non-supplemented soyabean control diet.

6.5 Conclusion

The results of this study clearly show no deterrent effect of including BSFL doses on bird performance. Although the group of birds given 1% dose BSFL showed poor performance in the grower phase, the higher dose and the lower dose showed performance comparable to that of control group. Moreover, the nitrogen digestibility from the results of the current study showed the positive effect of BSFL supplementation on nitrogen digestibility for broilers which might confirm the theory.

The results of AME, AMEn, and nitrogen retention were interesting and agreed with other studies. AME and AMEn are considered crucial factors in production efficiency and the

supplementation of BSFL did not negatively affect energy yielding nutrients. They also confirmed that black soldier fly larvae could be used as feed additive in broiler diet at level of 0.3% to improve nitrogen retention reducing nitrogen excretion to the environment.

Generally, the results have proven the viability of using black soldier fly larvae at a supplementary level rather than having to use higher inclusions not due to any negative impact of black soldierfly larvae on the boilers health integrity but rather economically. That also can pave the way towards the use of black soldierfly larvae as an additive in poultry industry.

Chapter 7- Discussion

Soyabean meal is considered the main source of protein in feed for the poultry industry, however soya bean growth and cultivation have developed at the expense of exploiting a vast amount of wild land, altering the land cover and accordingly disturbing the natural balance of soil by altering soil nutrients and causing catastrophic changes in biochemistry of watersheds (Neill *et al.*, 2013). While Brazil is one of the major exporters of soyabeans, the overcultivation of soyabean is threatening the land biodiversity in Brazil (Fearnside, 2001). In addition to the negative impact of soyabean on wild land, there is also the high carbon footprint resulting from the substantial carbon emissions of soyabean production, and from long distance transportation (Guisti *et al*., 2023). That this has led to the need for a more sustainable feed option for feed protein, to encourage sustainability in poultry production.

Insects have emerged among the poultry feed options as an alternative to soyabean. Insects can be farmed on a small scale such as on local poultry farms, as well as on a larger industrial scale. Both options have no requirement for a large area, and this reduces the great environmental impacts caused by exploitation of agricultural land (or wild land in case of growing soyabean) and can remove the need for high transportation costs since insects can be bred locally. In addition, in free range poultry farming, insects are considered a conventional feed of choice, and a good enrichment for the birds.

The regulations in Europe are strict regarding the use of insect meal and also regarding the type of substrate used for insects destined for feed. According to EU animal health law regulation (EU No. 2016/429) on transmissible animal diseases, it is forbidden to use slaughterhouse wastes, manure or catering food waste in feeding of livestock to prevent the spread of disease, with emphasis on mad cow disease because of Europe's historic issues with this particular disease. Additionally, regulations in Europe No.1143/2014 limits foreign insects brought to the

EU for farming purposes, as this might affect the natural biodiversity in the area and may cause uncontrollable outbreaks of these insects in crops. By 2017, the EU listed seven insect species that are approved for feeding as a protein meal, including black soldierfly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) (EU regulation No.2017/893). However, the current UK legislation prevents the utilization of processed insect meal for poultry feed, and only live larvae are allowed as these are considered a common feed for birds farmed in a free-range environment. While in some African countries, for example Kenya, the restrictions in the use of edible insects are being mitigated, with a vast number of commercial insect farms being developed day by day, and feed traders showing over 80% acceptability towards using insect meal for feed as a step towards creating more sustainable protein alternatives for livestock (Tanga and Kababu, 2023). In warm environments like in Africa, Australia, and south America, insect farming could be more cost efficient as there would be less need mechanical control of environmental conditions compared to Europe and the UK. In these temperate regions, insects can flourish in certain seasons when the temperature is favourable but might need expensive measures to control the insect breeding environment. Apart from that, some types of insects need specific expertise in growing and handling to achieve the greatest value, not to mention the long-life cycle of some insects destined for feed which might take up to a year per cycle, therefore there are several limitations when considering raising the mealworm for example. In this thesis, two types of insects were studied from different insect orders, with each having its own characteristics.

This research has shown substrate plays a crucial role in the rearing of insect larvae, impacting their growth, development, survival rates, and overall health. Different insect species have specific substrate requirements that need to be met to optimize their rearing conditions. In data chapter3 for both trials T1 and T2, mealworms (*Tenebrio molitor* larvae), which are considered a popular pet food across Europe and the UK (Payne *et al.,* 2016), were brought from an insect

farm were nearly 7 to 8 weeks of age and it took another two to three weeks (depending on the age, for T1 trial larvae were brought about 7 weeks and were reared for another 3 weeks to reach the prepupae while in T2 larvae were 8 weeks and reared for 2 weeks) to reach the prepupae because typically the larvae can take up to 12 weeks in ±30ºC temperature to reach an appropriate size for feed (www.breedinginsects.com/2020), which is considered costly in cold European countries where the temperature can drop to under 0ºC in winter and in some occasion can't exceed 20ºC. As well as certain environmental conditions of humidity (studies have shown mealworm thrive in up to 75% humidity) If conditions of either temperature or humidity of the substrate are not favourable for mealworm, this tends to slow their developmental process and elicit a cannibalistic behaviour which affects the final larvae yield and mass gain. (Kotsou *et al*., 2021). In addition to this, although mealworm can valorise a range of waste substrates, several studies including T1 and T2 trials in the current thesis showed that mealworm feeding substrates require certain processing conditions. In T1 trial when mealworm were raised on banana peel substrate, the group of larvae fed the banana peel couldn't survive the first week of trial although the environmental conditions were fixed for both substrates (banana peels and DDGS), the mealworms fed on banana peels haven't survived the first week of the trial with 100% mortality, and these results have led to supposing that banana peels should have been dried and ground as a processing method before being introduced as mealworm substrate or banana peel itself was detrimental to mealworm. Mealworm is also considered as pest for grains and therefore during rearing several measures should be considered to avoid any escape or outbreak of these insects into farmland which could be compromising to crops. These above-mentioned obstacles need to be overcome mealworm farming for feed as cost effective as possible especially when the protein content of mealworm can exceed 50% and some mealworm feeding substrates can raise the mealworm protein content up to 60%. That was well evidenced in T2 trial of this thesis when protein

content of mealworm experimental group fed the seawaste reached 635 g/kg protein. In summary, these findings show substrate is a pivotal factor in insect larvae rearing, influencing nutritional intake, environmental conditions, microbial interactions, and overall larval health. Optimizing substrate conditions tailored to specific insect species can significantly enhance rearing efficiency and productivity.

Black soldierfly (*Hermetia illucens*) of the order Diptera is the second insect studied and there are a few members of this order which can also be considered for insect farming, especially housefly (*Musca domestica*). However, Black soldierfly is preferred over the housefly for several reasons such as, adults of black soldierfly do not interact with humans, do not feed and therefore have not been recorded to transmit any diseases, unlike housefly which is the main vector for mechanical disease transmission of pathogenic *Entamoeba histolytica* (El-Salem *et al*., 2021). The life cycle of black soldierfly can take only two weeks under ±25ºC temperature, and the larvae can take few days more to reach the final appropriate size to be ready for use as feed. Black soldierfly larvae do not require specific conditions for their substrate and can valorise a range of waste substrates, from animal manure to food wastes, converting less valuable waste substrates into a high-quality protein. In addition, the post-feeding substrate (frass) has been shown to be an excellent natural fertilizer, to restore the useful nutrients to the soil and enhance plant growth (Poveda, 2021). It is difficult to say which substrate is better for growth of black soldierfly larvae, especially since BSFL has a strong ability to utilise any substrate. The problems faced during the insect studies included the efficiency of separation of post-feeding larvae from frass was a limiting factor to the protein yield, where the larvae that with very low percentage of frass gave higher protein than the larvae suspected of containing high amount of frass although this speculation has not been confirmed. For mealworm, the type of substrate proved to be important as during the mealworm study with feeding on different substrates, the conventional grain substrate (in the form of wheat distillers grains with solubles

(DDGS)), was the most favourable for mealworm however, the larvae with the highest protein content were those that fed on the sea waste substrate. It is worth mentioning that it is very important to perform further investigation on other nutritional parameters beside the protein since this study was limited to investigating protein content. Therefore, to highlight the effect of feeding substrate on insect nutritional profile, and how different substrates can manipulate insect protein content as well as amino acids concentrations. mealworm fed distillers grains with solubles had higher protein content than soya and black soldierfly fed brewery wastes had protein content almost compatible to soya. That is good evidence on how well insects utilise the substrate and how it reflects on the nutritional profile, also this can be a good indication on how larvae will be able to utilise whatever additives included in the feeding substrate whether it is an amino acid that could enhance larvae performance. In T3 study of data chapter 4 BSFL raised on fruit waste and bran were brought from insect rearing farm were suspected of contamination with frass. Although that did not affect the digestibility of the broilers however, it affected the protein content for BSFL fruitwaste, and BSFL bran yielding 313.4 g/kg, and 399.4 g/kg protein respectively.

Many studies have shown that total protein content of mealworm is higher than that of black soldierfly larvae and the later has relatively higher fat content (Bußler *et al*., 2016; Melenchón *et al*., 2021), including the current studies in this thesis. Probably due to the nature of black soldierfly larvae diet versus mealworm diet, mealworm have been reported to survive best on variety of grains and cereals while BSFL have the tendency to valorise a wider range of substrates. In data chapter 4, in trials T3, and T4, BSFL raised on fruit wastes had the lowest protein content (313.4 g/kg) but showed the highest fat yield (371.49 g/kg), probably the fruit waste as a substrate was characterised by less protein and higher sugar content and that probably reflected on the larvae nutritional profile, on the other hand BSFL raised on brewery waste showed high protein content (485.2 g/kg) that is compatible to soya protein (488.4 g/kg),

and also compatible to mealworm raised on DDGS (499.8 g/kg), and mealworm raised on bran (461.9 g/kg). concluding from the above that BSFL protein content can be manipulated through diet to be a suitable source of protein. Nevertheless, the positive effect of both mealworm or black soldierfly larvae on enhancing the digestibility of broilers have been reported in this thesis in data chapter 4 as the first study to investigate both black soldierfly and mealworm both from various raising substrates on the broilers' amino acids digestibility. From the two studies T3, and T4 conducted in this thesis it has been evidenced that insect meal regardless of the raising substrate, can improve overall nitrogen and amino acids digestibility of broilers, although the substrate effect is obvious on the insect nutritional profile like protein and fat content. It can also be concluded from comparing the time needed for mealworm to complete one life cycle to black soldierfly that only takes to weeks to complete a life cycle, in addition to the certain requirements of mealworm regarding certain conditions of substrate, and environmental conditions, while blacksoldierfly larvae practically feed on any type of substrate. This balances out the difference in protein content since black soldierfly can still yield as high-quality protein as mealworm and that can also make BSFL a more attractive candidate for soybean meal or fish meal replacement, it can also at some point solve the high cost of insect meal by promoting the idea of more local insect farms to save costs of transportation, and if BSFL to feed on food wastes from farms or local restaurants etc.,…, leading to increase in production of insect meal as expected by the feed producers that could overcome the high price of insect meal and force the prices to drop down against other feed products like fishmeal (Rabobank, 2021). All the above-mentioned advantages are also met by some obstacles of legislations and consumers acceptance to insects-fed chicken. Therefore, more research heading towards releasing restrictions on insects as feed for poultry.

As mentioned in data chapter 5 that the feed intake results varied among experimental groups of broilers that were feed mealworm from different sources. As mentioned before, avian palate

responds positively to omami taste which is translated by the presence of different amino acids, in human it is mono sodium glutamate (Roura *et al*., 2008). In 1996 Moran and stillborn discovered that L-Glutamate have elevated feed intake in broilers fed on protein diet with suitable amino acids. That might explain the increase in feed intake results of the broilers fed 5% MW ENTEC over the broilers group fed 5 % MW COMP, and probably the MW ENTEC product have been subjected to amino acids supplementation.

While is can be concluded from these studies there are few nutritional barrier to update of BSFL or MW in poultry feed, there remain other economic, legislative, and logistical barriers challenges that must be overcome before widespread adoption as these challenges impact the scalability and acceptance of insect-based feed solutions within the poultry industry. In terms of economic barriers, the primary consideration is market competition: traditional feed ingredients such as soybean meal and fishmeal are well-established and often cheaper due to economies of scale. However, while competing with these conventional feeds on price is currently a significant hurdle, emerging considerations of carbon taxation schemes that will likely affect animal feed, will rebalance the economics in favour of insect larvae, as long as producers focus on use of waste streams such as those evaluate in this thesis as feeding substrates. Other economic barriers relate to the cost of producing insect larvae at scale due to the need for specialized infrastructure, controlled environments, and labour.

Developing technical solutions to increase scale of production are also key to offering a viable feed material to the poultry sector which is a highly integrated business model requiring extremely high volumes of consistently available, consistently profiled material. Scaling up production to meet the demands of large poultry operations requires substantial capital investment and technological innovation, which can be prohibitive for many startups and small companies currently working in the insect farming sector.

In addition to the EU legislative barriers described above, there are global concerns over food safety than must be addressed robustly. Regulatory bodies for differing global regions may have stringent requirements that insect producers must meet, which can be challenging and costly to achieve. This is difficult for any new feed or food materials, due to the lack of harmonized regulations: regulatory standards for insect-based feeds vary widely between countries, creating challenges for producers looking to operate or export internationally. This lack of harmonization can lead to additional compliance costs and complexity. Regardless of this, ensuring that insect larvae are free from pathogens, contaminants, and harmful substances is critical particularly as consumer perception can influence legislative action. Public scepticism or lack of knowledge about the safety and benefits of insect-based feeds can slow legislative progress and market acceptance. Some of these challenges can be addressed by established robust Quality Control systems: maintaining consistent quality of insect larvae in terms of nutritional content and safety is crucial. This requires stringent quality control measures and protocols, which can be difficult to standardize across different production facilities.

Alongside scale up is the need for logistical supply chain development. Establishing a reliable and efficient supply chain for insect larvae, from production to processing to distribution, is complex. This includes the need for specialized transportation and storage conditions to maintain product quality. Beyond transporting the larvae destined for animal feed, producing insect larvae at scale requires advanced technology for breeding, rearing, and processing. Many producers may lack access to or the expertise required to implement these technologies effectively.

The growing desire to achieve net zero food production is strongly driving efforts to overcome these barriers, as insects present an unparalleled opportunity to circularize food production. Many governments and NGO funding bodies have therefore created economic incentives.

Subsidies, grants, and investment in research and development can help reduce the financial burden on producers and stimulate growth in the sector. In parallel to this, other governing bodies are working to develop regulatory frameworks. By developing clear, science-based regulations and harmonizing standards internationally can facilitate smoother market entry and expansion. Adoption of responsible research and innovation frameworks (RIFs) is also helpful for increasing awareness and acceptance of insect-based feeds among consumers. By addressing these barriers, the industry can move towards more sustainable and economically viable insect-based poultry feed solutions.

Future work

The results reported in this thesis have profoundly illustrated how substrate affects product quality and composition insect larvae rearing. The substrate often provides essential nutrients required for the larvae's growth. However, there are a number of substrate features that were not considered in this research which greatly impact on larvae rearing. For example, the moisture content of the substrate is critical for maintaining hydration levels in larvae but too much moisture can lead to fungal growth and bacterial infections. The physical texture of the substrate can also affect larvae mobility and their ability to burrow. Soft substrates might be better for delicate larvae, while more robust larvae might prefer coarser materials. Beyond these considerations, the microbial composition of the substrate can influence larval health and development. Beneficial microbes can enhance digestion and nutrient absorption, while harmful microbes can cause disease. All these elements need further investigation to support decision-making over optimum substrate for BSFL and MW rearing for poultry feed.

It seems now more important than ever for the rest of the globe to relieve the restrictions of use of insects as a protein source for feed and focus on developing strategies towards investigating the effect of feeding insect meal on meat quality. It would be important in future work to

investigate more novel insect feeding substrates from local sources, which will have a role in minimizing carbon cost. For example, just as wheat DDGS has been investigated as potential feeding substrate for both mealworm, and black soldierfly larvae, there are other by-products which have potential for use as insect feed substrates. These include the end product of the corn fermented protein process (Hossain *et al*., 2023; Kilburn-Kappeler and Aldrich, 2023) or waste substrates like material from the production of algae (Saadaoui *et al*., 2021). As well as investigating the safety aspects of waste substrate use to reduce legislative issues, more attention should be directed towards investigating methods to improve the quality and nutritional content of insect larvae meal by supplementing the insect feeding substrate. Vitamins, minerals or amino acids could be utilised as feed substrate additives, which in turn could increase the amino acid content or general nutritional quality of post feeding larvae, which could result in an improved nutritional profile of poultry. This could reduce the requirement for synthetic addition of costly synthetic amino acids in poultry diets and this could be also useful in organic farming where synthetic additives are limited.

Conclusions

Insect production is a promising market that could introduce a more sustainable high quality protein alternative to soya or fishmeal.

Insect nutritional profile could be manipulated through feeding substrate, in fact, it was well evidenced in some of the studies of this thesis that larvae raised on different substrates can differ in total protein content, fat content and amino acids content.

Mealworm has an advantage over BSFL in expressing higher protein content, however, BSFL have extremely shorter life cycle, can feed on any sort of substrate, can still yield high quality protein, and protein content can be manipulated through diet, that means the time taken for

mealworm to complete one life cycle practically equals the time needed for BSFL to go through six cycles, that means BSFL can yield six times more protein than mealworm in the same time frame.

Insect meal inclusions from either mealworms or BSFL and from whatever feeding substrate, did significantly enhance digestibility of broilers compared to soyabean meal inclusions.

Insect protein is more expensive than other protein feed like fish meal or soya however, that might be due to small scale production and high demand with scarcity practically means high prices. That could be solved by increasing insect meal production and better choice of type of insect that could yield more protein in less time.

References

Abramson, M.B., 2004, 'Mad cow disease: an approach to its containment'. *J. Health Care L. & Pol'y*, vol.7, p.316.

Al-Mnaser, A., Dakheel, M., Alkandari, F., & Woodward, M., 2022, 'Polyphenolic phytochemicals as natural feed additives to control bacterial pathogens in the chicken gut'. *Archives of Microbiology*, vol. 204 no. 5, pp. 253.

Azagoh, C., Ducept, F., Garcia, R., Rakotozafy, L., Cuvelier, M. E., Keller, S., Lewandowski, R., & Mezdour, S., 2016, 'Extraction and physicochemical characterization of Tenebrio molitor proteins'. *Food Research International*, vol. *88*, pp. 24–31.

Akpomie, K. G., & Conradie, J., 2020, 'Banana peel as a biosorbent for the decontamination of water pollutants', A review. In *Environmental Chemistry Letters*, Vol. 18, no. 4, pp. 1085–1112.

AHDB, U., 2017. Nutrient management guide (RB209). *Agriculture & Horticulture Development Board.*

Abd El-Hack, M.E., Shafi, M.E., Alghamdi, W.Y., Abdelnour, S.A., Shehata, A.M., Noreldin, A.E., Ashour, E.A., Swelum, A.A., Al-Sagan, A.A., Alkhateeb, M. and Taha, A.E., 2020, 'Black soldier fly (Hermetia illucens) meal as a promising feed ingredient for poultry: A comprehensive review', *Agriculture*, Vol. 10, no.8, pp.339.

Abdel-Raheem, S.M., Leitgeb, R. and Iben, C., 2011. 'Effects of dietary inclusion level of distillers' dried grains with solubles (DDGS) from wheat and corn on amino acid digestibilities in broilers', *International Journal of Poultry Science*, Vol.*10*, no.12, pp.952-958.

Bußler, S., Rumpold, B.A., Jander, E., Rawel, H.M. and Schlüter, O.K., 2016. 'Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (Tenebrio molitor) and black soldier fly (Hermetia illucens) larvae.', *Heliyon*, Vol.*2*, no.12.

Bush, K., & Page, M. G. P., 2017, 'What we may expect from novel antibacterial agents in the pipeline with respect to resistance and pharmacodynamic principles', In *Journal of Pharmacokinetics and Pharmacodynamics*,Vol. 44, no. 2, pp. 113–132.

Beskin, K. v., Holcomb, C. D., Cammack, J. A., Crippen, T. L., Knap, A. H., Sweet, S. T., & Tomberlin, J. K., 2018, 'Larval digestion of different manure types by the black soldier fly (Diptera: Stratiomyidae) impacts associated volatile emissions', *Waste Management*, Vol. *74*, pp. 213–220.

Barnes, E. M., Impey, C. S., & Stevens, B. J. H., 1979, 'Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick', *Journal of Hygiene*, Vol. 82 no. 2, pp. 263–283.

Biasato, I., de Marco, M., Rotolo, L., Renna, M., Lussiana, C., Dabbou, S., Capucchio, M. T., Biasibetti, E., Costa, P., Gai, F., Pozzo, L., Dezzutto, D., Bergagna, S., Martínez, S., Tarantola, M., Gasco, L., & Schiavone, A., 2016, 'Effects of dietary Tenebrio molitor meal inclusion in free-range chickens', *Journal of Animal Physiology and Animal Nutrition*, Vol., 100 no. 6, pp. 1104–1112.

Biasato, I.; Ferrocino, I.; Grego, E.; Dabbou, S.; Gai, F.; Gasco, L.; Cocolin, L.; Capucchio, M.T.; Schiavone, A. 2019, 'Gut microbiota and mucin composition in female broiler chickens fed diets including yellow mealworm (Tenebrio molitor, L.)', *Animals*, , no.9, pp.213.

Biasato, I.; Gasco, L.; De Marco, M.; Renna, M.; Rotolo, L.; Dabbou, S.; Capucchio, M.T.; Biasibetti, E.; Tarantola, M.; Sterpone, L.; et al. 2018, 'Yellow mealworm larvae (Tenebrio molitor) inclusion in diets for male broiler chickens: Effects on growth performance, gut morphology, and histological findings', *Poultry Science*., no.97, pp.540–548.

Bulet, P. and Stocklin, R., 2005, 'Insect antimicrobial peptides: structures, properties and gene regulation', *Protein and peptide letters*, Vol. 12 no. 1, pp.3-11.

Benzertiha, A., Kierończyk, B., Kołodziejski, P., Pruszyńska–Oszmałek, E., Rawski, M., Józefiak, D. and Józefiak, A., 2020, 'Tenebrio molitor and Zophobas morio full-fat meals as functional feed additives affect broiler chickens' growth performance and immune system traits', *Poultry science*, Vol. 99, no. 1, pp.196-206.

Belyea, R.L., Rausch, K.D., Clevenger, T.E., Singh, V., Johnston, D.B. and Tumbleson, M.E., 2010, 'Sources of variation in composition of DDGS', *Animal feed science and technology*, Vol. 159 no. 3-4, pp.122-130.

Belforti, M., Gai, F., Lussiana, C., Renna, M., Malfatto, V., Rotolo, L., De Marco, M., Dabbou, S., Schiavone, A., Zoccarato, I. and Gasco, L., 2015, 'Tenebrio molitor meal in rainbow trout (Oncorhynchus mykiss) diets: effects on animal performance, nutrient digestibility and chemical composition of fillets', *Italian Journal of Animal Science*, Vol. 14, no. 4, p.4170.

Belyea, R.L., Rausch, K.D., Tumbleson, M.E., 2004, 'Composition of corn and distillers dried grains with solubles from dry grind processing', *Bioresource technology*, Vol. 94, pp. 293-298.

Belyea, R.L., Rausch, K.D., Clevenger, T.E., Singh, V., Johnston, D.B., Tumbleson, M.E., 2010, 'Sources of variation in composition of DDGS', *Animal Feed Science and Technology*, Vol. 159 pp. 122- 130.

Bovera, F., Piccolo, G., Gasco, L., Marono, S., Loponte, R., Vassalotti, G., Mastellone, V., Lombardi, P., Attia, Y.A. and Nizza, A., 2015, 'Yellow mealworm larvae (Tenebrio molitor, L.) as a possible alternative to soybean meal in broiler diets', *British poultry science*, Vol. 56, no. 5, pp. 569-575.

Banks, I.J., Gibson, W.T. and Cameron, M.M., 2014, 'Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation', *Tropical medicine & international health*, Vol. 19, no. 1, pp.14-22.

Brehélin, M. and Boemare, N., 1988, 'Immune recognition in insects: conflicting effects of autologous plasma and serum', *Journal of Comparative Physiology B*, Vol. 157, pp.759-764.

Bordiean, A., Krzyżaniak, M., Stolarski, M. J., Czachorowski, S., & Peni, D. (2020). Will yellow mealworm become a source of safe proteins for Europe? In *Agriculture (Switzerland)* (Vol. 10, Issue 6, pp. 1–30). MDPI AG. https://doi.org/10.3390/agriculture10060233

Ballitoc, D.A. and Sun, S., 2013, 'Ground yellow mealworms (Tenebrio molitor L.) feed supplementation improves growth performance and carcass yield characteristics in broilers', *Open Science Repository Agriculture*, Vol.*18*, p.e23050425.

Biasato, I., Gasco, L., de Marco, M., Renna, M., Rotolo, L., Dabbou, S., Capucchio, M. T., Biasibetti, E., Tarantola, M., Bianchi, C., Cavallarin, L., Gai, F., Pozzo, L., Dezzutto, D., Bergagna, S., & Schiavone, A., 2017, 'Effects of yellow mealworm larvae (Tenebrio molitor) inclusion in diets for female broiler chickens: implications for animal health and gut histology', *Animal Feed Science and Technology*, Vol.234, pp.253–263.

Biasato, I., Gasco, L., de Marco, M., Renna, M., Rotolo, L., Dabbou, S., Capucchio, M. T., Biasibetti, E., Tarantola, M., Sterpone, L., Cavallarin, L., Gai, F., Pozzo, L., Bergagna, S., Dezzutto, D., Zoccarato, I., & Schiavone, A., 2018, 'Yellow mealworm larvae (Tenebrio molitor) inclusion in diets for male broiler chickens: Effects on growth performance, gut morphology, and histological findings', *Poultry Science*, Vol.97, no.2, pp.540–548.

Breeding insects. (2020) *Yellow Mealworm Life Cycle*. Available at: www.breedinginsects.com (accessed: 26 January 2023).

Britannica. (2023) *Exarate pupa.* Available at: www.britannica.com (accessed: 26 January 2023).

Clarke, E.J. and Wiseman, J., 200, 'Developments in plant breeding for improved nutritional quality of soya beans II. Anti-nutritional factors', *The Journal of Agricultural Science*, Vol.134, no.2, pp.125-136.

Cammack, J.A., Miranda, C.D., Jordan, H.R. and Tomberlin, J.K., 2021, 'Upcycling of manure with insects: current and future prospects', *Journal of Insects as Food and Feed*, Vol.7, no.5, pp.605-619.

Cromwell, G.L., Herkelman, K.L. Stahly, T.S., 1993, 'Physical, Chemical and Nutritional characteristics of distillers dried grains with solubles for chicks and pigs', *Journal of Animal Science*, Vol. 71, pp. 679-686.

Cammack, J.A. and Tomberlin, J.K., 2017, 'The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly Hermetia illucens (L.)(Diptera: Stratiomyidae)', *Insects*, Vol.8, no.2, p.56.

Castanon, J.I.R., 2007, 'History of the use of antibiotic as growth promoters in European poultry feeds', *Poultry science*, Vol.86, no.11, pp.2466-2471.

Crossley, A.C.S., 1964, 'An experimental analysis of the origins and physiology of haemocytes in the blue blow-fly Calliphora erythrocephala,(Meig)', *Journal of Experimental Zoology*, Vol.157, no.3, pp.375-397.

Cao, X., He, Y., Hu, Y., Wang, Y., Chen, Y.R., Bryant, B., Clem, R.J., Schwartz, L.M., Blissard, G. and Jiang, H., 2015, 'The immune signaling pathways of Manduca sexta', *Insect biochemistry and molecular biology*, Vol.62, pp.64-74.

Clayton, A.M., Dong, Y. and Dimopoulos, G., 2014, 'The Anopheles innate immune system in the defense against malaria infection', *Journal of innate immunity*, Vol.6, no.2, pp.169-181.

Cutrignelli, M. I., Messina, M., Tulli, F., Randazzi, B., Olivotto, I., Gasco, L., Loponte, R., & Bovera, F., 2018, 'Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens', *Research in Veterinary Science*, Vol.117, pp.209–215.

Caligiani, A., Marseglia, A., Leni, G., Baldassarre, S., Maistrello, L., Dossena, A., & Sforza, S., 2018, 'Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin', *Food Research International*, Vol.105, pp.812–820.

Darfour, B 2019, 'Controlling the deterioration of harvested grain/seed to improve food security', PhD thesis, Lowa state University, USA.

Daniela Alvarez, Kevin A Wilkinson, Michel Treilhou, Nathan Téné, Denis Castillo, Michel Sauvain, 2019, 'Prospecting Peptides Isolated From Black Soldier Fly (Diptera: Stratiomyidae) With Antimicrobial Activity Against *Helicobacter pylori* (Campylobacterales: Helicobacteraceae)', Journal *of Insect Science*, Vol.19, Issue 6.

De Jong, I. C., & van Emous, R. A., 2017, '*Broiler breeding flocks: management and animal welfare*, pp. 211–230.

Denbow, D.M., 2015, 'Gastrointestinal anatomy and physiology', In *Sturkie's avian physiology*, Academic Press, pp. 337-366.

De Marco, M., Martínez, S., Hernandez, F., Madrid, J., Gai, F., Rotolo, L., Belforti, M., Bergero, D., Katz, H., Dabbou, S., Kovitvadhi, A., Zoccarato, I., Gasco, L., & Schiavone, A., 2015, 'Nutritional value of two insect larval meals (Tenebrio molitor and Hermetia illucens) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy', *Animal Feed Science and Technology*, Vol.209, pp.211–218.

Dortmans, B.; Diener, S.; Verstappen, B.; Zurbrügg, C., 2017, '*Black Soldier Fly Biowaste Processing—A Step-by-Step Guide Eawag*' Swiss Federal Institute of Aquatic Science and Technology: Dübendorf, Switzerland,; pp. 6–54. Available online: www.researchgate.net/publication/353923113_Black_Soldier_Fly_Biowaste_Processing_-_A_Step-by-Step_Guide_2nd_Edition (accessed on 26 August 2022).

Duke, G.E., 1986, 'Alimentary canal: anatomy, regulation of feeding, and motility', In *Avian physiology*, pp. 269-288.

Duggan, B.M., 2018, 'Genetic improvement of skeletal architecture and locomotion in domestic poultry, PhD thesis, The University of Edinburgh, Edinburgh.

Derler, H., Lienhard, A., Berner, S., Grasser, M., Posch, A. and Rehorska, R., 2021, 'Use them for what they are good at: Mealworms in circular food systems', *Insects*, Vol.12, no.1, pp.40.

Diener, S., Zurbrügg, C. and Tockner, K., 2009, 'Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates', *Waste Management & Research*, Vol.27, no.6, pp.603-610.

Dunkley, C.S., McReynolds, J.L., Dunkley, K.D., Kubena, L.F., Nisbet, D.J. and Ricke, S.C., 2007, 'Molting in Salmonella Enteritidis-challenged laying hens fed alfalfa crumbles. III. Blood plasma metabolite response', *Poultry science*, Vol.86, no.12, pp.2492-2501.

Donachie, W.D. and Begg, K.J., 1970, 'Growth of the bacterial cell', *Nature*, Vol.227, no.5264, pp.1220-1224.

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E. and Weltzien, E., 2018, 'The oil fraction and partially defatted meal of black soldier fly larvae (Hermetia illucens) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (Oncorhynchus mykiss)', *Aquaculture*, Vol.492, pp.24-34.

Dabbou, S., Gai, F., Biasato, I., Capucchio, M. T., Biasibetti, E., Dezzutto, D., Meneguz, M., Plachà, I., Gasco, L., & Schiavone, A., 2018, 'Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features', *Journal of Animal Science and Biotechnology*, Vol.9, no.1.

Dekruyff RH, Durkin HG, Gilmour DG, Thorbecke GJ., 1975, 'Migratory patterns of B lymphocytes: II. Fate of cells from central lymphoid organs in the chicken.' *Cell Immunology*.; Vol.16:301, no.14.

EFSA Scientific Committee, More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Hougaard Bennekou, S., Koutsoumanis, K., Lambré, C. and Machera, K., 2021, 'Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: Human and animal health', *Efsa Journal*, Vol.19, no.8, p.e06768.

Ewing, W.H., 1986, 'Edwards and Ewing's identification of Enterobacteriaceae', *Edwards and Ewing's Identification of Enterobacteriaceae.*, (Edition 4).

El-Salem, R.M., Lahwal, M.M. and Alqdari, H., 2021, 'Detection of Intestinal Parasites Transmitted Mechanically by House Flies (Musca domestica, Diptera: Muscidae) Infesting in Southern City of Libya', *Journal of Medical Sciences*, Vol.16, no.1, pp.13-15.

Evans, E., & Patterson, R. J., 2007, 'Is lysine:methionine always important?', *Feedstuffs Reprint*, Vol.79, no.15.

Elahi, U., Xu, C. C., Wang, J., Lin, J., Wu, S. G., Zhang, H. J., & Qi, G. H., 2022, 'Insect meal as a feed ingredient for poultry', In *Animal Bioscience*, Vol.35, no.2, pp.332–346.

EU legisilations. (2013). insect producers for food and feed. Available at: https://ipiff.org (accessed: November 2020).

Foley, J. A., Ramankutty, N., Brauman, K. A., Cassidy, E. S., Gerber, J. S., Johnston, M., Mueller, N. D., O'Connell, C., Ray, D. K., West, P. C., Balzer, C., Bennett, E. M., Carpenter, S. R., Hill, J., Monfreda, C., Polasky, S., Rockström, J., Sheehan, J., Siebert, S., … Zaks, D. P. M., 2011, 'Solutions for a cultivated planet', *Nature*, Vol.478, no.7369, pp.337–342.

Finke, M.D., DeFoliart, G.R. and Benevenga, N.J., 1989, 'Use of a four-parameter logistic model to evaluate the quality of the protein from three insect species when fed to rats', *The Journal of nutrition*, Vol.119, no.6, pp.864-871.

FAO statistics. (2017). *Poultry products production.* Available at: www.fao.org (accessed: 11 June 2023).

FAO statistics. (2020). *Poultry products production.* Available at: www.fao.org (accessed: 19 November 2023).

FAO statistics. (2023). *Poultry products production*. Available at: www.fao.org (accessed: 19 November 2023).

Fearnside, P. M., 2001, 'Soybean cultivation as a threat to the environment in Brazil', *Environmental Conservation*, Vol.28, no.1, pp.23–38.

Feed navigator. (2021) *Demand for insect protein could hit 500000 tons by2030.* Available at: www.feednavigator.com (accessed: 19 November 2023).

Fletcher, R. 2021., 'Why insect production may have minimal impact on aquaculture sustainability', *In: The Fish Site*. Cited 9 November 2021. www.thefishsite. com/articles/why-insect-production-may-have-minimal-impact-on-aquaculturesustainability

Food Ethics Council. (2007) *Meat consumption: Trends and environmental implications*. Available at: www.foodethicscouncil.org (accessed: 29 November 2023).

Godley, A. and Williams, B., 2007, 'The chicken, the factory farm and the supermarket: the emergence of the modern poultry industry in Britain', Department of Economics, University of Reading, Reading.

Gasco, L., Józefiak, A., & Henry, M., 2021, 'Beyond the protein concept: health aspects of using edible insects on animals', *Journal of Insects as Food and Feed*, Vol.7, no.5, pp.715–741.

Gao, Z., Wang, W., Lu, X., Zhu, F., Liu, W., Wang, X., & Lei, C., 2019, 'Bioconversion performance and life table of black soldier fly (Hermetia illucens) on fermented maize straw', *Journal of Cleaner Production*, Vol.230, pp.974–980.

Gasco, L., Biasato, I., Dabbou, S., Schiavone, A., & Gai, F., 2019, 'Animals fed insect-based diets: State-of-the-art on digestibility, performance and product quality', In *Animals*, Vol. 9, no.4.

Garcia, M.A., García, C. and Markides, K., 2019, 'Demography of aging', *Handbook of Population, Poston*, pp.143-144.

Gong, J., Forster, R.J., Yu, H., Chambers, J.R., Sabour, P.M., Wheatcroft, R., and Chen, S., 2002, 'Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen', *FEMS Microbiol. Lett.*, no.208, pp.1-7.

Gabriel, I., Lessire, M., Mallet, S. and Guillot, J.F., 2006, 'Microflora of the digestive tract: critical factors and consequences for poultry', *World's poultry science journal*, Vol.62, no.3, pp.499-511.

Gandhe, A.S., John, S.H. and Nagaraju, J., 2007, 'Noduler, a novel immune up-regulated protein mediates nodulation response in insects', *The Journal of Immunology*, Vol.179, no.10, pp.6943-6951.

Ge, Y., Hu, K. di, Wang, S. S., Hu, L. Y., Chen, X. Y., Li, Y. H., Yang, Y., Yang, F., & Zhang, H., 2017, 'Hydrogen sulfide alleviates postharvest ripening and senescence of banana by antagonizing the effect of ethylene', *PLoS ONE*, Vol.12, no.6.

Giusti, G., Galo, N.R., Pereira, R.P.T., Silva, D.A.L. and Filimonau, V., 2023, 'Assessing the impact of drought on carbon footprint of soybean production from the life cycle perspective', *Journal of cleaner production*, Vol.425, p.138843.

Gordon J, Manley NR. 2011, 'Mechanisms of thymus organogenesis and morphogenesis. Development', Vol.138:38 p.65–78.

Green, T.R. and Popa, R., 2012, 'Enhanced ammonia content in compost leachate processed by black soldier fly larvae', *Applied biochemistry and biotechnology*, Vol.*166*, pp.1381-1387.

Green Eco Technology, 2020 'How it works', viewed online through: https://www.greenecotec.com/how-it-works, accessed at: (16 April 2024)

Harrigan, G.G., Glenn, K.C. and Ridley, W.P., 2010, 'Assessing the natural variability in crop composition', *Regulatory Toxicology and Pharmacology*, Vol.58, no.3, pp.S13-S20.

Hrncir T, Stepankova R, Kozakova H, Hudcovic T, Tlaskalova-Hogenova H., 2008, 'Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice', BMC Immunol. Vol.6, no.9:65.

Howe, R.W., 1956, 'The effect of temperature and humidity on the rate of development and mortality of Tribolium castaneum (Herbst)(Coleoptera, Tenebrionidae)', *Annals of Applied Biology*, Vol.44, no.2, pp.356-368.

Hong, J., Han, T., & Kim, Y. Y., 2020, 'Mealworm (Tenebrio molitor larvae) as an alternative protein source for monogastric animal: A review', *Animals*, Vol.10, no.11, pp.1–20.

Henry, M.A., Golomazou, E., Asimaki, A., Psofakis, P., Fountoulaki, E., Mente, E., Rumbos, C.I., Athanassiou, C.G. and Karapanagiotidis, I.T., 2022, 'Partial dietary fishmeal replacement with full-fat or defatted superworm (Zophobas morio) larvae meals modulates the innate immune system of gilthead seabream, Sparus aurata', *Aquaculture Reports*, Vol.27, p.101347.

Henry, M.A., Gasco, L., Chatzifotis, S. and Piccolo, G., 2018, 'Does dietary insect meal affect the fish immune system? The case of mealworm, Tenebrio molitor on European sea bass, Dicentrarchus labrax', *Developmental & Comparative Immunology*, Vol.81, pp.204-209.

Hoeft, R.G., Aldrich, S.R., Nafziger, E.D. and Johnson, R.R., 2000, 'Modern corn and soybean production'.

Homberger, D.G., 2017, 'The avian lingual and laryngeal apparatus within the context of the head and jaw apparatus, with comparisons to the mammalian condition: functional morphology and biomechanics of evaporative cooling, feeding, drinking, and vocalization', *The biology of the avian respiratory system: evolution, development, structure and function*, pp.27-97.

Hird, S.M., 2017, 'Evolutionary biology needs wild microbiomes', *Frontiers in microbiology*, Vol.8, p.725.

Hikasa, Y., Baba, E., Fukata, T. and Arakawa, A., 1982, 'The invasion of Salmonella typhimurium into the cecal wall of chickens infected with Eimeria tenella', *Zentralblatt Für Bakteriologie, Mikrobiologie Und Hygiene. 1. Abt. Originale. a, Medizinische Mikrobiologie, Infektionskrankheiten Und Parasitologie*, Vol.253, no.3), pp.344-354.

Hillyer, J.F., 2016, 'Insect immunology and hematopoiesis', *Developmental & Comparative Immunology*, Vol.58, pp.102-118.

Huang, R. L., Deng, Z. Y., Yang, C. B., Yin, Y. L., Ming, Y. X., Wu, G. Y., Li, T. J., Li, L. L., Tang, Z. R., Kang, P., Hou, Z. P., Deng, D., Xiang, H., Xiang, F. K., & Guo, Y. M., 2007, 'Dietary oligochitosan supplementation enhances immune status of broilers', *Journal of the Science of Food and Agriculture*, Vol.87, no.1, pp.153–159.

Hartinger, K., Greinix, J., Thaler, N., Ebbing, M. A., Yacoubi, N., Schedle, K., & Gierus, M., 2021, 'Effect of graded substitution of soybean meal by Hermetia illucens larvae meal on animal performance, apparent ileal digestibility, gut histology and microbial metabolites of broilers' *Animals*, Vol.11, no.6.

Hossain, M.S., Zhang, Y. and Small, B.C., 2023, 'Evaluation of a corn fermented protein with solubles (CFPS) as a complete soybean meal replacer in practical diets for Atlantic salmon (Salmo salar)', *Aquaculture*, Vol.566, p.739198.

Iaconisi, V., Marono, S., Parisi, G., Gasco, L., Genovese, L., Maricchiolo, G., Bovera, F., & Piccolo, G., 2017, 'Dietary inclusion of Tenebrio molitor larvae meal: Effects on growth performance and final quality treats of blackspot sea bream (Pagellus bogaraveo)', *Aquaculture*, Vol.476, pp.49–58.

Ibadurrohman, K., Gusniani, I., Hartono, M.D. and Suwartha, N., 2020, 'The potential analysis of food waste management using bioconversion of the organic waste by the black soldier fly (Hermetia illucens) larvae in the cafeteria of the faculty of engineering, universitas indonesia', *Research and Education Center for Green Technologies*, Kyushu University, Vol. 7, no.1, pp.61-66.

Józefiak, A., & Engberg, R. M., 2017, 'Insect proteins as a potential source of antimicrobial peptides in livestock production. A review', *Journal of Animal and Feed Sciences*, Vol.26, no.2, pp.87–99.

Jankowski, J., Kozłowski, K., Zduńczyk, Z., Stępniowska, A., Ognik, K., Kierończyk, B., Józefiak, D., & Juśkiewicz, J., 2021, 'The effect of dietary full-fat Hermetia illucens larvae meal on gut physiology and growth performance in young turkeys', *Animal Feed Science and Technology*, Vol.275.

Jessica de Souza Vilela, Nicholas M. Andronicos, Manisha Kolakshyapati, Matthew Hilliar, Terence Z. Sibanda, Nigel R. Andrew, Robert A. Swick, Stuart Wilkinson, Isabelle Ruhnke, 2021, 'Black soldier fly larvae in broiler diets improve broiler performance and modulate the immune system', *Animal Nutrition*, Vol.7, Issue 3, pp.695-706,

Jukes, T.H. and Williams, W.L., 1953, 'Nutritional effects of antibiotics', *Pharmacological reviews*, Vol.5, no.4, pp.381-420.

Jackson, S. and Duke, G.E., 1995, 'Intestine fullness influences feeding behaviour and crop filling in the domestic turkey', *Physiology & behavior*, Vol.58, no.5, pp.1027-1034.

Kilburn-Kappeler, L.R., Lema Almeida, K.A., Paulk, C.B. and Aldrich, C.G., 2023, 'Comparison of corn fermented protein (CFP) to distillers dried grains with solubles (DDGS) fed to healthy adult dogs', *Frontiers in Animal Science*, Vol.4, p.1210144.

Kulshreshtha, G., Hincke, M. T., Prithiviraj, B., & Critchley, A., 2020, 'A review of the varied uses of macroalgae as dietary supplements in selected poultry with special reference to laying hen and broiler chickens', *Journal of Marine Science and Engineering*, Vol.8, no.7.

Klasing, K. C.,1999, 'Avian Gastrointestinal Anatomy and Physiology', *Seminars in Avian and Exotic Pet Medicine*, Vol. 8, no.2.

Khojely, D. M., Ibrahim, S. E., Sapey, E., & Han, T., 2018, 'History, current status, and prospects of soybean production and research in sub-Saharan Africa', *Crop Journal*, Vol. 6, no. 3, pp. 226–235.

Kim, Y., Mosier, N. S., Hendrickson, R., Ezeji, T., Blaschek, H., Dien, B., Cotta, M., Dale, B., & Ladisch, M. R., 2008, 'Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage', *Bioresource Technology*, Vol.99, no.12, pp.5165–5176.

Kim, E.J., 2010, *Amino acid digestibility of various feedstuffs using different methods*. University of Illinois, Urbana-Champaign.

Koutsos, L., McComb, A. and Finke, M., 2019, 'Insect composition and uses in animal feeding applications: a brief review', *Annals of the Entomological Society of America*, Vol. *112*, no.6, pp.544-551.

Kierończyk, B., Rawski, M., Mikołajczak, Z., Homska, N., Jankowski, J., Ognik, K., Józefiak, A., Mazurkiewicz, J. and Józefiak, D., 2022, 'Available for millions of years but discovered through the last decade: Insects as a source of nutrients and energy in animal diets', *Animal Nutrition*, Vol.11, pp.60-79.

Kunitz, M., 1947, 'Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor', *The Journal of general physiology*', Vol.30, no.4, p.311.

Kumar, V., Sinha, A.K., Makkar, H.P. and Becker, K., 2010, 'Dietary roles of phytate and phytase in human nutrition: A review', *Food chemistry*, Vol.120, no.4, pp.945-959.

M Klonick, A., (2017) *Bug ideas: Assessing the market potential and regulation of insects.* Available at: www.dukespace.lib.duke.edu (accessed: 11 June 2022).

Kirchhelle, C., 2018, 'Swann song: Antibiotic regulation in British livestock production (1953–2006)', *Bulletin of the History of Medicine*, Vol.92, no.2, pp.317-350.

Kogut, M.H., 2019, 'The effect of microbiome modulation on the intestinal health of poultry', *Animal feed science and technology*, Vol.250, pp.32-40.

Kaldhusdal, M. & Hofshagen, M. 1992, 'Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis', *Poultry Science*, Vol.71, pp.1145-53.

Kaya, H.K., 2002, 'Natural enemies and other antagonists', In *Entomopathogenic nematology* Wallingford UK: CABI publishing, pp. 189-203.

Kulshreshtha, G., Hincke, M.T., Prithiviraj, B. and Critchley, A., 2020, 'A review of the varied uses of macroalgae as dietary supplements in selected poultry with special reference to laying hen and broiler chickens', *Journal of Marine Science and Engineering*, Vol.8, no.7, p.536.

Kim, S. Y., Park, J. bin, Lee, Y. B., Yoon, H. J., Lee, K. Y., & Kim, N. J., 2015, 'Growth characteristics of mealworm Tenebrio molitor', *Journal of Sericultural and Entomological Science*, Vol.53, no.1, pp.1–5.

Kotsou, K., Rumbos, C. I., Baliota, G. v., Gourgouta, M., & Athanassiou, C. G., 2021, 'Influence of temperature, relative humidity and protein content on the growth and development of larvae of the lesser mealworm, alphitobius diaperinus (Panzer)', *Sustainability (Switzerland)*, Vol.13(19).

Khan, S., Khan, R. U., Alam, W., & Sultan, A., 2018, 'Evaluating the nutritive profile of three insect meals and their effects to replace soya bean in broiler diet', *Journal of Animal Physiology and Animal Nutrition*, Vol.102, no.2, pp. e662–e668.

Khosravi, S., Kim, E., Lee, Y. S., & Lee, S. M., 2018, 'Dietary inclusion of mealworm (Tenebrio molitor) meal as an alternative protein source in practical diets for juvenile rockfish (Sebastes schlegeli)' *Entomological Research*, Vol.48, no.3, pp.214–221.

Kumar, V., Fawole, F. J., Romano, N., Hossain, M. S., Labh, S. N., Overturf, K., & Small, B. C., 2021, 'Insect (black soldier fly, Hermetia illucens) meal supplementation prevents the soybean meal-induced intestinal enteritis in rainbow trout and health benefits of using insect oil', *Fish and Shellfish Immunology*, Vol.109, pp.116–124.

Kluth, H. and Rodehutscord, M., 2010. 'Effect of the duration of prefeeding on amino acid digestibility of wheat distillers dried grains with solubles in broiler chicken', *Poultry science*, Vol.*89*, no.4, pp.681-687.

Lähteenmäki-Uutela, A., Grmelová, N., Hénault-Ethier, L., Deschamps, M.H., Vandenberg, G.W., Zhao, A., Zhang, Y., Yang, B. and Nemane, V., 2017, 'Insects as food and feed: laws of the European union, United States, Canada, Mexico, Australia, and China', *European Food and Feed Law Review*, pp.22-36.

Lemme, A., Ravindran, V., & Bryden, W. L., 2004, 'Ileal digestibility of amino acids in feed ingredients for broilers' *World's Poultry Science Journal*, Vol.60, no.4, pp.423–437.

Li, Y., Kortner, T.M., Chikwati, E.M., Belghit, I., Lock, E.J. and Krogdahl, Å., 2020, 'Total replacement of fish meal with black soldier fly (Hermetia illucens) larvae meal does not compromise the gut health of Atlantic salmon (Salmo salar)', *Aquaculture*, Vol.520, p.734967.

Langston, K., Selaledi, L., & Yusuf, A., 2023, 'Evaluation of alternative substrates for rearing the yellow mealworm Tenebrio molitor (L).', *International Journal of Tropical Insect Science*, Vol.43, no.5, pp.1523–1530.

Lock, E.R., Arsiwalla, T. and Waagbø, R., 2016, 'Insect larvae meal as an alternative source of nutrients in the diet of A tlantic salmon (S almo salar) postsmolt', *Aquaculture nutrition*, Vol.22, no.6, pp.1202-1213.

Leinonen, I., Williams, A.G., Wiseman, J., Guy, J. and Kyriazakis, I., 2012, 'Predicting the environmental impacts of chicken systems in the United Kingdom through a life cycle assessment: Broiler production systems', *Poultry science*, Vol.91, no.1, pp.8-25.

Liener, I.E. and Kakade, M.L., 1980, ‚Protease inhibitors', *Toxic constituents of plant foodstuffs*, Vol.2, pp.7-71.

Li J, Hegeman CE, Hanlon RW et al., 1997, 'Secretion of active recombinant phytase from soybean ceii-suspension cuitures', *Plant Physiol*, Vol.114, pp.1103–1111.

Lum, K.K., Kim, J. and Lei, X.G., 2013, 'Dual potential of microalgae as a sustainable biofuel feedstock and animal feed', *Journal of animal science and biotechnology*, Vol.4, pp.1-7.

Lähteenmäki-Uutela, A., Hénault-Ethier, L., Marimuthu, S.B., Talibov, S., Allen, R.N., Nemane, V., Vandenberg, G.W. and Józefiak, D., 2018, 'The impact of the insect regulatory

system on the insect marketing system', *Journal of Insects as Food and Feed*, Vol.4, no.3, pp.187-198.

Lu, Y., Zhang, S., Sun, S., Wu, M., Bao, Y., Tong, H., Ren, M., Jin, N., Xu, J., Zhou, H. and Xu, W., 2021, 'Effects of different nitrogen sources and ratios to carbon on larval development and bioconversion efficiency in food waste treatment by black soldier fly larvae (Hermetia illucens)', *Insects*, Vol.12, no.6, p.507.

Lee, M.D. and Newell, D.G., 2006, 'Campylobacter in poultry: filling an ecological niche', *Avian diseases*, Vol.50, no.1, pp.1-9.

Leutenegger, R., 1967, 'Early events of Sericesthis iridescent virus infection in hemocytes of Gallerio mellonella (L.)', *Virology*, Vol.32, no.1, pp.109-116.

Lu, S., Taethaisong, N., Meethip, W., Surakhunthod, J., Sinpru, B., Sroichak, T., Archa, P., Thongpea, S., Paengkoum, S., Purba, R.A.P. and Paengkoum, P., 2022, 'Nutritional composition of black soldier fly larvae (Hermetia illucens L.) and its potential uses as alternative protein sources in animal diets: A review', *Insects*, Vol.13, no.9, p.831.

Murphy, M. M., Barraj, L. M., & Higgins, K. A., 2022, 'Healthy U.S.-style dietary patterns can be modified to provide increased energy from protein', *Nutrition Journal*, Vol.21, no.1.

Mattioli, S., Paci, G., Fratini, F., Dal Bosco, A., Tuccinardi, T., & Mancini, S., 2021, 'Former foodstuff in mealworm farming: Effects on fatty acids profile, lipid metabolism and antioxidant molecules', *LWT*, Vol.147, pp.111644

Makkar, H.P., Tran, G., Heuzé, V. and Ankers, P., 2014, 'State-of-the-art on use of insects as animal feed', *Animal feed science and technology*, Vol.197, pp.1-33.

Mackenzie, S.G., Leinonen, I., Ferguson, N. and Kyriazakis, I., 2016, 'Can the environmental impact of pig systems be reduced by utilising co-products as feed?', *Journal of Cleaner Production*, Vol.115, pp.172-181.

Manno, N., Estraver, W.Z., Tafur, C.M., Torres, C.L., Schwarzinger, C., List, M., Schoefberger, W., Coico, F.R.M., Leon, J.M., Battisti, A. and Paoletti, M.G., 2018, 'Edible insects and other chitin-bearing foods in ethnic Peru: Accessibility, nutritional acceptance, and food-security implications', *Journal of Ethnobiology*, Vol.38, no.3, pp.424-447.

Morales-Ramos, J.A., Kelstrup, H.C., Rojas, M.G. and Emery, V., 2019, 'Body mass icrease induced by eight years of artificial selection in the yellow mealworm (Coleoptera: Tenebrionidae) and life history trade-offs', *Journal of Insect Science*, Vol.19, no.2, p.4.

McLelland, J., 1975, 'Aves digestive system', *The anatomy of the domestic animals*, pp.1857-1882.

McNab, J.M., (1989). 'Measuring availability of amino acids from digestibility experiments', In *Proc. Proc. 7th Eur. Symp. Poult. Nutr*, Spain, pp. 45-53.

Mitsuoka, T., 1974, 'Die Faekalflora bei Menschen II. Mitteilung: Die Zusammensetzung der Bifidobacterienflora der verschiedenen Altersgruppen', *Zbl Bakt Hyg I Orig A*, Vol.226, pp.469-478.

MacConkey, A., 1905, 'Lactose-fermenting bacteria in faeces', *Epidemiology & Infection*, Vol.*5*, no.3, pp.333-379.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V., 1999, 'Food-related illness and death in the United States', *Emerging infectious diseases*, Vol.5, no.5, p.607.

Miller, R.W., Skinner, J., Sulakvelidze, A., Mathis, G.F. and Hofacre, C.L., 2010, 'Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens', *Avian diseases*, Vol.54, no.1, pp.33-40.

Manniello, M.D., Moretta, A., Salvia, R., Scieuzo, C., Lucchetti, D., Vogel, H., Sgambato, A. and Falabella, P., 2021, 'Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance', *Cellular and Molecular Life Sciences*, Vol.78, no.9, pp.4259-4282.

Matsuzaki, K., 1999, 'Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes', *Biochimica et Biophysica Acta (BBA)-Biomembranes*, Vol.1462, no.1-2, pp.1-10.

Melenchón, F., Larrán, A.M., De Mercado, E., Hidalgo, M.C., Cardenete, G., Barroso, F.G., Fabrikov, D., Lourenço, H.M., Pessoa, M.F. and Tomás-Almenar, C., 2021. 'Potential use of black soldier fly (Hermetia illucens) and mealworm (Tenebrio molitor) insectmeals in diets for rainbow trout (Oncorhynchus mykiss)', *Aquaculture Nutrition*, Vol.*27*, no.2, pp.491-505.

Muleya, M., Li, D., Chiutsi-Phiri, G., Botoman, L., Brameld, J.M. and Salter, A.M., 2023, 'In vitro determination of the protein quality of maize varieties cultivated in Malawi using the INFOGEST digestion method', *Heliyon*, Vol.9, no.9.

Norhisham, A.R., Abood, F., Rita, M. and Hakeem, K.R., 2013, 'Effect of humidity on egg hatchability and reproductive biology of the bamboo borer (Dinoderus minutus Fabricius)', *SpringerPlus*, Vol.2, pp.1-6.

Nischalke, S., Wagler, I., Tanga, C., Allan, D., Phankaew, C., Ratompoarison, C., Razafindrakotomamonjy, A. and Kusia, E., 2020, 'How to turn collectors of edible insects into mini-livestock farmers: Multidimensional sustainability challenges to a thriving industry', *Global Food Security*, Vol.26, p.100376.

Newton, L.A.R.R.Y., Sheppard, C.R.A.I.G., Watson, D.W., Burtle, G.A.R.Y. and Dove, R.O.B.E.R.T., 2005, 'Using the black soldier fly, Hermetia illucens, as a value-added tool for the management of swine manure', *Animal and Poultry Waste Management Center, North Carolina State University, Raleigh, NC*, Vol.17, no.2005, p.18.

Nichols, K., Bannink, A. and Dijkstra, J., 2019, 'Energy and nitrogen balance of dairy cattle as affected by provision of different essential amino acid profiles at the same metabolizable protein supply', *Journal of dairy science*, Vol.102, no.10, pp.8963-8976.

Nyachoti, C.M., Haouse, J.D., Slominski, B.A., Seddon, I.R., 2005, 'Energy and nutrient digestibilities in wheat dried distillers grains with solubles fed to growing pigs', *Journal of the Science of Food and Agriculture,* Vol.85, pp.2581-2586.

Neill, C., Coe, M. T., Riskin, S. H., Krusche, A. v., Elsenbeer, H., Macedo, M. N., McHorney, R., Lefebvre, P., Davidson, E. A., Scheffler, R., e Silva Figueira, A. M., Porder, S., & Deegan, L. A., 2013, 'Watershed responses to Amazon soya bean cropland expansion and intensification', *Philosophical Transactions of the Royal Society B: Biological Sciences*, Vol.368, no.1619.

O'Malley, R. C., Wallauer, W., Murray, C. M., & Goodall, J., 2012, 'The appearance and spread of ant fishing among the Kasekela chimpanzees of Gombe: A possible case of intercommunity cultural transmission', *Current Anthropology*, Vol.53, no.5, pp.650–663.

Oonincx, D.G.A.B., Van Huis, A. and Van Loon, J.J.A., 2015, 'Nutrient utilisation by black soldier flies fed with chicken, pig, or cow manure', *Journal of Insects as Food and Feed*, Vol.1, no.2, pp.131-139.

Oba, P.M., Utterback, P.L., Parsons, C.M. and Swanson, K.S., 2020, 'True nutrient and amino acid digestibility of dog foods made with human-grade ingredients using the precision-fed cecectomized rooster assay', *Translational Animal Science*, Vol.4, no.1, pp.442-451.

Oonincx, D. G. A. B., van Broekhoven, S., van Huis, A., & van Loon, J. J. A., 2015, 'Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products', *PLoS ONE,* Vol.10, no.12.

Parsons, C. M., 2020, 'Unresolved issues for amino acid digestibility in poultry nutrition', In *Journal of Applied Poultry Research,* Vol. 29, no.1, pp. 1–10.

Poveda, J., 2021, 'Insect frass in the development of sustainable agriculture. A review', *Agronomy for Sustainable Development*, Vol.41, no.1, p.5.

Pelletier, N. and Tyedmers, P., 2010, 'Forecasting potential global environmental costs of livestock production 2000–2050', *Proceedings of the National Academy of Sciences*, Vol.107, no.43, pp.18371-18374.

Parodi, A., Gerrits, W.J., Van Loon, J.J., De Boer, I.J., Aarnink, A.J. and Van Zanten, H.H., 2021, 'Black soldier fly reared on pig manure: Bioconversion efficiencies, nutrients in the residual material, greenhouse gas and ammonia emissions', *Waste Management*, Vol.126, pp.674-683.

Pan, D. and Yu, Z., 2014, 'Intestinal microbiome of poultry and its interaction with host and diet', *Gut microbes*, Vol.5, no.1, pp.108-119.

Parsons, C.M., Potter, L.M. and Brown Jr, R.D., 1982, 'Effects of dietary protein and intestinal microflora on excretion of amino acids in poultry', *Poultry Science*, Vol.61, no.5, pp.939-946.

Porter Jr, R.E., 1998, 'Bacterial enteritides of poultry', *Poultry Science*, Vol.77, no.8, pp.1159-1165.

Panini, R. L., Freitas, L. E. L., Guimarães, A. M., Rios, C., da Silva, M. F. O., Vieira, F. N., Fracalossi, D. M., Samuels, R. I., Prudêncio, E. S., Silva, C. P., & Amboni, R. D. M. C., 2017, 'Potential use of mealworms as an alternative protein source for Pacific white shrimp: Digestibility and performance', *Aquaculture*, Vol.473, pp.115–120.

Payne, C. L. R., Dobermann, D., Forkes, A., House, J., Josephs, J., McBride, A., Müller, A., Quilliam, R. S., & Soares, S., 2016, 'Insects as food and feed: European perspectives on recent research and future priorities', *Journal of Insects as Food and Feed*, Vol.2, no.4, 269–276.

Qin, L., Wang, Z., Sun, Y., Shu, Q., Feng, P., Zhu, L., Xu, J. and Yuan, Z., 2016, 'Microalgae consortia cultivation in dairy wastewater to improve the potential of nutrient removal and biodiesel feedstock production', *Environmental science and pollution research*, Vol.23, pp.8379-8387.

Rabobank. 2021., 'No longer crawling: Insect protein to come of age in the 2020s. In: RaboResearch: Food & Agribusiness.', Cited 9 November 2021. Viewed online at: www.researchrabobank.com.

Randazzo, B., Zarantoniello, M., Cardinaletti, G., Cerri, R., Giorgini, E., Belloni, A., Contò, M., Tibaldi, E., & Olivotto, I., 2021, 'Hermetia illucens and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (Sparus aurata) diet: A multidisciplinary study on fish gut status', *Animals*, Vol.11, no.3, pp.1–22.

Randall, L.P., Ridley, A.M., Cooles, S.W., Sharma, M., Sayers, A.R., Pumbwe, L., Newell, D.G., Piddock, L.J.V. and Woodward, M.J., 2003, 'Prevalence of multiple antibiotic resistance in 443 Campylobacter spp. isolated from humans and animals', *Journal of Antimicrobial Chemotherapy*, Vol.52, no.3, pp.507-510.

Ravindran, V, and Wayne L. Bryden, 1999, 'Amino acid availability in poultry—in vitro and in vivo measurements*', Australian Journal of Agricultural Research* Vol.50, no.5, pp. 889 - 908

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., Prearo, M., Capucchio, M. T., Biasato, I., Biasibetti, E., de Marco, M., Brugiapaglia, A., Zoccarato, I., & Gasco, L., 2017, 'Evaluation of the suitability of a partially defatted black soldier fly (Hermetia illucens L.) larvae meal as ingredient for rainbow trout (Oncorhynchus mykiss Walbaum) diets', *Journal of Animal Science and Biotechnology*, Vol.8, no.1.

Rehman, K. ur, Ur Rehman, R., Somroo, A. A., Cai, M., Zheng, L., Xiao, X., Ur Rehman, A., Rehman, A., Tomberlin, J. K., Yu, Z., & Zhang, J., 2019, 'Enhanced bioconversion of dairy and chicken manure by the interaction of exogenous bacteria and black soldier fly larvae', *Journal of Environmental Management*, Vol.237, pp.75–83.

Rumbos, C. I., Bliamplias, D., Gourgouta, M., Michail, V., & Athanassiou, C. G., 2021, 'Rearing Tenebrio molitor and Alphitobius diaperinus larvae on seed cleaning process byproducts', *Insects*, Vol.12, no.4.

Rovai, D., Ortgies, M., Amin, S., Kuwahara, S., Schwartz, G., Lesniauskas, R., Garza, J., & Lammert, A., 2021, 'Utilization of Carrot Pomace to Grow Mealworm Larvae (Tenebrio molitor)', *Sustainability*, Vol.13, no.16, 9341.

Ribeiro, J. C., Lima, R. C., Maia, M. R. G., Almeida, A. A., Fonseca, A. J. M., Cabrita, A. R. J., & Cunha, L. M., 2019, 'Impact of defatting freeze-dried edible crickets (Acheta domesticus and Gryllodes sigillatus) on the nutritive value, overall liking and sensory profile of cereal bars', *LWT*, Vol.113, pp.108335.

Ruschioni, S., Loreto, N., Foligni, R., Mannozzi, C., Raffaelli, N., Zamporlini, F., Pasquini, M., Roncolini, A., Cardinali, F., Osimani, A., Aquilanti, L., Isidoro, N., Riolo, P., & Mozzon, M., 2020, 'Addition of olive pomace to feeding substrate affects growth performance and nutritional value of mealworm (Tenebrio molitor L.) larvae', *Foods*, Vol.*9*, no.3, pp.317.

Rumpold, B. A., & Schlüter, O. K., 2013, 'Nutritional composition and safety aspects of edible insects', In *Molecular Nutrition and Food Research*, Vol. 57, no.5, pp. 802–823.

Rodehutscord, M., Abel, H. J., Friedt, W., Wenk, C., Flachowsky, G., Ahlgrimm, H. J., Johnke, B., Kühl, R., & Breves, G., 2002, 'Consequences of the ban of by-products from terrestrial animals in livestock feeding in Germany and the European Union: Alternatives, nutrient and energy cycles, plant production, and economic aspects', In *Archives of Animal Nutrition*, Vol.56, no.2, pp. 67–91.

Ritter, K. S., 1990, 'Cholesterol and insects', *The Food Insects Newsletter,* Vol.3, no.1.

Ruiz, N., Parsons, C.M., Stein, H.H., Coon, C.N., Eys, J. and Miles, R.D., 2020, 'A review: 100 years of soybean meal', *ADM: Chicago, IL, USA.*

Rachel, K.V. and Sirisha, G.V., 2014, 'A review of protease inhibitors from different sources', *International Journal of Applied, Physical and Bio-Chemistry Research*, Vol.4, pp.1-18.

Ravindran, V., Hew, L.I., Ravindran, G. And Bryden, W.L., 2005, 'Apparent ileal digestibility of amino acids in dietary ingredients for broiler chickens', *Animal Science* Vol.81, pp. 85-97.

Ravzanaadii, N., Kim, S.-H., Choi, W.-H., Hong, S.-J., & Kim, N.-J., 2012, 'Nutritional Value of Mealworm, Tenebrio molitor as Food Source', *International Journal of Industrial Entomology*, Vol.25, no.1, pp.93–98.

Remus, A., Peres, F. M., Hauschild, L., Andretta, I., Kipper, M., de Paula Gobi, J., & Pomar, C., 2015, 'Exploratory study on the utilization of different dietary methionine sources and methionine to lysine ratio for growing-finishing pigs', *Livestock Science*, Vol.181, pp.96–102.

Ryan CA, 1990, 'Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens', *Annu. Rev. Phytopathol,* Vol.28, pp.425-449.

Reddy, N.R., 2001, 'Occurrence, distribution, content, and dietary intake of phytate', In *Food phytates*, pp. 41-68).

Rothman, J.M., Raubenheimer, D., Bryer, M.A., Takahashi, M. and Gilbert, C.C., 2014, 'Nutritional contributions of insects to primate diets: implications for primate evolution', *Journal of Human Evolution*, Vol.71, pp.59-69.

Regassa, A. and Nyachoti, C.M., 2018, 'Application of resistant starch in swine and poultry diets with particular reference to gut health and function', *Animal Nutrition*, Vol.4, no.3, pp.305-310.

Rattana, M., Yongyut, C. and Supachet, P., 2023, 'Drying treatment impact on oil yield extracted from black soldier fly larvae using supercritical carbon dioxide and its biological activities', *Journal of Insects as Food and Feed*, Vol.1, pp.1-21.

Roura, E., Baldwin, M.W. and Klasing, K.C., 2012, 'The avian taste system: an update', In *23 rd ANNUAL AUSTRALIAN POULTRY SCIENCE SYMPOSIUM*, p. 97.

Rossi, J. R., Baraldi-Artoni, S. M., Oliveira, D., Cruz, C. D., Franzo, V. S., & Sagula, A., 2005, 'Morphology of glandular stomach (Ventriculus glandularis) and muscular stomach (Ventriculus muscularis) of the partrigde Rhynchotus rufescens', *Ciência Rural*, Vol.35, pp.1319-1324.

Ravindran, V. and Bryden, W.L., 1999, 'Amino acid availability in poultry—In vitro and in vivo measurements', *Australian Journal of Agricultural Research*, Vol.50, no.5, pp.889-908.

Saadaoui, I., Rasheed, R., Aguilar, A., Cherif, M., Al Jabri, H., Sayadi, S. and Manning, S.R., 2021, 'Microalgal-based feed: promising alternative feedstocks for livestock and poultry production', *Journal of Animal Science and Biotechnology*, Vol.12, no.1, p.76.

Samar, M.E., Ávila, R.E., Esteban, F.J., Olmedo, L., Dettin, L., Massone, A., Pedrosa, J.A. and Peinado, M.A., 2002, 'Histochemical and ultrastructural study of the chicken salivary palatine glands', *Acta histochemica*, Vol.104, no.2, pp.199-207.

Staley M, Conners MG, Hall K, Miller LJ. Linking stress and immunity: Immunoglobulin A as a non-invasive physiological biomarker in animal welfare studies. Horm Behav. 2018 Jun;102:55-68. doi: 10.1016/j.yhbeh.2018.04.011. Epub 2018 May 10. PMID: 29705025.

Stein, H.H., Sève, B., Fuller, M.F., Moughan, P.J. and De Lange, C.F.M., 2007, 'Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: Terminology and application', *Journal of animal science*, Vol.85, no.1, pp.172-180.

Short, F.J., Gorton, P., Wiseman, J. and Boorman, K.N., 1996, 'Determination of titanium dioxide added as an inert marker in chicken digestibility studies', *Animal feed science and technology*, Vol.59, no.4, pp.215-221.

Smith, H.W., 1965, 'The development of the flora of the alimentary tract in young animals', *Journal of Pathology and Bacteriology*, Vol.90, pp.495-513.

Shrivastava, S.K., PRAKASH, A. and RAO, J., 2018, 'Insect for nutraceutical-an unexplored wealth of India', *Applied Zoologists Research Association,(AZRA)*, Vol.29, no.1, pp.1-22.

Setti, L., Francia, E., Pulvirenti, A., Gigliano, S., Zaccardelli, M., Pane, C., Caradonia, F., Bortolini, S., Maistrello, L., & Ronga, D., 2019, 'Use of black soldier fly (Hermetia illucens (L.), Diptera: Stratiomyidae) larvae processing residue in peat-based growing media', *Waste Management*, Vol.95, pp.278–288.

Sheehan, G., Farrell, G., & Kavanagh, K., 2020, 'Immune priming: the secret weapon of the insect world', In *Virulence*, Vol. 11, no.1, pp. 238–246.

Sharma, D., Choudhary, M., Vashistt, J., Shrivastava, R., & Bisht, G. S., 2019, 'Cationic antimicrobial peptide and its poly-N-substituted glycine congener: Antibacterial and antibiofilm potential against A. baumannii', *Biochemical and Biophysical Research Communications*, Vol.518, no.3, 472–478.

Svihus, B., Choct, M., & Classen, H. L., 2013, 'Function and nutritional roles of the avian caeca: A review', In *World's Poultry Science Journal*, Vol.69, no.2, pp. 249–264.

Scholey, D. v., Morgan, N. K., Riemensperger, A., Hardy, R., & Burton, E. J., 2018, 'Effect of supplementation of phytase to diets low in inorganic phosphorus on growth performance and mineralization of broilers', *Poultry Science*, Vol.97, no.7, pp.2435–2440.

Schneeman, B.O., Chang, I., Smith, L.B. and Lyman, R.L., 1977, 'Effect of dietary amino acids, casein, and soybean trypsin inhibitor on pancreatic protein secretion in rats', *The Journal of Nutrition*, Vol.107, no.2, pp.281-288.

Stull, V. J., Kersten, M., Bergmans, R. S., Patz, J. A., & Paskewitz, S., 2019, 'Crude Protein, Amino Acid, and Iron Content of Tenebrio molitor (Coleoptera, Tenebrionidae) Reared on an Agricultural Byproduct from Maize Production: An Exploratory Study', *Annals of the Entomological Society of America*, Vol.112, no.6, pp.533–543.

Schiavone, A., de Marco, M., Martínez, S., Dabbou, S., Renna, M., Madrid, J., Hernandez, F., Rotolo, L., Costa, P., Gai, F., & Gasco, L., 2017, 'Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (Hermetia illucens L.) meal for broiler chickens: Apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility', *Journal of Animal Science and Biotechnology*, Vol.8, no.1.

Strid Eriksson, I., Elmquist, H., Stern, S. and Nybrant, T., 2005, 'Environmental systems analysis of pig production-the impact of feed choice (12 pp)', *The International Journal of Life Cycle Assessment*, Vol.10, pp.143-154.

Stein, H.H., Berger, L.L., Drackley, J.K., Fahey Jr, G.C., Hernot, D.C. and Parsons, C.M., 2008, 'Nutritional properties and feeding values of soybeans and their coproducts. In *Soybeans*, pp. 613-660.

Schneeman , BO , Chang , I , Smith , L and Lyman , LR . 1977, 'Effect of dietary amino acids, casein and soybean trypsin inhibitors on pancreatic protein secretion in rats', *Journal of Nutrition*, Vol.107, pp. 281 – 288.

Selaledi, L., Mbajiorgu, C.A. and Mabelebele, M., 2020, 'The use of yellow mealworm (T. molitor) as alternative source of protein in poultry diets: a review', *Tropical animal health and production*, Vol.52, pp.7-16.

Spiehs, M.J., Whitney, M.H., Shurson, G.C., 2002, 'Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota', *Journal of Animal Science*, Vol.80, pp.2639-2645.

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P. and De Smet, S., 2017, 'Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates', *Journal of the Science of Food and Agriculture*, Vol.97, no.8, pp.2594-2600.

Siddiqui, S.A., Ristow, B., Rahayu, T., Putra, N.S., Yuwono, N.W., Mategeko, B., Smetana, S., Saki, M., Nawaz, A. and Nagdalian, A., 2022, 'Black soldier fly larvae (BSFL) and their affinity for organic waste processing', *Waste Management*, Vol.140, pp.1-13.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D. and Biavati, B., 2010, 'Characterization of probiotic strains: an application as feed additives in poultry against Campylobacter jejuni', *International journal of food microbiology*, Vol.141, pp.S98-S108.

Su, L.H., Chiu, C.H., Chu, C. and Ou, J.T., 2004, 'Antimicrobial resistance in nontyphoid Salmonella serotypes: a global challenge', *Clinical infectious diseases*, Vol.39, no.4, pp.546-551.

Schmitt, E. and de Vries, W., 2020, ' Potential benefits of using Hermetia illucens frass as a soil amendment on food production and for environmental impact reduction' *Current Opinion in Green and Sustainable Chemistry*, Vol.*25*, p.100335.

Schmit, A.R. and Ratcliffe, N.A., 1977, 'The encapsulation of foreign tissue implants in Galleria mellonella larvae', *Journal of Insect Physiology*, Vol.23, no.2, pp.175-184.

Shar, Z. H., Fletcher, M. T., Sumbal, G. A., Sherazi, S. T. H., Giles, C., Bhanger, M. I., & Nizamani, S. M., 2016, 'Banana peel: an effective biosorbent for aflatoxins', *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, Vol.33, no.5, pp.849–860.

Sánchez-Muros, M. J., Barroso, F. G., & Manzano-Agugliaro, F., 2014, 'Insect meal as renewable source of food for animal feeding: A review', In *Journal of Cleaner Production*, Vol. 65, pp. 16–27.

Sedgh-Gooya, S., Torki, M., Darbemamieh, M., Khamisabadi, H., & Abdolmohamadi, A., 2022, 'Growth performance and intestinal morphometric features of broiler chickens fed on dietary inclusion of yellow mealworm (Tenebrio molitor) larvae powder', *Veterinary Medicine and Science*, Vol.8, no.5, pp.2050–2058.

Sangiorgio, P., Verardi, A., Dimatteo, S., Spagnoletta, A., Moliterni, S. and Errico, S., 2021. Tenebrio molitor in the circular economy: a novel approach for plastic valorisation and PHA biological recovery. *Environmental Science and Pollution Research*, Vol.28, no.38, pp.52689-52701.

Surendra, K.C., Tomberlin, J.K., van Huis, A., Cammack, J.A., Heckmann, L.H.L. and Khanal, S.K., 2020, 'Rethinking organic wastes bioconversion: Evaluating the potential of the black

soldier fly (Hermetia illucens (L.))(Diptera: Stratiomyidae)(BSF)', *Waste Management*, Vol.*117*, pp.58-80.

Song, B., Tang, D., Yan, S. et al., 2021, 'Effects of age on immune function in broiler chickens', *Journal of Animal Science and Biotechnology* Vol.12, no.42.

Taglieri, I., Bedini, S., Zinnai, A., Sanmartin, C., Conti, B., Farina, P., Chiriboga Ortega, R.D., De Leo, M., Braca, A., Tavarini, S. and Venturi, F., 2022, 'Effects of flaxseed cake fortification on bread shelf life, and its possible use as feed for Tenebrio molitor larvae in a circular economy: preliminary results', *Journal of the Science of Food and Agriculture*, Vol.102, no.4, pp.1736-1743.

Testing. (2021) *Interleukin-6*. Available at: www.testing.com (accessed: 26 November 2023).

Tsochatzis, E.D., Berggreen, I.E., Nørgaard, J.V., Theodoridis, G. and Dalsgaard, T.K., 2021, 'Biodegradation of expanded polystyrene by mealworm larvae under different feeding strategies evaluated by metabolic profiling using GC-TOF-MS', *Chemosphere*, Vol.281, p.130840.

Tomberlin, J.K., Sheppard, D.C. and Joyce, J.A., 2002, 'Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. *Annals of the Entomological Society of America*, Vol.95, no.3, pp.379-386.

Tennie, C., Gilby, I. C., & Mundry, R., 2009, 'The meat-scrap hypothesis: Small quantities of meat may promote cooperative hunting in wild chimpanzees Pan troglodytes', *Behavioral Ecology and Sociobiology*, Vol.63, no.3, pp.421–431.

Tschirner, M. and Kloas, W., 2017, 'Increasing the sustainability of aquaculture systems: Insects as alternative protein source for fish diets', *GAIA-Ecological Perspectives for Science and Society*, Vol.26, no.4, pp.332-340.

Tomberlin, J.K., Sheppard, D.C. and Joyce, J.A., 2002, 'Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets', *Annals of the Entomological Society of America*, Vol.95, no.3, pp.379-386.

Tomberlin, J.K., Crippen, T.L., Tarone, A.M., Chaudhury, M.F., Singh, B., Cammack, J.A. and Meisel, R.P., 2017, 'A review of bacterial interactions with blow flies (Diptera: Calliphoridae) of medical, veterinary, and forensic importance', *Annals of the Entomological Society of America*, Vol.110, no.1, pp.19-36.

Tonu, N.S., Sufian, M.A., Sarker, S., Kamal, M.M., Rahman, M.H. and Hossain, M.M., 2011, 'Pathologicalstudy on colibacillosis in chickens and detection of Escherichia coli by PCR', *Bangladesh Journal of Veterinary Medicine*, Vol.9, no.1, pp.17-25.

Tsao, I.Y., Chen, J.W., Li, C.J., Lo, H.L., Christensen, B.M. and Chen, C.C., 2015, 'The dual roles of Armigeres subalbatus prophenoloxidase V in parasite melanization and egg chorion melanization in the mosquito Ar. Subalbatus', *Insect biochemistry and molecular biology*, Vol.64, pp.68-77.

Tlaskalovahogenova H, Stěpankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinovažadnikova R, et al., 2004, 'Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases.' Immunol Lett.; Vol.93:97, no.108.

Van Broekhoven, S., Oonincx, D. G. A. B., van Huis, A., & van Loon, J. J. A., 2015, 'Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products', *Journal of Insect Physiology*, Vol.73, pp.1–10.

Van Krimpen, M.M., Bikker, P., Van der Meer, I.M., Van der Peet-Schwering, C.M.C. and Vereijken, J.M., (2013), 'Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products (No. 662)', Research report, Wageningen UR Livestock Research, Netherlands.

Van Huis, A., 2013, 'Potential of insects as food and feed in assuring food security', In *Annual Review of Entomology*, Vol. 58, pp. 563–583.

Van der Ploeg, J., Albert, J., Apgar, M., Bennett, G., Boso, D., Cohen, P., Daokalia, C., Faiau, J., Harohau, D. and Iramo, E., (2016). *Learning from the lagoon: Research in development in Solomon Islands*. WorldFish.

Voora, V., Larrea, C. and Bermudez, S., (2020). *Global market report: soybeans*. Winnipeg, Manitoba: International Institute for Sustainable Development.

Vu, H. T., Scarlett, C. J., & Vuong, Q. v., 2019, 'Changes of phytochemicals and antioxidant capacity of banana peel during the ripening process; with and without ethylene treatment', *Scientia Horticulturae*, Vol.253, pp.255–262.

Wang, H., Rehman, K. ur, Feng, W., Yang, D., Rehman, R. ur, Cai, M., Zhang, J., Yu, Z., & Zheng, L., 2020, 'Physicochemical structure of chitin in the developing stages of black soldier fly', *International Journal of Biological Macromolecules*, Vol.149, pp.901–907.

Widyaratne, G.P. and Zijlstra, R.T., 2007, 'Nutritional value of wheat and corn distiller's dried grain with solubles: Digestibility and digestible contents of energy, amino acids and phosphorus, nutrient excretion and growth performance of grower finisher pigs', *Canadian Journal of Animal Science* Vol.87, pp.103-114.

Weigel, J.C., Loy, D. and Kilmer, L., 1997, 'Feed co-products of the dry corn milling process', *Renewable Fuels Association and National Corn Growers Association*. Washington DC and St. Louis, MO, pp.2-4.

Westrell, T., Ciampa, N., Boelaert, F., Helwigh, B., Korsgaard, H., Chríel, M., Ammon, A. and Mäkelä, P., 2009, 'Zoonotic infections in Europe in 2007: a summary of the EFSA-ECDC annual report', *Eurosurveillance*, Vol.14, no.3, p.19100.

Wiegel, J, 2015, 'Clostridiaceae. In: Bergey's manual of systematics of archaea and bacteria', *Encyclopedia of life sciences*, pp 1–5.

Xaviera-filho, J. and Campos, F.A.P., 1989, 'Proteinase inhibitors', *Toxicants of Plant Origin*, Cheeke. Ed., CRC Press pp. 1-27.

Yan, Y., Tan, F., Miao, H., Wang, H. and Cao, Y., 2019, 'Effect of shikonin against Candida albicans biofilms', *Frontiers in Microbiology*, Vol.10, p.1085.

Yang, S. S., Chen, Y. di, Kang, J. H., Xie, T. R., He, L., Xing, D. F., Ren, N. Q., Ho, S. H., & Wu, W. M., 2019, 'Generation of high-efficient biochar for dye adsorption using frass of yellow mealworms (larvae of Tenebrio molitor Linnaeus)fed with wheat straw for insect biomass production', *Journal of Cleaner Production*, Vol.227, pp.33–47.

Yang, Z., Pirgozliev, V. R., Rose, S. P., Woods, S., Yang, H. M., Wang, Z. Y., & Bedford, M. R., 2020, 'Effect of age on the relationship between metabolizable energy and digestible energy for broiler chickens', *Poultry Science*, Vol.99, no.1, pp.320–330.

Yamauchi, K.E. and Isshiki, Y., 1991, 'Scanning electron microscopic observations on the intestinal villi in growing White Leghorn and broiler chickens from 1 to 30 days of age', *British Poultry Science*, Vol.32, no.1, pp.67-78.

Yeoman, C. J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N. D., & White, B. A., 2012, 'The microbiome of the chicken gastrointestinal tract', *Animal health research reviews / Conference of Research Workers in Animal Diseases*, Vol. 13, no.1, pp. 89–99.

Youssef, I.M., Westfahl, C., Sünder, A., Liebert, F. and Kamphues, J., 2008, 'Evaluation of dried distillers' grains with solubles (DDGS) as a protein source for broilers. *Archives of Animal Nutrition*, Vol.*62*, no.5, pp.404-414.

Zaky, A.A., Chen, Z., Qin, M., Wang, M. and Jia, Y., 2020. Assessment of antioxidant activity, amino acids, phenolic acids and functional attributes in defatted rice bran and rice bran protein concentrate. *Prog. Nutr*, Vol.*22*, p.e2020069.

Zhang, L.L., Feng, R.J. and Zhang, Y.D., 2012. 'Evaluation of different methods of protein extraction and identification of differentially expressed proteins upon ethylene-induced early-ripening in banana peels', *Journal of the Science of Food and Agriculture*, Vol.*92*, no.10, pp.2106-2115.

Zasloff, M., 2002, 'Innate immunity, antimicrobial peptides, and protection of the oral cavity', *The Lancet*, Vol.360, no.9340, pp.1116-1117.

Zhou, F., Tomberlin, J.K., Zheng, L., Yu, Z. and Zhang, J., 2013, 'Developmental and waste reduction plasticity of three black soldier fly strains (Diptera: Stratiomyidae) raised on different livestock manures', *Journal of medical entomology*, Vol.50, no.6, pp.1224-1230.