EVALUATION OF STANDARDIZED ILEAL DIGESTIBILITY OF AMINO ACIDS AND METABOLIC AVAILABILITY OF METHIONINE IN PARTIALLY-DEFATTED BLACK SOLDIER FLY LARVAE MEAL FED TO GROWING PIGS

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ABSTRACT

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As food and animal protein demand increases, black soldier fly larvae (BSFL) meal has gained interest in the past couple of years for use in animal feed as an alternative protein ingredient. Black soldier fly larvae is an attractive protein source; however, it is important to evaluate its amino acid (AA) digestibility and metabolic availability (MA) before incorporation in feed formulation. The global objectives of this thesis were to determine the standardized ileal digestibility (SID) coefficient of AA and MA of methionine using the indicator AA oxidation (IAAO) method from BSFL meal, and to compare the results from the two protein quality methodologies. The SID coefficient of indispensable AA in BSFL meal was found to be above 83%, while the MA of methionine was found to be 53.33%. This finding further confirms that SID coefficient tends to overestimate AA bioavailability and that MA is a more accurate representation of bioavailability.

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LIST OF ABBREVIATIONS

AA	amino acid
AAFCO	Association of American Feed Control Official
ADF	acid detergent fiber
AID	apparent ileal digestibility
ATTD	apparent total tract digestibility
BSF	black soldier fly
BSFL	black soldier fly larvae
CP	crude protein
DL-Met	DL-Methionine
DM	dry matter
EAAL	endogenous amino acid losses
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FCR	feed conversion ratio
GE	gross energy
GHG	greenhouse gas
GIT	gastrointestinal tract
IAAO	indicator amino acid oxidation
IFIF	International Feed Industry Federation
MA	metabolic availability
MBM	meat and bone meal
ME	metabolizable energy

NDF	neutral detergent fiber
NFD	nitrogen-free diet
NPN	non-protein nitrogen
NRC	National Research Council
OECD	Organization for Economic Co-Operation Development
PD-BSFL	partially defatted black soldier fly larvae meal
SBM	soybean meal
SID	standardized ileal digestibility
SRGA	slope ratio growth assay
TID	true ileal digestibility

1 Chapter 1: Literature review

1.1 Introduction

Sustainable growth of the animal production industry is a crucial step to meet the growing food demand globally. By 2050, the global human population is projected to reach or exceed nine billion (FAO, 2011). Subsequently, the consumption of animal protein is anticipated to be 9% higher by 2030 as food demand shifts towards more animal-based products, especially in developing countries, where income and urbanization are increasing (Msangi and Rosegrant, 2011). Therefore, to continue feeding the growing population, livestock, poultry, and aquaculture production will need to double (International Feed Industry Federation [IFIF], 2012). In response to that, a 70% increase in global animal feed production is expected according to the Food and Agriculture Organization (FAO, 2012a). Unfortunately, increase in food demand and animal production will continue to put pressure on the environment such as higher food waste and greenhouse gas (GHG) emission (Foley et al., 2011). Based on a study conducted by Environment and Climate Change Canada, 2.94 million metric tonnes of food waste was produced by Canadian households in 2019, and that number is predicted to keep increasing as population size and food demand grow. Thus, an innovative approach to ensure food security globally by increasing animal production and promoting a more sustainable environment is warranted (Foley et al., 2011).

Feed is typically the most expensive component in animal production and comprises 60-70% of total cost (Van Huis et al., 2013; Pomar et al., 2011). The main drivers contributing to high feed costs are the price increase of ingredients, especially the common protein ingredients, competition of feedstuffs between human food and animal feed (Foley et al., 2011), and imprecise nutrient feed formulation and feeding management (Pomar et al., 2011). The prices of common feed protein ingredients such as soybean meal (SBM), fish meal, and animal meat meal, have

doubled in the past five years due to supply and demand imbalance (Veldkamp and Bosch, 2015). Additionally, the high cost of SBM and fish meal are expected to continue as these ingredients are becoming less environmentally sustainable and their long-term availability will be increasingly constrained (Van Huis et al., 2013; Makkar et al., 2014). Factors that lead to unsustainable soybean cultivation are reduced availability of arable land that can potentially lead to cropland expansion to highly biodiverse forest areas (Carvalho, 1999; Foley et al., 2011), high water requirement (Ercin et al., 2012; Steinfeld et al., 2006), environmental disturbance due to the use of pesticides and fertilizers (Carvalho, 1999), and increase in GHG emission due to long distance transport, as 90% of global soybean producers are located in South America, while importers are international (He et al., 2019). Furthermore, the use of soybean is highly competitive between human and animal feed. The United States Department of Agriculture (USDA; 2015) reported that 70% of soybeans that were grown in the US were used for animal feed, while the other 30% was used for human consumption and biodiesel. Additionally, fish meal, fish oil, and grain products are also competitive for use in both human food and animal feed (Asche et al., 2012; Van Huis et al., 2013). Therefore, the utilization of alternative protein ingredient is beneficial to expand protein choices for feed manufacturers and put less pressure on the human food chain.

Meanwhile, fish meal is considered unsustainable because it is sourced from the wild population that has been deteriorating in numbers due to overexploitation over the past years (FAO, 2012b). However, the growth of the aquaculture industry has been promising, as it might shift the sourcing of fish meal to farmed instead of wild fishing. In contrast to SBM and fish meal, rendered animal protein ingredients are considered sustainable because their raw materials (e.g. intestines, organs, bones) are derived from by-products of animal slaughter (Meeker and Hamilton, 2006). Unfortunately, these rendered ingredients are variable in nutritional content and quality,

have poor bioavailability of nutrients (Bureau et al., 1999), and also becoming more expensive (Ravindran and Blair, 1993). Rendered proteins are especially expensive for feed manufacturers that need to import, such as those in Asia, where animal by-products are also consumed by humans (Ravindran and Blair, 1993). Fluctuations in currency exchange and shipping fees add-on to the already expensive rendered products for these manufacturers. Thus, there is an urgent need to find alternative protein ingredients for animal feeds that are economically advantageous, environmentally sustainable, and highly nutritious.

Insect meal is possibly one of the most attractive alternative protein ingredients for animal feed because insects can be raised sustainably, are nutrient dense, and environmentally friendly (Van Huis et al., 2013; Veldkamp and Bosch, 2015). Previous studies have explored several insect species that are being considered as feed ingredients, such as the common housefly (Miller and Shaw, 1969), cricket (Makkar et al., 2014), mealworm (Aguilar-Miranda et al., 2002; Veldkamp and Bosch, 2015), and black soldier fly (BSF; *Hermetia illucens*) (Onsongo et al., 2018; St-Hilaire et al., 2007). Among these, BSF larvae (BSFL) is the most promising sustainable protein alternative for animal feed as BSFL possess relatively high activities of amylase, lipase, and protease enzymes in their digestive system that enable them to up-cycle organic or animal waste to high value protein biomass (Kim et al., 2011; Newton et al., 2005). Additionally, BSFL are suitable for large scale production (Spranghers et al., 2016), have been demonstrated to support excellent growth in poultry (Schiavone et al., 2017a; Dabbou et al., 2018) and aquaculture species (St-Hilaire et al., 2007), have better feed conversion ratio (FCR) (Oonincx et al.,2015) and protein yield per m^2 compared to cricket and mealworm (Koutsos et al., 2019). However, before incorporating new protein sources into animal feed, it is imperative to carefully assess their protein quality to prevent over- or under-estimation of the amino acid (AA) availability (Adeola et al.,

2016). Protein quality is based on two concepts: (1) the protein's AA composition relative to the animal's AA requirement and (2) the degree of AA digestion, absorption, and metabolic availability for protein synthesis (Nosworthy and House, 2017). To assess protein quality, several methodologies exist and will be discussed further.

In addition to the prices of protein ingredients, imprecise nutrient feed formulation can also lead to high feed costs and other economic losses such as reduced animal productivity. When animals are fed a nutrient-deficient diet, in particular diet that is low in protein, it can lead to AA imbalances, reduced growth performance, and decreased productivity (Pomar et al., 2011). However, providing excess nutrients in the diet is similarly undesirable as it increases feed cost, can lead to nutrient inefficiency, and increase nutrient excretion (Hauschild et al., 2010; Pomar et al., 2011; NRC, 2012). For example, feeding excessive levels of AAs (nitrogen) and phosphorus can further increase urinary nitrogen and phosphorus excretion (Jongbloed and Lenis, 1992; Burdett et al., 2018), which negatively impact the environment (e.g. soil acidification and water eutrophication; Swanson et al., 2013). This nutrient excretion issue is concerning as currently the levels of nitrogen and phosphorus excretion in most intensive pig production farms are critically high (Garcia-Launay et al., 2014). Andretta et al. (2016) observed a minimum of 8% reduction in feed cost and 40% decrease in nitrogen and phosphorus excretion when pigs were fed with tailored-diet specific to the nutrient requirement of each individual based on their growth and feed intake patterns. Thus, individual precision feeding is desirable. However, this practice is currently not viable as it requires advanced technology such as continuous automatic data collection and processing of each individual animal on the farm (Banhazi et al., 2012) and there is still limited data on the bioavailability of nutrients in ingredients (Pomar et al., 2009). Therefore, precision feeding should focus on a group of animals that have been controlled for variations (e.g. grouping

based on life stage, weight, and physiological conditions; Pomar and Remus, 2019). To implement precision feeding, improved diet efficiency, information on the animal's nutrient requirement, protein quality measurement of feed ingredients, accurate formulation, and integration of data collection, processing, and system control are required (Pomar et al., 2009; Banhazi et al., 2012).

This review will examine the production, sustainability attributes, nutritional characteristics, and feed application of BSF. Furthermore, it will also focus on the overview, advantages, and disadvantages of various protein quality methodologies.

1.2 Black soldier fly (*Hermetia illucens*)

The BSF originate from tropical and subtropical parts of America, but today can be found in any tropical region across the globe (Diener et al., 2009). Naturally, its larvae can be found in the manure of cattle, poultry, and swine (Veldkamp and Bosch, 2015). Moreover, it can also be found in the waste of fish processing by-products, and reared on organic side streams such as coffee bean pulp, food waste (Diener et al., 2009; Van Huis et al., 2013). BSF is resistant to extreme environmental conditions such as drought and low oxygen level. An optimal rearing condition of BSFL should be approximately 29–31^{o}C with 50-70% humidity (Diener et al., 2011).

One of the most attractive attributes for incorporating BSFL into animal feed is the capability for sustainable rearing. The BSFL are able to utilize organic substrates, such as livestock manure, fermented organic digestate, and vegetable and fruit scraps for growth (Spranghers et al., 2016; Meneguz et al., 2018). This provides a sustainable alternative waste disposal method as BSFL subsequently convert the organic waste to valuable protein and fat biomass (Van Huis et al., 2013; Makkar et al., 2014). A 50% reduction in swine and poultry manure has been reported by rearing BSFL on these substrates and consequently, reducing the amount of energy and cost

associated with manure removal (Newton et al., 1977; Meneguz et al., 2018). Field trials in Costa Rica reported a 65-75% reduction of organic household waste when used as substrate for the production of BSFL (Diener et al., 2011). Unfortunately, due to health and safety concerns, currently BSFL are only approved for use in animal feed if they are raised on feed-grade materials; thus, no animal manure, human food waste, or other types of waste are accepted (European Commission, 2017; AAFCO, 2016). Additionally, this will ensure consistent nutrient composition of BSFL for commercial production (Klonick, 2017). This limits the potential benefit of environmental sustainability of BSFL; however, this rearing practice might change in the future as more research becomes available on BSFL rearing, nutrition, and feed/food safety. Other environmentally-friendly properties of rearing BSFL include low GHG emissions and ammonia, minimal water requirement, and low risk of zoonotic disease transmission (Van Huis et al., 2013; Rumpold and Schluter, 2013).

Additionally, BSF is considered a non-pest and non-disease transmitting insect (Sheppard et al., 2002). It is not considered as pest because the adult flies are not attracted to human food or environment. Additionally, BSF also assists in reducing the population of housefly, which is considered as pest and disease vector, by means of inhibiting the oviposition of housefly (Makkar et al., 2014). As mentioned before, BSFL can naturally be found on livestock manure and as the larvae grow and feed on it, manure becomes less solid and more liquid, which is unfavorable for housefly larvae to grow and multiply (Sheppard et al., 1994; Newton et al., 2005). BSFs only feed during their larval stage and once transformed into prepupae, their gastrointestinal tract (GIT) will be emptied and they will seek a dry, sheltered environment to finally metamorphosize to an adult fly. This self-harvest strategy by BSFL, along with proper processing techniques, greatly reduce

the risk of carrying pathogenic microorganisms (Sheppard et al., 2002; Klunder et al.,2012; Spranghers et al., 2016).

1.2.1 Nutritional characteristics of black soldier fly larvae and prepupae

The BSFL is a nutritionally-attractive protein source with an average crude protein (CP) of 40% - 50% (as-fed) (Renna et al., 2017; Crosbie et al., 2020; Enviroflight, KY, USA). Leucine, valine, and lysine are three of the most predominant indispensable AAs found in BSFL meal, while methionine is the lowest (Enviroflight, KY, USA; Bosch et al., 2014; Yu et al., 2019; Crosbie et al., 2020). The overall AA profile of BSFL meal has been reported to be similar to SBM, canola meal, and meat bone meal (Table 1.1; NRC, 2012; Enviroflight, KY, USA). Lysine, leucine, valine, histidine, and tryptophan contents of BSFL meal are particularly similar to those of SBM. However, when compared to fish meal, the AA profile of BSFL meal is overall lower (NRC, 2012; Tschirner and Simon, 2015; Enviroflight, KY, USA).

The final nutritional composition of BSFL or prepupae has been suggested to depend not only on its life stage, but also on the feeding substrates and processing methods (Tschirner and Simon, 2015; Veldkamp and Bosch, 2015). The notion that feeding substrates may influence the nutritional composition of BSFL arose from the variable fat and ash contents of BSFL reared on different manure types (Newton et al., 1977; Newton et al., 2005). Thus, many studies have investigated the impact of different feeds on the BSFL nutrient composition (e.g., Tomberlin et al., 2002; Danieli et al., 2019). Spranghers et al. (2016) evaluated the effects of feeding BSFL poultry feed, fresh and fermented vegetable waste, and restaurant waste on the AA and fatty acid contents of the prepupae. Regardless of the AA variations in the feeding substrates, the AA levels of BSF prepupae were relatively constant. Similar results were also reported by others, where the AA pattern of BSFL fed cereal middlings and swine manure were comparable and not significantly

different (St-Hilaire et al., 2007; Tschirner and Simon, 2015). In addition to the AA, the CP content of the prepupae was also comparable among different feeding substrates (Spranghers et al., 2016). Therefore, rearing or feeding substrates appear to have no substantial influence on the AA composition and CP level of BSFL and prepupae.

Unlike AA and CP, the crude fat and fatty acid content of BSF prepupae vary depending on the nutrient composition of the feeding substrates (Spranghers et al., 2016). For example, the ether extract (EE) of BSF prepupae fed chicken feed (EE chicken feed = 5.3% DM basis), fermented vegetable digestate (EE vegetable digestate = 6.2% DM basis), and vegetable waste (EE vegetable waste = 2.1% DM basis) were 33.6, 21.8, and 37.1% (DM basis), respectively (Spranghers et al., 2016). Depending on its crude fat level, BSFL meal is currently marketed as defatted (DF), partially-defatted (PD), and full fat (FF). However, currently there is no universally established threshold range of crude fat level to which BSFL meal can be categorized into. Defatted, PD, and FF BSFL meal have been reported to have a range of crude fat level of 1-6% (Mwaniki and Kiarie, 2019 Wang et al., 2019), 14 - 17% (Renna et al., 2017; Schiavone et al., 2017b), and 32% (Crosbie et al., 2020; Weththasinghe et al., 2021) on as-fed basis, respectively.

In regard to fatty acid profile, BSF prepupae are mainly comprised of saturated fatty acids, with lauric acid having the highest concentration among all other fatty acids that were measured. Interestingly, lauric acid content stays relatively consistent, regardless of its level in the feed substrates (Spranghers et al., 2016). A major benefit of high lauric acid content is its antimicrobial activities against gram-positive bacteria (Skrivanova et al., 2006; Spranghers et al., 2018). Thus, in addition to being an excellent source of protein, BSFL and prepupae can potentially serve as an in-feed antibiotic alternative (Newton et al., 2008; Spranghers et al., 2018). The quest for substitutes of the current in-feed antibiotics is important, as several countries (e.g. European Union

countries and Canada) have banned the use of in-feed antibiotics as growth promoters due to increase in antimicrobial-resistant bacteria (Michiels et al., 2009; Health Canada, 2018).

Another nutritional aspect to consider when incorporating BSFL or prepupae in feed is the chitin content. Chitin, a non-protein nitrogen (NPN) polysaccharide, is the main component of the insect exoskeleton (Newton et al., 1977) and makes up the majority, if not all of fiber content in insects (Finke, 2007). Structurally, chitin and cellulose share similarities; chitin is a linear polymer of β-(1-4) N-acetyl-D-glucosamine units, while cellulose is a linear polymer of β-(1-4) N-acetyl-D-glucopyranose units (Finke, 2007). Further, when analyzing acid detergent fiber (ADF) content in insects, nitrogen is present in the ADF fraction, which further supports that ADF content is mainly chitin (Barker et al., 1998; Finke, 2002). Chitin can negatively influence the apparent protein digestibility of an ingredient or feed and lead to decreased feed efficiency (Sanchez-Muros et al., 2014). High chitin content can also overestimate the CP in BSF meal and BSF-containing feed (Diener et al., 2009). For example, a 20-25 g/kg overestimation of CP level can be seen in BSF prepupae due to the presence of chitin (Spranghers et al., 2016). Therefore, when analyzing nutrient content of BSF for diet formulation, NPN from chitin needs to be accounted for.

1.2.2 Incorporation of black soldier fly larvae meal in animal feed

Currently, the regulatory status of BSFL is approved by the Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO), for use in poultry and aquaculture species (Enviroflight, Ohio, USA). Additionally, BSFL protein meal has been approved by the FDA for use in adult maintenance dog food and treats; however, it is only tentatively approved by AAFCO as of September 2021 (Enterra, 2021). Application of BSFL meal or its prepupae in animal feed has been extensively studied in poultry and aquaculture production (Kroeckel et al., 2012; Dabbou et al., 2018; Kawasaki et al., 2019; Mwaniki et al., 2020).

Compared to its larval stage, BSF prepupae have a higher level of the non-protein nitrogen, chitin, in its exoskeleton (Smets et al., 2020; Soetemans et al., 2020). Onsongo et al. (2018) reported no significant effects on feed intake, FCR, and average daily gain of broiler chickens when SBM and fish meal were partially replaced with BSF prepupae meal. Additionally, conventional broiler feed with no BSF was 19% more expensive compared to the treatment diet, which had 15% BSF prepupae meal (Onsongo et al., 2018). Mwaniki et al., (2020) reported that BSFL can replace SBM 100% in the diet for Shaver White hens with no negative effects on hen-day egg production; however, FCR was observed to be lower, which warrant further investigations. For aquaculture, St-Hilaire et al. (2007) indicated that partially replacing fish meal and fish oil with BSFL meal in rainbow trout feed had no significant effect on FCR. However, omega-3 fatty acid content in the fillet was significantly reduced in BSF-fed trout because unlike fish oil, BSF contains little to no omega-3 (St-Hilaire et al., 2007).

There are also studies which have investigated the application of BSF in livestock feed (Newton et al., 2005; Jayanegara et al., 2017; Yu et al., 2019; Biasato et al., 2019; Crosbie et al., 2020). Newton et al. (2005) reported that BSF prepupae meal might need further processing (e.g. heat treatment or cuticle removal) to make it more suitable for feeding young piglets as 100% substitution of spray-dried plasma with BSF prepupae reduced weight gain by 3% compared to the control diet. Another study on pigs, used the apparent total tract digestibility (ATTD) method and reported that the CP digestibility of BSFL meal and SBM (76.0 and 77.2% respectively) was similar, while crude fat digestibility was significantly higher in BSFL (83.6 and 73.0%; Newton et al., 1977). Standardized ileal digestibility of BSFL meal and prepupae in pigs were also determined by Crosbie et al., (2020) and Tan et al., (2020), respectively. Despite the growing

interest, more research is still needed on the practical application, AA bioavailability, and economic feasibility of BSFL inclusion in livestock feed (Koutsos et al., 2019).

1.3 Protein quality methodologies

1.3.1 The protein quality concept

Protein quality is defined as the capacity of AA profile of a food source or mixture of sources to fulfill the essential AA requirement of an individual (Schaafsma, 2005; Nosworthy and House, 2017). In principle, protein quality refers to the measure of AA bioavailability, which is the proportion of AAs that are digested and absorbed in a metabolically available form (Batterham, 1992; Fan, 1994; Stein et al., 2007a). The concepts of digestibility and bioavailability are important to distinguish as not all digested AAs are absorbed in a form that will allow the animals to metabolically utilize them (NRC, 2012). To evaluate the protein quality of an ingredient and precisely formulate feed, a critical assessment of AA bioavailability is essential (NRC, 2012; Columbus and de Lange, 2012; Van Milgen and Dourmad, 2015). Some factors that affect protein quality are the processing methods of ingredients (e.g., extrusion, fermentation, acid treatment), cooking method of protein sources in feed (Khattab et al., 2009), and the physiology of individual animals (e.g., life stages, species, genetics; NRC, 2012).

The concept of protein quality is more frequently used in human instead of animal nutrition because animals (i.e. livestock, companion, or aquaculture animals) are usually fed a mixture of ingredients in formulated diets that are nutritionally balanced (Mansilla et al., 2020). Meanwhile, humans have a more diverse dietary option and the dietary habit of each individual cannot be controlled (Mansilla et al., 2020). Therefore, protein quality information on ingredients can be used as a guidance for humans to make a nutritionally-informed decision when it comes to choosing protein sources. Despite of not being commonly used, the determination of protein

quality of ingredients in animal nutrition is also important to understand the bioavailability of AA from an ingredient and to utilize the results as guidance or insights for human nutrition (Rutherfurd et al., 2015; Hodgkinson et al., 2018).

There are several methodologies that can be categorized into *in-vitro,* indirect *in-vivo,* and direct *in-vivo,* to determine protein quality of an ingredient (Lewis and Bayley, 1995). *In-vitro* methods are relatively cheaper, less variable, faster, and are not restricted by ethics (Minekus et al., 1995). Some *in-vitro* approaches include the dynamic TNO Gastro Intestinal Model (TIM-1) (Minekus et al., 1995), the static pH-drop (Hsu et al., 1977), and pH-stat (Pedersen and Eggum, 1983), while indirect *in-vivo* methods include the use of plasma AA concentrations and nitrogen digestibility measurement (Lewis and Bayley, 1995). In general, *in-vivo* methods are perceived as a more accurate representation of the animals' metabolic processes; however, these methods are more expensive, laborious, and may be constrained by ethical considerations (Fernandez-Garcia et al., 2009; NRC, 2012). Protein digestibility-corrected amino acid score (PDCAAS), digestible indispensable amino acid score (DIAAS), slope-ratio growth assay, and indicator amino acid oxidation (IAAO) are some of the notable *in-vivo* protein quality methods. The PDCAAS and DIAAS values are more frequently used in human nutrition (Mansilla et al., 2020); however, to calculate PDCAAS and DIAAS, total tract protein digestibility and ileal AA digestibility values, respectively, need to first be determined (FAO/WHO, 1991; Mansilla et al., 2020).

1.3.2 PDCAAS and DIAAS

Protein-digestibility-corrected AA score (PDCAAS) has been well accepted as the standard to measure the quality of protein ingredients by the FAO and WHO (Codex Alimentarius Commission, 1989; FAO, 1991). The PDCAAS is calculated as the product of indispensable AA with the lowest AA score and the total tract protein digestibility. The AA score is calculated as a

ratio of indispensable AA content per gram of total protein and the reference AA pattern of either infant, child, or adult human population (FAO/WHO, 1991). However, following a 2012 FAO meeting, DIAAS was advocated as a preferred method to measure protein quality as it addresses several shortcomings of the PDCAAS (FAO, 2013). First, DIAAS is calculated based on ileal digestibility value of individual indispensable AA rather than total tract protein digestibility (reviewed on Mansilla et al., 2020). Ileal digestibility is perceived as a more accurate measure of AA digestibility as the majority of AA digestion and absorption occur in the small and not in the large intestine (Stein et al., 2007b). Second, DIAAS value is not truncated to 1.0 like the PDCAAS, this truncation underestimates ingredient with higher protein quality value and therefore, accurate comparison of protein quality between ingredients cannot be made (Sarwar, 1997). However, a major limitation of the DIAAS method is the limited availability of data on ileal digestibility of AA in humans; therefore, ileal digestibility data on animals, especially the pigs are commonly used as model for humans (Rutherfurd et al., 2015; Hodgkinson et al., 2018). As of 2020, no regulatory agency has adopted the application of DIAAS as a standard to determine protein quality, mainly because of the limited ileal digestibility data (Mansilla et al., 2020). Hopefully, as more ileal digestibility studies on new protein ingredients in swine are emerging, more ileal digestibility data will be available for use in DIAAS calculation.

1.3.3 Digestibility methods: Total tract and ileal digestibility

During the digestion process, dietary AAs along with other nutrients will be digested and absorbed in the GIT. The amount of AA that has disappeared determines the absorptive efficiency and indicates how digestible and absorbable the dietary AAs are (Stein et al., 2007b). Due to practicality, results from AA digestibility studies are often used to estimate bioavailability (Mosenthin et al., 2000; Columbus and de Lange, 2012); this will be further elaborated in the

upcoming sections. Two commonly-used methodologies to measure AA digestibility are total tract and ileal digestibility. The total tract is recognized as the simplest digestibility method and assumes that AA absorption occurs throughout all parts of the GIT (Stein et al., 2007b). This technique measures the amount of AA absorbed by subtracting the AA intake with the amount recovered in the feces (Stein et al., 2007b). There are several shortcomings of this method that makes it less suitable to estimate AA bioavailability (Sauer and Ozimek, 1986). First, the majority of AA absorption occurs in the small intestine and only negligible amounts are absorbed in the colon (Stein et al., 2007b). This has been confirmed by previous studies where there was no significant increase in nitrogen balance, body protein accretion, and protein synthesis when different levels of AAs were deliberately infused into the colon of cecal-cannulated pigs (Darragh et al., 1994; Fuller and Reeds, 1998). Second, the absorption of NPN (primarily ammonia, traces of amines, and amides) is significant in the large intestine (Darragh et al., 1994). Absorption of nitrogen as NPN in the large intestine has been thought to have no nutritional value and to not contribute to protein synthesis in the animals (Darragh et al., 1994; Fuller and Reeds, 1998). However, a study by Columbus et al., 2014 concluded that absorbed nitrogen in the form of NPN in the colon does contribute to protein synthesis. This is due to NPN being returned to the small intestine in the form of urea and utilized by microbes for AA production (Columbus et al., 2014). However, nitrogen that is absorbed in the large intestine is less efficiently used (20% efficiency; Columbus et al., 2014) for protein deposition compared to nitrogen that is absorbed in the small intestine as AAs (75% efficiency; NRC, 1998). Therefore, accounting for NPN absorption in the colon can affect the accuracy of dietary protein digestibility. Last, fermentation of nutrients by gut microorganisms occurs primarily in the colon. Fermentation of undigested or endogenous proteins leads to the production of ammonia that can be reabsorbed for protein synthesis or eliminated as urea (Low,

1990; Zebrowska and Buraczewksi, 1998). These colonic microbes can also lead to de novo synthesis of microbial proteins (Columbus and de Lange, 2012) or further breakdown of dietary AAs that can underestimate or overestimate the AA content of the digesta, respectively, and further affect digestibility values (Zebrowska and Buraczewski, 1998). Therefore, to more accurately quantify AA digestibility, digesta collection at the terminal ileum is considered more appropriate.

The ileal digestibility method is more complex, as the procedure is more invasive to accommodate digesta collection at the distal ileum of an animal (Sauer and de Lange, 1992), but is believed to be more accurate for quantifying AA digestibility and estimating its bioavailability compared to the total tract approach (Sauer and Ozimek, 1986; Rademacher et al., 1995). The justifications for increased accuracy of the ileal digestibility method in regards to bioavailability estimation are because growth performance is associated to a greater degree with ileal digestibility coefficients (Low et al., 1982; Just et al., 1985) and disappearance of nitrogen beyond the distal ileum is most likely caused by losses during fermentation that have little to no nutritional benefits to the animal (Columbus and de Lange, 2012). Dierick et al. (1988) demonstrated that ileal digestibility coefficients are more closely correlated to the growth performance of monogastric animals compared to the total tract coefficients (i.e. 0.76 vs 0.34). Furthermore, Rademacher et al. (1995) reported that ileal digestibility and growth performance are correlated. The majority of absorbed AA or nitrogen beyond the distal ileum is believed to have no nutritional benefits and is intended as substrates for urinary ammonia production (Libao-Mercado et al., 2009). This is in agreement with Fuller and Reeds (1998), no change in fecal digestibility was observed when casein was infused into the cecum of pigs. Additionally, Wunsche et al. (1982) reported that urinary ammonia increased as AAs were supplied to the colon. Thus, AA absorption in the hindgut does not contribute to protein synthesis (Sauer, 1976; Sauer and Ozimek, 1986).

Ileal digestibility can be expressed as apparent, standardized, and true depending on how endogenous AA losses (EAAL) are accounted for (Stein et al., 2007a; Adeola et al., 2016). The EAAL are defined as AAs that are not of dietary origin and will not be reabsorbed into the digestive lumen. Main sources of EAAL are digestive enzymes, sloughed intestinal epithelial cells, microbially-synthesized AAs, and mucin (Moughan et al., 1992; Nyachoti et al., 1997). Ileal EAAL can be further differentiated into basal and specific losses, which can be measured by several methods (Nyachoti et al., 1997).

A major limitation of both total tract and ileal digestibility methods is they do not accurately measure AA bioavailability. This is because digestibility coefficients only reflect the amount of AA that has disappeared from the GIT and do not account for the AA form in which it is absorbed (Batterham, 1992; Stein et al., 2007a). Since routine quantification of AA bioavailability is non-practical, complex, time consuming, and costly, SID results are widely accepted as reliable estimates of AA bioavailability (Mosenthin et al., 2000; Moehn et al., 2005).

1.3.3.1 Apparent, standardized, and true ileal digestibility

Apparent ileal digestibility (AID) is the simplest digestibility technique to conduct; however, it is not the most ideal method to calculate digestibility coefficient because it does not account for the contribution of EAAL (Stein et al., 2007a; Adeola et al., 2016) and AID values are not additive in diets with multiple protein ingredients (Fan and Sauer, 1995). Overlooking the EAAL can lead to underestimation of AA digestibility in feed ingredients, especially in low-protein sources such as cereal grains (Rademacher et al., 2001). This occurs because the ileal digesta will appear to have a high amount of undigested dietary AA, when in reality, the digesta is also comprised of a substantial amount of EAAL (Rademacher et al., 2001; Stein et al., 2005). The AID coefficient is not additive and this is largely due to the curvilinear relationship between AID

values with dietary AA levels (Fan et al., 1994) and the failure to adjust for EAAL (Stein et al., 2007b). The curvilinear relationship is seen in AID because at a certain point of high dietary AA levels, endogenous AA decreases and only represents a smaller proportion of AA in the ileal digesta; therefore, the initial sharp increase of AID following increase in dietary AA eventually reached a plateau (Fan et al., 1994). This non-linear relationship would not be observed if EAAL was accounted for and this would lead to a relatively stable digestive values that are independent of the dietary AA concentrations (Fan et al., 1994; Columbus and de Lange, 2012). The additivity concept in a mixed diet refers to the proportional quantities of each AA digestibility value per ingredient added with values from other ingredients collectively. Together, this addition represents the digestibility of each AA in the diet as a whole (Stein et al., 2005). Additivity in AA digestibility coefficients are more useful and favorable because it will improve precision in diet formulation on a digestible AA basis. Subsequently, the AAs supplied in the feed can be matched with the animal's estimated AA requirements and growth performance can then be accurately predicted (Fan et al., 1994; Stein et al., 2005; Jansman et al., 2002).

To overcome the limitations of AID, the SID method was developed. The SID calculates the ileal AA digestibility coefficient of an ingredient after the total AA in the digesta is corrected for basal EAAL (Stein et al., 2007b). The advantages of the SID method are additivity of SID coefficients in a mixture of feed ingredients, the simplicity of basal EAAL quantification (Stein et al., 2007b), and the simultaneous provision of digestibility values for all AAs (Columbus and de Lange, 2012). Furthermore, SID values are not influenced by diet-related nutrients such as protein concentration or chitin content (Stein et al., 2007b), making SID the best approach for formulating diets. Additionally, the NRC reports the AA requirement of swine and nutrient analysis of feed ingredient on an SID basis (NRC, 2012).

Despite the clear advantages, there are also limitations associated with the SID method. Foremost, the SID coefficients tend to overestimate the bioavailability of AAs in heat-treated ingredients (Stein et al., 2007b; NRC, 2012). For example, when exposed to intense heat and pressure, lysine reacts with reducing sugars to form a Maillard complex (Eklund et al., 2015). Once the Maillard complex is digested and absorbed, it can lead to the overestimation of lysine bioavailability because bonded-lysine, though absorbed, is unavailable for protein synthesis (Finot and Magnenat, 1981; Moughan and Rutherfurd, 1996). Next, SID values are influenced by feed intake level and highly dependent upon accurate estimation of basal EAAL (Stein et al., 2007a). Another limitation to the method is it assumes that the application of absorbed AA for protein synthesis and metabolic pathways are not influenced by protein level in the diet (Columbus and de Lange, 2012). However, caution needs to be taken with this assumption, especially when the feed ingredients used are contributing to more EAAL, have been heat-treated, or contain high fiber, which will affect the flow of DM in the GIT (Columbus and de Lange, 2012).

The third type of ileal digestibility measurement is TID which calculates the digestibility values of AA by correcting for total EAAL (basal and specific). The digestibility coefficient derived from TID is considered the most accurate to measure digestion and absorption as it accounts for both basal and diet-specific EAAL (Stein et al., 2007a). However, one major limitation of applying TID in practical feed formulation is the inadequacy of available data on total EAAL measurements of various commonly-used feed ingredients (Stein et al., 2007b). Additionally, quantifying total EAAL for each ingredient is not practical for routine assessment, as it is expensive, laborious, and technically difficult to complete (Stein et al., 2007b). The TID coefficients are also considered non-additive, as TID does not differentiate between ingredients that might stimulate the secretion of specific EAAL (Stein et al., 2007a), and formulation does not

occur on a TID basis but rather on an SID. Therefore, the SID remains the most reliable, practically useful, and widely-used method to quantify AA digestibility and estimate bioavailability in feed ingredients and complete feed (Jansman et al., 2002; Stein et al., 2007b).

1.3.3.2 Measurement of basal and total endogenous amino acid losses

Basal or non-specific EAAL are the minimum inevitable and anticipated AA loss that occurs in an animal's body due to normal metabolic processes when feed flows throughout the GIT (Stein et al., 2007b). Accurate measurement of basal EAAL is important when conducting the SID method, as it drives the accuracy and reliability of the SID coefficient (Levesque et al., 2011). The amount of basal EAAL is fairly constant regardless of the types of feed ingredients or protein levels in the diet (Stein et al., 2007b). There are multiple different methods to measure basal EAAL such as the regression method, nitrogen free diet (NFD), and highly digestible purified diet (casein-based diet) approaches (Stein et al., 2007b; Fan and Sauer, 1995; Nyachoti et al., 1997).

The regression method is a traditional approach that has been used to determine basal EAAL in several studies (Fan et al., 1995; Eklund et al., 2015). In this method, the animals are fed isonitrogenous diets with a minimum of three graded dietary AA levels to achieve a regression line (Eklund et al., 2015). The line is then mathematically extrapolated to a 0% nitrogen or AA intake, and the amount of nitrogen or AA recovered at this intercept is considered the basal EAAL estimation (Nyachoti et al., 1997). Eklund et al. (2015) measured the basal EAAL of growing barrows with the regression method and found that the amount of AA recovered to be within the basal EAAL values estimated by the NFD approach, with the exception of lower proline and glycine (Stein et al., 2005; Eklund et al., 2015). Some limitations of the regression method are the requirement of multiple dietary treatments in order to develop the slope and it is labor-intensive

and may be statistically inaccurate as the intercept at 0% protein intake is achieved through extrapolation and errors could be contributed by the interval of regressor (Adeola et al., 2016).

The NFD method, also known as the protein-free diet approach, assumes that when a nitrogen-free diet is given to an animal for several days, all nitrogen recovered in the ileal digesta will be of endogenous origin (Nyachoti et al., 1997). A study by Jansman et al. (2002) compared different methodologies to quantify and determine the AA composition of basal EAAL by assessing data from 33 ileal digestibility studies in pigs. Basal endogenous CP flow in pigs fed highly digestible protein diet, such as casein, is only 20% higher compared to the NFD results (Jansman et al., 2002). The higher endogenous losses in pigs fed the high protein diets is believed to be caused by the stimulation of digestive enzymes secretion as a response to high protein intake (Jansman et al., 2002). This concept is further supported by other studies, where feeding NFD has resulted in reduced overall total protein excretions into the gut lumen and decreased pancreatic secretions of digestive enzymes such as trypsin and chymotrypsin (Ozimek et al., 1985; Darragh et al., 1990). Thus, NFD method underestimates basal EAAL due to its non-stimulatory effects on endogenous secretions (Butts et al., 1993). The major constraint with the NFD method is how feeding NFD induces an abnormal physiological state in the animal due to the deprivation of essential AAs and dietary nitrogen, leading to a catabolic state or a negative nitrogen balance (Low, 1980; de Lange et al., 1989). In response, the body will catabolize muscle protein to maintain normal physiological conditions (Jansman et al., 2002) and this catabolism causes a surge in alanine and glutamine concentration as products of protein breakdown (Rodwell, 1985). High concentration of glutamine eventually reaches the lumen of the GIT and it is further metabolized to glutamate, ammonia, proline, and glycine there (Rodwell, 1985; Rogers and Phang, 1985). Thus, NFD method tends to overestimate proline and glycine in the basal EAAL. Overestimation of

proline and glycine would become a problem if EAAL is expressed as a percentage of total endogenous losses as this will decrease the relative concentration of other AAs and affect the SID calculation (de Lange et al., 1989). Despite these limitations, basal EAAL measurement by the NFD method is similar and consistent with results of the regression method, which has been considered as a more precise technique to quantify basal EAAL (Fan et al., 1995). The NFD is also known to be the most practical and widely-used method for basal EAAL quantification in animal studies (Stein et al., 2007b).

Since the NFD method can lead to an abnormal physiological state, feeding a highly digestible or purified intact protein source is seen as an alternative approach (Stein et al., 2007b). The ileal AA outflow of animals fed highly digestible protein ingredients such as casein, egg protein, or crystalline AAs, consists solely of endogenous origin (Nyachoti et al., 1997). This is because these ingredients are assumed to be 100% digested and absorbed in the small intestine, prior to the terminal ileum; thus, they will not contribute significantly to the AA content of ileal digesta (Adeola et al., 2016). Additionally, it is also assumed that they will not induce specific secretion of EAAL (Jansman et al., 2002; Adeola et al., 2016). These assumptions are the main limitations of the method, as the true digestibility of those ingredients are assumed to be 100% (Nyachoti et al., 1997). Despite the limitations, measurement of basal EAAL using this method is considered to be the most physiologically-normal because the animal is not deprived of dietary nitrogen or AAs (Jansman et al., 2002). Additionally, ileal digesta recovered from this method has a significantly lower level of proline and glycine compared to the NFD method (Jansman et al., 2002). The basal EAAL estimate results vary between different methodologies, experimental conditions, or digesta sampling methods. Furthermore, variations can also be due to the animal's

genotype and gut health (Seve et al., 2001). Therefore, it is important to routinely measure basal EAAL for each ileal digestibility study (Stein et al., 2007b).

Total EAAL consists of the basal plus specific EAAL. Specific EAAL is the endogenous AA secretion that is stimulated by the consumption of diets with certain nutrient compositions or feed ingredient characteristics, and this loss is additional to the basal losses (Stein et al., 2007b). High protein diets can induce more specific EAAL as it triggers the secretion of additional digestive enzymes (Nyachoti et al., 1997). However, when an animal is given a highly digestible or purified protein ingredient, the specific EAAL is negligible (Souffrant, 1991). The type and characteristics of fiber may also affect the level of EAAL. For example, neutral detergent fiber-(NDF) rich sources such as wheat bran, stimulates higher EAAL in pigs compared to ingredients that are low or free from NDF (Schulze et al., 1995). Anti-nutritional factor (ANF) such as protease inhibitor, tannins, and lectins (Huisman et al., 1992; Jansman et al., 1995) tend to increase endogenous nitrogen in the digesta by stimulating more EAAL secretion or reducing EAAL reabsorption in the gut lumen (Schulze, 1994). Accurate measurement of total EAAL is especially important when conducting a TID study and it requires more sophisticated and complicated procedures compared to measuring basal EAAL (Stein et al., 2007b). Two methods that are commonly used to determine total EAAL are the homoarginine (Rutherfurd and Moughan, 1990) and isotope dilution technique (de Lange et al., 1990).

The homoarginine technique estimates total endogenous losses by separating the dietary and endogenous lysine sources by guanidinating all dietary lysine to homoarginine (Hagmeister and Erbersdobler, 1985). Some limitations of the method are risk of incomplete dietary lysine guanidination (Rutherfurd and Moughan, 1990), risk of homoarginine hydrolyzation in the gut leading to the breakdown of homoarginine to lysine and urea, and this method can only be used to

directly measure endogenous lysine losses (Marty et al., 1994). The main advantage of this method is that it can be conducted in animals in a normal physiological state (Moughan et al., 1998). Additionally, there are several estimates of total endogenous lysine losses using guanidinated feedstuffs (Rutherfurd and Moughan, 1990; Nyachoti et al., 1997) that are available for reference.

The isotope dilution technique involves labeling nitrogen in the diet (Moughan et al., 1998) or the endogenous pool (de Lange et al., 1990) with stable 15N isotope. However, this method receives a lot of criticisms and may not be the most suitable approach to estimate total EAAL. Limitations of this approach are determining the most suitable pre-cursor pool, difficulty in maintaining steady state (Moughan et al., 1992), non-uniform nitrogen labeling, underestimation of EAAL because this technique does not account for the EAAL contribution from mucosal cells, and the cost of isotope and the subsequent analysis (de Lange et al., 1992).

1.3.4 Methods to quantify amino acid bioavailability

Despite being more practical for routine feed formulation purposes, ileal digestibility does not accurately quantify AA bioavailability, which is a reliable protein quality indicator. Further, TID, which is considered as the most accurate ileal digestibility method for estimating protein quality (Stein et al., 2007b) fails to account for first-pass metabolism and only measures the disappearance of AA in the GIT regardless of the form (Stein et al., 2007b; Levesque et al., 2010). Due to the intricacy of bioavailability measurement, ileal digestibility results are well-accepted as estimates for AA bioavailability (Levesque et al., 2010; Moehn et al., 2005). However, effort is still needed to develop and implement a practical method to directly quantify AA bioavailability. The benefits of having such methodology include improved feed formulation accuracy, reduced feed cost, and the ability to bypass the need to measure EAAL because bioavailability

measurements account for all metabolic losses (Ball et al., 2004). Two well-known *in-vivo* methods to quantify AA bioavailability in pigs are discussed below.

1.3.4.1 Slope ratio growth assay

Slope ratio growth assay (SRGA) is considered to be the gold standard to quantify AA bioavailability in swine nutrition (Lewis and Bayley, 1995; Levesque et al., 2010). This assay involves feeding all subject animals a common basal diet before the start of treatment period. Then, the animals are fed a reference diet, which is basal diet supplied with graded levels of crystalline AAs or an ingredient known to have 100% bioavailability, such as casein, to achieve graded levels of the test AA in the diet (Batterham et al., 1990). The test AA must be the first limiting AA in the diet and the highest level tested should not meet recommended requirement to ensure a linear response (Batterham, 1992). The experimental diets will also have the same levels of graded test AA as the reference diet with the test protein ingredient as the sole source of AA (Batterham et al., 1990). To achieve equal AA concentrations between two different ingredients (e.g. casein in reference diet and BSFL in test diet), the test protein inclusion is adjusted at the expense of another ingredient such as corn starch (Batterham et al., 1990). A minimum of three AA levels are necessary to obtain a linear response with graded intake of test ingredients (Batterham, 1992). Parameters measured in SRGA are growth rate, carcass gain per day, feed conversion efficiency, or whole-body protein retention (Batterham et al., 1990). The changes in parameter measurements from animals fed the test ingredient are compared to the response of those fed the reference diet in a slope ratio approach. Thus, the measured AA bioavailability of the test ingredient is known as relative bioavailability (Batterham, 1992; Stein et al., 2007b).

One main advantage of the SRGA is that the bioavailability results contribute to practical and economical insights in the swine industry because SRGA utilizes animal growth as the

response parameter (Lewis and Bayley, 1995). However, the AA bioavailability values obtained from SRGA are not additive in a mixture of ingredients because they are highly variable and dependent on the experimental conditions (Gabert et al., 2001). Other concerns with the growth assay are the bioavailability values are less suitable for feed formulation (Fan, 1994), tedious, and expensive to conduct because the SRGA requires a large number of animals for a prolonged period to measure bioavailability of one AA (Batterham, 1992). Thus, a more practical and rapid AA bioavailability technique is beneficial.

1.3.4.2 Indicator amino acid oxidation (IAAO)

The indicator amino acid oxidation (IAAO) method has been widely used to determine the AA requirements in pigs and humans (Bertolo et al., 2005; Humayun et al., 2007; Moehn et al., 2008; Elango et al., 2012); however, previous studies have demonstrated that IAAO is also a promising technique to quantify AA bioavailability of protein ingredients in swine (Moehn et al., 2005; Shoveller et al., 2010; Levesque et al., 2011). The principle of IAAO as a bioavailability measurement method is that changes in the oxidation of the indicator AA can be observed as the test AA becomes more or less bioavailable for the animal (Moehn et al., 2005; Humayun et al., 2007). As the test AA (i.e. the first limiting AA in the diet) becomes more bioavailable, oxidation of indicator AA decreases and protein synthesis increases (Ball and Bayley, 1986; Moehn et al., 2005). This is the result of one essential AA being deficient in the diet, which does not optimize protein synthesis and all other excess AAs, including the indicator AA, are oxidized as they cannot be stored in the body or further incorporated to synthesize protein (Holt et al., 1962; Pencharz and Ball, 2003; Moehn et al., 2005). The indicator AA is usually a carbon-labeled indispensable AA which will be irreversibly catabolized into carbon dioxide (CO_2) and urea when oxidized (Levesque et al., 2010). The most commonly-used indicator AA in pig studies is the carbon-labeled

phenylalanine, stable L-[1-^{13}C]-Phenylalanine or radioactive L-[1-^{14}C]-Phenylalanine, in the presence of excess tyrosine (Moehn et al., 2005; Shoveller et al., 2010; Levesque et al., 2011). The main advantages of using phenylalanine as an indicator AA are the tightly regulated intracellular phenylalanine concentrations, short half-life, and small size of the phenylalanine pool (Neale and Waterlow, 1974; Flaim et al., 1982). Consequently, phenylalanine can respond quickly to changes in the limiting AA level based on the test AA intake (Ball and Bayley, 1984). The purpose of having a labeled AA is to monitor and measure the appearance of labeled CO_2 as a result of whole-body protein oxidation. Higher labeled CO_2 production indicates higher indicator AA oxidation and lower bioavailability of the limiting AA in the feed (Moehn et al., 2005). The production of labeled CO_2 will decrease and eventually plateau with increasing intake of the test AA. Once the limiting AA requirement is satisfied, protein synthesis will occur instead of AA oxidation (Moehn et al., 2005). The indicator AA can be delivered orally or through intravenous infusion. Moehn et al. (2005) demonstrated that the indicator oxidation response to graded test AA diets was similar between oral and intravenous delivery. Thus, oral delivery is favored as it is less invasive (i.e. no surgery needed to set up the catheter for intravenous infusion; Moehn et al., 2005).

The IAAO technique is considered an accurate, reliable, and rapid method to quantify AA bioavailability (Moehn et al., 2005). Moehn et al. (2005) reported that the bioavailability of lysine in raw peas obtained from the IAAO method was 88.8% which was comparable to the 85% value reported by Van Barneveld et al. (1995) using the SRGA method. This was an exciting finding because SRGA is considered as the gold standard for bioavailability measurement (Lewis and Bayley, 1995). There are four key conditions to be fulfilled in IAAO study to measure AA bioavailability. First, the test AA must be the first limiting in the reference and experimental diets to drive indicator AA oxidation. Second, changes in oxidation rate response towards intake of test

AA must be linear (Littell et al., 1995). To achieve this linear response, a minimum of three graded levels of the test AA below the NRC recommended requirement is required (Baker, 1986). Third, to allow precise determination of the AA bioavailability, the rate of indicator oxidation must not be variable (Moehn et al., 2005). Lastly, the indicator AA must achieve and be maintained at an isotopic steady state in the duration of the breath collection day. Therefore, continuous delivery of isotope through small meal portions is needed to maintain steady state (Moehn et al., 2005; Levesque et al., 2010).

When used to measure bioavailability, the IAAO adopts the slope-ratio assay principle (Moehn et al., 2005; Shoveller et al., 2010), where the change or reduction in indicator AA oxidation in response to incremental concentration of test AA from a test ingredient is compared relative to the oxidation change in response to the incremental test AA from crystalline or highly digestible protein ingredient (Moehn et al., 2005). The slopes represent the change of oxidation rate (y-axis) of the indicator AA in response to unit change intake (x-axis) of the test AA (Shoveller et al., 2010). For example, an IAAO study in pigs by Moehn et al. (2005) observed an increase in phenylalanine oxidation as lysine becomes less bioavailable in heated compared to raw peas due to the Maillard reaction. The change in phenylalanine oxidation per gram of lysine is referred as the slope. To calculate the bioavailability of lysine, the slope for peas-lysine (feedstuff) is divided by the slope for free lysine, which was obtained by feeding pigs a reference diet. The reference diet was a basal diet supplemented with crystalline lysine to reach 80% of the NRC lysine requirements. The lysine bioavailability results were found to be 88.8% and 54.8% for raw and heated peas, respectively (Moehn et al., 2005). Therefore, phenylalanine oxidation directly reflects lysine bioavailability. Additionally, increases or decreases in phenylalanine oxidation was contributed solely by the changes in lysine intake and bioavailability (Moehn et al., 2005).

Compared to the SRGA that involves many subjects and a long study period, the IAAO only requires a short diet adaptation period (2 days), which can reduce the number of animals required as multiple breath collection can be repeated on the same subject (Levesque et al., 2010) and different AAs can be tested relatively rapidly one after the other when the animals are at a similar physiological stage (Moehn et al., 2008). Moehn et al. (2004) found that the rate of phenylalanine oxidation and CO_2 production in IAAO did not differ significantly between various diet adaptation periods of 2 – 10 days (Moehn et al., 2004). Furthermore, unlike the SRGA, AA bioavailability results from IAAO are considered to be additive in a mixture of ingredients (Ball and Bayley, 1986; Levesque et al., 2010). Amino acid bioavailability results that are additive will be highly beneficial to improve the practicality and optimize the accuracy of nutrient formulation in feed (Fan, 1994), especially because most commercial feed incorporates at least one low-protein ingredient (e.g. cereal grains; Stein et al., 2005).

When compared to other protein quality methods such as the ileal digestibility, IAAO is less invasive, as no surgical procedure is required (Levesque et al., 2010). Additionally, IAAO can circumvent the need to measure or assume the highly variable EAAL as IAAO results account for all losses during digestion, absorption, and utilization of the test AA (Elango et al., 2008). However, there are also some disadvantages associated with the IAAO method. First, IAAO is expensive and requires more sophisticated equipment. Additionally, IAAO can only provide bioavailability values for one AA at a time, while ileal digestibility can provide digestibility values as estimates for bioavailability for all AAs simultaneously (Levesque et al., 2010). Regardless of these limitations, IAAO is still one of the leading and advanced methodologies to quantify AA bioavailability and has been considered as the gold standard for AA bioavailability measurement in humans by the World Health Organization (Pencharz and Ball, 2003; WHO, 2007).

1.4 Summary and conclusion

The purpose of this literature review was to describe and compare the concept, advantages, and disadvantages of different protein quality methodologies with the main emphasis on SID and IAAO and to also introduce BSFL meal as a valuable protein ingredient in animal feed. Assessing protein quality is especially important when introducing a new protein ingredient in animal feed, such as the highly nutritious and sustainable BSFL meal. The SID method has been utilized and acknowledged in swine nutrition studies to estimate the AA bioavailability of feed ingredients (Batterham, 1992; Levesque et al., 2010). However, the development of IAAO is seen as a more promising method to directly quantify AA bioavailability. To the best of current knowledge, no studies to date have successfully compared the two methods on the same test ingredient and the same population of animals. Many published studies are investigating each method separately and therefore are prone to discrepancies in results due to different study designs. One of the most relevant research studies where AA digestibility and bioavailability methods were compared together was conducted by Batterham et al. (1990). The study compared TID with SRGA; however, the animal subjects used were very different (20 kg female and male growing pigs in the SRGA, while the TID used 65 kg male pigs) (Batterham et al., 1990). Hence, the results are highly variable and not comparable. Comparison of SID and IAAO can provide insight on how different the digestibility values are from the bioavailability measurement. Once the discrepancies between the SID and IAAO results are well understood, utilization of AA digestibility values as a reflection of bioavailability can be used more effectively in feed formulation (Columbus and de Lange, 2012). Therefore, prior to incorporating BSFL meal in swine feed, it is important to determine its protein quality by examining the SID results in contrast to the IAAO on the same subject population.

1.5 Rationale and research objectives

The quest for alternative protein ingredients in feed for livestock, poultry, aquaculture, and pet food is growing because of two main reasons. First, the demand for food and animal products is anticipated to increase by 2050, when the human population is estimated to reach and exceed nine billion (FAO, 2011). Second, commonly used feed protein ingredients [e.g. soybean meal (SBM) and fish meal] are becoming more expensive, less environmentally sustainable, and constrained for continuous use as an ingredient, resulting in uncertainty in the sustainability of the ingredient supply chain [International Monetary Fund (IMF), 2010; Sanchez-Muros, 2014]. In the past few decades, due to the increased demand of soybean for food products, animal feed, and industrial usage, soybean cultivation has expanded rapidly (Jia et al., 2020; WWF, 2014). Expansion of soybean cultivation has led to increased land-use conflict (i.e. deforestation) (Ferreira et al., 2016; Cohn and O'Rourke, 2011) and greenhouse gas emissions due to long-distance transport (He et al., 2019), mainly because 90% of the global soybean producers are only concentrated in Argentina and Brazil (Mathews and Goldsztein, 2009). Meanwhile, fish meal production is quantitatively variable as its production depends on the population of wild-caught fish, which continues to decline (FAO, 2012b). As a result, fish meal price continues to increase due to supply and demand imbalance (IMF, 2010).

Black soldier fly larvae (BSFL) meal is one of the most attractive, nutritious, and sustainable alternative feed protein ingredients. Black soldier fly larvae can be reared on organic waste or livestock manure (Meneguz et al., 2018; Spranghers et al., 2016), its farming requires minimal water, produces low greenhouse gas emission, and low ammonia (Van Huis et al., 2013). This presents a unique added benefit to using BSFL meal as it is able to provide sustainable alternative for waste disposal and can potentially assist in resolving the mounting food waste issue

(Diener et al., 2011). Black soldier fly larvae meal is a protein-rich ingredient, with an average crude protein (CP) content of 40 – 58% and first limiting in the sulphur amino acid, methionine (Matin et al., 2021; Biasato et al., 2019; Renna et al., 2017). The crude fat level of BSFL varies depending on its feeding substrates (Spranghers et al., 2016) and it is currently marketed as defatted, partially-defatted, and full fat (Mwaniki and Kiarie, 2019; Schiavone et al., 2017b). The extracted BSFL oil is rich in lauric acid and is also available in the market as an alternative fat source in animal feed (personal communication with Enviroflight).

Assessment of the protein quality of BSFL meal is needed to accurately incorporate this alternative protein ingredient in swine feed formulations because analysis of proximate crude protein (CP) alone does not accurately represent AA bioavailability for net protein gain in the animal. First, CP analysis only measures the nitrogen content of the ingredient with the assumption that all nitrogen present is part of a protein (Merrill and Watt, 1973). However, BSFL contains chitin, a non-protein nitrogen, that could lead to the overestimation of CP (Newton et al., 1977). Additionally, not all of the protein nitrogen from BSFL is used for protein synthesis within the animal. Therefore, protein quality measurement, which analyzes the AA digestibility or metabolic availability of an ingredient, are more useful for feed formulation to accurately meet the AA requirements and produce a more cost-efficient feed as AAs are not formulated in excess. Additionally, when AA digestibility or bioavailability data of novel alternative protein ingredients are readily available, feed manufacturers are more likely to adopt and incorporate those alternative ingredients (Woyengo et al., 2014).

Two main protein quality methodologies that are going to be investigated in this thesis are standardized ileal digestibility (SID) and indicator amino acid oxidation (IAAO). The apparent ileal digestibility (AID) on AAs and apparent total tract digestibility (ATTD) method on energy

and fiber will also be conducted as an add-on to the SID study, as many researchers conduct the AID method to make conclusions that can only be gained by SID measures. The SID method measures the AA digestibility and absorption through collection of ileal digesta at the terminal ileum and when both test diet and nitrogen-free diet (NFD) are fed to account for basal endogenous AA losses. The SID is well-accepted as an estimate of AA bioavailability value (Jansman et al., 2002; Stein et al., 2007a). The AID method is a similar concept as the SID; however, it is a simpler approach as the basal endogenous AA losses is not accounted for (Stein et al., 2007b; Adeola et al., 2016). Thus, AID values are often regarded as less accurate compared to SID for protein quality measurement (Stein et al., 2007a). Meanwhile, IAAO measures the metabolic availability of AA in a test ingredient relative to an ingredient known to be 100% bioavailable or other reference ingredients (Moehn et al., 2005; Shoveller et al., 2010). Therefore, understanding the result discrepancies between the two methods will improve the applicability of SID coefficients as an estimate for AA bioavailability. To the best of our knowledge, there is currently no published literature investigating or comparing the two methods on the same test ingredient and the same animal population. Further, there are a limited number of studies that focuses on evaluating the SID (Crosbie et al., 2020; Tan et al., 2020) and none has been done on the AA bioavailability of BSFL meal in growing swine feed.

Therefore, the global objectives of the studies conducted in this thesis are as follows:

1. To determine the SID coefficient of crude protein and AA for partially-defatted BSFL meal fed to growing pigs,
2. To quantify the relative metabolic availability of methionine from partially-defatted BSFL meal using the IAAO method for growing pigs, and

3. To compare the SID coefficient with the metabolic availability value of methionine in partially-defatted BSFL meal in growing pigs

The sub-objectives of the SID study (chapter 2) are to also compare and evaluate the digestibility coefficients obtained from AID and SID methodologies and to determine the ATTD coefficient of neutral detergent fiber (NDF), acid detergent fiber (ADF), and gross energy (GE) of BSFL meal. While the determination of both SID and metabolic availability from IAAO are single outcomes, we hypothesized that the SID coefficient of methionine obtained from the SID method will be greater compared to the metabolic availability of methionine obtained from the IAAO method in partially-defatted BSFL meal.

1.6 Table

Table 1.1: Comparison of analysed AA values of black soldier fly larvae (BSFL) meal, soybean meal (SBM), canola meal, and meat and bone meal (MBM) (%, as-fed).

Item	BSFL meal[1]	SBM[2]	Canola meal[2]	MBM[2]
Indispensable AA, %				
Arginine	2.32	3.45	2.28	3.53
Histidine	1.38	1.28	1.07	0.91
Isoleucine	1.90	2.14	1.42	1.47
Leucine	3.15	3.62	2.45	3.06
Lysine	2.64	2.96	2.07	2.59
Methionine	0.47	0.66	0.71	0.69
Phenylalanine	1.79	2.40	1.48	1.65
Threonine	1.26	1.86	1.55	1.63
Tryptophan	0.60	0.66	0.43	0.30
Valine	2.71	2.23	1.78	2.19
Dispensable AA, %				
Alanine	2.96	2.06	1.61	3.87
Aspartic Acid	3.97	5.41	2.56	3.74
Cystine	0.38	0.70	0.86	0.46
Glutamic Acid	5.64	8.54	6.35	6.09
Glycine	2.62	1.99	1.80	7.06
Proline	2.75	2.53	2.02	4.38
Serine	1.93	2.36	1.49	1.89
Tyrosine	2.84	1.59	1.06	1.08

[1] Enviroflight, Maysville, KY, USA
[2] NRC, 2012 (SBM – dehulled, solvent extracted; canola meal – solvent extracted)

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2 Chapter 2: Standardized ileal digestibility of crude protein and amino acids and apparent total tract digestibility of energy and fiber in partially-defatted black soldier fly larvae meal fed to growing pigs

2.1 Abstract

Black soldier fly larvae (BSFL) meal is one of the most attractive and promising protein alternatives in swine feed due to its favourable nutrient profile and sustainability attributes. As a novel protein ingredient, the protein quality of BSFL meal needs to be evaluated prior to incorporation in feed. One of the methodologies to evaluate protein quality is by determining the ileal digestibility of the ingredient's amino acids (AA). The objective of the present study was to determine the standardized ileal digestibility (SID, %) of CP and AA of partially defatted black soldier fly larvae (PD-BSFL) meal (49.7% CP; 14.4% crude fat, as fed). Subsequently, these results were compared to the SID AA coefficient and SID AA content of other common protein ingredients in swine feed. Additionally, sub-objectives of this study were to compare the digestibility coefficients from apparent ileal digestibility (AID) and SID and to determine the apparent total tract digestibility (ATTD) of NDF, ADF, and GE of the PD-BSFL meal. Six ileal-cannulated barrows (18.03 ± 0.67 kg BW) were used in a 2x2 Latin square design and fed either a nitrogen-free diet (NFD) or PD-BSFL-containing diet over two 11-d experimental periods. In each period, barrows were adapted for 7-d to the diet and ileal digesta collection was conducted on day 10 and 11 for 8h per day. Basal endogenous AA losses were calculated from pigs fed the NFD and as expected, the ileal digesta AA contents were greater ($P<0.05$) for pigs fed the BSFL diet than NFD. For indispensable AA, the SID of PD-BSFL were all above 83%, the highest being for Arg (93.0%) and followed by Met (90.4%). When compared to other common protein ingredients, the SID AA content of PD-BSFL were comparable to soybean and canola meal but lower compared

to fishmeal. The SID coefficient of indispensable AA of PD-BSFL were similar to that of full fat BSFL meal and overall higher than BSF prepupae. The SID AA values were higher than the AID as SID corrects for basal endogenous AA losses. The ATTD of NDF, ADF, and GE in PD-BSFL meal were found to be 45.25, 44.68, and 77.46%, respectively. These results suggest that PD-BSFL meal is a promising alternative protein ingredient in growing pig diets and our ingredient characteristics align closely with other protein ingredients.

2.2 Introduction

Global population growth, demographic changes, and rise in global income are the main determinants of increased food consumption and demand for agricultural commodities [Organization for Economic Co-Operation Development (OECD), 2021]. In the last decade, global pork production has increased by 36.8% and is predicted to increase by a total of 40% by 2050 (OECD, 2018; FAO 2011a). Additionally, by 2030, overall global meat consumption is projected to increase by 14% (OECD, 2021). However, due to the economic impact of the COVID-19 pandemic, the demand and international prices of meat products have temporarily declined in some regions (OECD, 2021). The OECD-FAO 2021 outlook projects that gross domestic product (GDP) will rebound by early 2022 and continue to grow at 2.9% rate over the next decade. Thus, the demand and consumption of pork and other animal products are expected to bounce back and increase in the coming years (OECD, 2021). The Food and Agriculture Organization (FAO) predicts that a 70% increase in global animal feed production is needed to fulfill the increased demand for animal protein consumption by 2050 (FAO, 2012a). To achieve this, feed production needs to increase in a sustainable manner, which can be achieved through incorporation of sustainable protein ingredients in livestock diets and precise feed formulation. The price of

frequently used protein ingredients (e.g. soybean meal and fish meal) in feed have doubled in the past five years and are expected to continue to increase in the future (Veldkamp and Bosch, 2015; Biasato et al., 2019). Additionally, soybean cultivation and the sourcing of wild fish for fish meal have become less environmentally sustainable in long term predictions (Carvalho, 1999; Foley et al., 2011; FAO, 2012b). Thus, an alternative feed protein ingredient is warranted.

Black soldier fly larvae (BSFL) meal is a promising protein ingredient alternative in swine feed because it is sustainable, rich in protein, has functional health benefits, and is palatable. The BSFL can be reared sustainably on organic waste, livestock manure, or other food waste; thus, providing a waste valorization strategy or a low carbon footprint method of waste disposal (Salomone et al., 2016; Spranghers et al., 2016; Meneguz et al., 2018). The nutrient profile, more specifically the AA profile, of BSFL meal is comparable to SBM, canola meal, and meat bone meal (Table 1.1; NRC, 2012; Enviroflight, Maysville, KY). Moreover, BSFL is rich in lauric acid and chitin, which have been shown to have functional antimicrobial activity (Skrivanova et al., 2006) and prebiotic properties (Selenius et al., 2018), respectively. Therefore, BSFL can also be considered as an alternative to in-feed antibiotic (Spranghers et al., 2018). In regards to acceptability and palatability, Newton et al. (1977) demonstrated that feed intake was greater for pigs that were fed BSFL meal-containing diet than the SBM diet with no additional fat. Further, average daily feed intake increased significantly with increasing BSFL meal inclusion in piglet diets, but this significant difference was not seen in weight gain, average daily gain, and FCR (Biasato et al., 2019). Thus, BSFL meal is a promising novel protein ingredient that could complement other currently-used protein ingredients and expand options for feed manufacturers.

The standardized ileal digestibility (SID) method is a common and gold standard method to measure disappearance of AA in the small intestine, as a proxy for digestibility and

bioavailability of novel protein ingredients, prior to its incorporation in animal feed (Stein et al., 2007). Evaluation of protein quality in novel ingredients is important to prevent under or over estimation of the ingredient's bioavailable AA composition; thus, improving the precision of feed formulation (Pomar et al., 2009; Adeola et al., 2016). The SID method has been used to determine digestibility of multiple feed ingredients used in swine (Stein et al., 2001; Rho et al., 2017; Petersen et al., 2014) and poultry feeds (Leung and Kiarie, 2020; Mwaniki and Kiarie, 2018; Adedokun et al., 2008; Szczurek, 2009). The method involves ileal-cannulated pigs that are usually fed a nitrogen-free diet to measure basal endogenous AA losses (EAAL) and a test diet that has the ingredient of interest as the only source of protein. Though considered to be an invasive approach, AA SID values are widely used in swine feed formulation due to the additivity among ingredients (NRC, 2012) and accepted to predict AA bioavailability, which is a more accurate determinant of protein quality (Mosenthin et al., 2000; Moehn et al., 2005). The AID AA value was also calculated as it is a similar method to SID; however, the basal EAAL is not being accounted for and AID values are not additive in a mixture of feed ingredients (Stein et al., 2007). Another digestibility method in this study is the ATTD. Apparent total tract digestibility (ATTD) method is the simplest digestibility method to conduct, it involves fecal collection and subtraction of the amount of nutrient intake to the amount of nutrient recovered in the feces (Stein et al., 2007). However, ATTD assumes that digestion occurs in all parts of the GIT, which is not accurate as most if not all of digestion happens in the small intestine (Stein et al., 2007). There is currently limited available literature that evaluates the ATTD of energy, fiber, and the SID of AA in BSFL meal, more specifically partially-defatted BSFL (PD-BSFL), in pig diets. Therefore, the objective of this study is to determine the SID values of CP and AA of PD-BSFL meal in growing pig diets. Additionally, the ATTD coefficient of neutral detergent fiber (NDF), acid detergent fiber (ADF), and gross

energy (GE) of the PD-BSFL meal will also be determined. To truly measure the protein quality of PD-BSFL meal, DIAAS value was further calculated using the SID coefficient. DIAAS is more applicable as reference for human nutrition and determination of ileal digestibility coefficient in humans is challenging and the procedure is considered invasive. Fortunately, pigs are excellent model to determine the AA metabolism in humans as both pigs and humans are monogastric and omnivorous species that share similar physiology, GIT, and metabolism capacity (Miller and Ullrey, 1987; Deglaire and Moughan, 2012; Roura et al., 2016).

2.3 Materials and methods

The experimental protocol and study design were reviewed and approved by the University of Guelph Animal Care Committee (AUP# 4439). Handling and caring for the animals were in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009).

2.3.1 Animals, housing, and ileal cannulation surgery

Six healthy Yorkshire barrows with an average initial body weight (BW) of 18.03 kg ± 0.34 were obtained from the Arkell Swine Research Station in Guelph, Ontario. During the first seven-day adaptation period, the barrows were pair-housed in plexi-glass pens with tenderfoot flooring, in a temperature-controlled room (23 - 24^{o}C) at the Department of Animal Biosciences, University of Guelph. They were provided with ad libitum access to commercial diet [FFM#1 Sew Pig Starter Ration (Floradale Feed Mill Limited, Floradale, ON)] and water, and the environment was enriched with age-appropriate toys (e.g. chains, rubber balls). The commercial diet consisted of a minimum of 22.9% CP, 4.6% crude fat, and a maximum of 1.4% crude fiber. After the adaptation period, all pigs were then fasted for approximately 15 hours prior to the ileal cannulation

surgery. A simple T-cannula was surgically fitted at the distal ileum following the procedure described in Sauer et al. (1983).

A six-day recovery period followed after the surgery. During this time, pigs were housed individually in plexi-glass pens adjacent to one another to allow nose-to-nose contact, where they received the commercial diet, Pig Starter No.3 Ration (Floradale Feed Mill Limited, Floradale, ON), ad libitum water, and supplemental heat for recovery. The surgical site was cleaned twice daily with warm water and zinc oxide cream was applied once the site was dry to eliminate skin irritation. A health monitoring checklist was used to record the recovery progress of each pig and to observe if there were any health concerns post-surgery that required veterinary attention.

2.3.2 Dietary formulation and feeding

Partially-defatted BSFL (PD-BSFL) meal was supplied for the study by Enviroflight, LLC (1118 Progress Way, Maysville, KY, USA). Enviroflight provided the complete proximate nutrient, AA, and fatty acid analysis of the BSFL meal (Table 2.1) following these methods. Briefly, the analysis of crude protein, crude fat (acid hydrolysis), crude fiber, and mineral content followed the AOAC 990.03 (2006), AOAC 954.02 (1977), AOCS Ba 6a-05, and AOAC 985.01 (1996) method, respectively. The AA and fatty acid analysis followed the AOAC 994.12 (2005) and AOAC 996.06 (1996) method, respectively. The BSFL was reared on a formulated diet with AAFCO-defined pre-consumer by-products to ensure consistent nutritional value. Oil from the BSFL meal was removed by mechanical pressing (personal communication with Enviroflight).

The SID study design was a replicated 2 × 2 Latin square design, where all six barrows were randomly assigned to one of two dietary treatments in each period. There were two 11-day experimental periods, the first seven days were the diet adaptation, followed by fecal collection on days eight and nine, and ileal digesta collection on days 10 and 11. To determine daily feeding

allowance, pigs' weight was extrapolated from initial BW right before the cannulation surgery as frequent weighing was avoided as it could lead to increased risk of a dislodged cannula. The daily ration was offered as a wet mash with feed-to-water ratio of 1:2 in two equal meals at 0830 and 1630 h. Total energy intake per day or daily feed allowance (kcal) was calculated based on 2.8 × maintenance digestible energy (DE) requirement, where the maintenance DE was 197 × (measured BW)$^{0.6}$ (NRC, 2012). All pigs finished their meals and always had access to ad libitum water throughout all experimental periods.

Dietary treatments in this study were nitrogen-free diet (NFD) and BSFL-containing test diets. The purpose of the NFD was to quantify the basal endogenous AA losses (EAAL) and it was formulated according to Rho et al. (2017). The BSFL-containing test diet had 36.5% of PD-BSFL meal added as the sole source of AA, at the expense of corn starch, sucrose, and corn oil (Table 2.2). Proximate nutrient and AA composition of the two experimental diets were analyzed (Table 2.3) and the methodologies used can be found in the following section. Additionally, titanium dioxide was added at 0.20% to both diets as an indigestible marker to quantify nutrient digestibility (Kiarie et al., 2016).

2.3.3 Ileal digesta collection, fecal collection, processing, and sample analysis

Fecal samples were collected through grab sampling from pen floors throughout days eight and nine of each experimental period. Collected fecal samples were then stored at -20^{o}C until further analysis. On days 10 and 11 of each experimental period, ileal digesta was collected from each pig for a continuous eight-hour duration per day (0830 to 1630). A plastic bag that was pre-filled with 10 ml of 10% formic acid, to inhibit microbial activity and growth, was attached to the cannula opening with an elastic band. Inhibition of microbial growth is important as microbes can lead to synthesis of de novo proteins or further breakdown of dietary AAs, which can

underestimate or overestimate the AA content of the digesta, respectively (Zebrowska and Buraczewski, 1998). The bags were checked periodically throughout the collection day to ensure that they were not dislodged from the cannula and were replaced when full. The collected digesta was then placed in a 4^{o}C fridge until the end of 8-hour collection period on both days. Digesta samples were pooled per pig per period and divided into several aliquots (approx. 250 grams) in aluminum tins. These tins were then stored in -20^{o}C freezer until further analysis.

Prior to chemical analysis, ileal digesta and fecal samples were freeze dried, finely ground, and mixed thoroughly. Experimental diets, ileal digesta, and fecal samples were all analyzed for dry matter (DM), crude protein and titanium dioxide contents. The DM analysis followed AOAC method 930.15 (2005), with slight modifications depending on the type of samples. Six to eight grams of feed samples were dried in the oven at 135^{o}C for 3 hours. Meanwhile, two grams of fecal and digesta samples were dried in the oven at 135^{o}C for 2 hours. Nitrogen content of samples was determined using a LECO analyzer (LECO Corporation, St. Joseph, MO), which adopts the combustion method (Method 968.06; AOAC, 2005). Next, crude protein was calculated by multiplying the nitrogen content by a factor of 6.25. Titanium dioxide content was analyzed following Myers et al. (2004) method with minor adjustments. Specifically, anhydrous Na_2SO_4 was added to each ash sample instead of the catalyst K_2SO_4 and $CuSO_4$ to the digestion tubes. Samples were then digested with H_2SO_4 at 120^{o}C for at least 20 hours instead of at 420^{o}C for 2 hours. The addition of 30% H_2O_2 was done after samples were settled, diluted, and pipetted to the 96-well plate, instead of added in the digestion tubes before sample settling and water addition. Lastly, titanium concentration was determined through absorbance reading using a UV spectrophotometer at 410 nm (Myers et al., 2004).

Feed, ileal digesta, and the BSFL meal samples were also analyzed for AA contents following the acid hydrolysis and performic acid oxidation with acid hydrolysis – sodium metabisulfite method (Method 994.12; AOAC, 2005), prior to being analyzed using ultra-performance liquid chromatography (UPLC). Gross energy, NDF and ADF contents were determined for fecal, experimental diets, and BSFL meal. Gross energy was determined using a bomb calorimeter (IKA Calorimeter System C 5000; IKA Works Inc., Wilmington, NC). The NDF and ADF analysis followed the filter bag technique (ANKOM A200 and A200I with 650 rpm agitation) and the two procedures were performed in sequence. NDF analysis was done first, then the same filter bags were used for the ADF analysis. Only the BSFL meal sample was fat extracted using acetone as the fat content was more than 5%. Furthermore, feed and BSFL meal were also analyzed for crude fiber, crude fat, macro, and microminerals by SGS Canada Inc (Guelph, Ontario). The crude fiber analysis followed the fritted glass crucible (Method 978.10; AOAC, 1996). Crude fat analysis followed the petroleum ether extraction method (Method 2 01-30-09; ANKOM Technology 2009). Lastly, the mineral analysis followed the atomic absorption spectrophotometric method (Method 968.08; AOAC 1969).

2.3.4 Calculations

Prior to calculating SID coefficients, basal EAAL (g/kg DMI) was first calculated according to Stein et al. (2007):

$$(1)\ \text{Basal EAAL} = \text{AA}_{\text{digesta}} \times \frac{\text{M}_{\text{feed}}}{\text{M}_{\text{digesta}}}$$

where $AA_{digesta}$ (g/kg DM) was the concentration of AA in the ileal digesta after being fed NFD and M_{feed} and $M_{digesta}$ (g/kg DM) were the concentrations of titanium dioxide marker in the NFD and ileal digesta, respectively (Stein et al., 2007).

The AID and SID coefficients were calculated according to equations 2 and 3, respectively:

$$(2)\ \text{AID}\ (\%) = (1 - \left(\frac{\text{AA}_{\text{digesta}}}{\text{AA}_{\text{feed}}} \times \frac{\text{M}_{\text{feed}}}{\text{M}_{\text{digesta}}}\right)) \times 100$$

$$(3)\ \text{SID}\ (\%) = \left((1 - \left(\frac{\text{AA}_{\text{digesta}}}{\text{AA}_{\text{feed}}} \times \frac{\text{M}_{\text{feed}}}{\text{M}_{\text{digesta}}}\right) \times 100) + \frac{\text{basal EAAL}}{\text{AA}_{\text{feed}}}\right) \times 100$$

where $AA_{digesta}$ and AA_{feed} (g/kg DM) were the AA concentrations in ileal digesta after being fed test diet and AA concentration in the test diet, respectively, M_{feed} and $M_{digesta}$ (g/kg DM) were the titanium dioxide marker contents in test diet and ileal digesta, respectively, and basal EAAL as defined by equation 1 (Stein et al., 2007)

Digestible indispensable amino acid score (DIAAS) for the PD-BSFL meal was calculated following the equations below (Marinangeli and House, 2017; Herreman et al., 2020) and using the reference AA scoring pattern for child age group (6 months to 3 year; FAO, 2011b):

$$(4)\ \text{DIAA}_x\ \text{ratio} = \frac{\text{IAA}_x \times \text{SID}_x}{\text{Reference pattern score IAA}_x}$$

where x represents individual indispensable AA, total sulfur AA (Met + Cys) and total aromatic AA (Phe + Tyr); IAA_x is the indispensable AA concentration in the PD-BSFL meal in mg/g CP unit, and SID_x is the SID coefficient of each indispensable AA, average SID coefficient of Met +

Cys and average SID coefficient of Phe + Tyr. Reference pattern score IAA_x (mg/g protein requirement) of growing child was used to reflect the growing pigs used in this study.

The DIAAS was calculated only based on the lowest DIAAx ratio obtained in equation (4):

(5) DIAAS = 100 x lowest DIAA ratio among all indispensable AA

The ATTD of NDF and ADF were calculated following the index method (Adeola, 2001).

(6) Digestibility (%) = $100 - [100 \times \left(\frac{\text{TiO}_2 \text{ concentration in feed} \times \text{A in feces}}{\text{TiO}_2 \text{ concentration in feces} \times \text{A in feed}}\right)]$

where A is the concentration of the test component (i.e. NDF or ADF)

The ATTD GE was calculated following the difference (indirect) method using the index method for sample collection as explained in Adeola (2001):

(7) ATTD GE (%) = $100 \times \left[\frac{(\text{T} \times \text{T}_\text{p}) - (\text{B} \times \text{B}_\text{p})}{\text{A}_\text{p}}\right]$

where T = digestibility of GE in the test diet (NFD plus the test feedstuff); B = digestibility of GE in the NFD; Bp = is the proportion (%) of GE in the test diet contributed by the NFD; Ap = the proportion (%) of GE in the test diet contributed by the test feedstuff; Tp = Bp + Ap = 100%; B and T were calculated following the digestibility equation (4).

The digestible energy of the diet and the PD-BSFL meal were calculated following the equation:

(8) Digestible energy (DE; kcal/kg) = GE sample x digestibility

where GE sample of the diet or the PD-BSFL meal were obtained from the bomb calorimeter (kcal/kg) and the digestibility (%) was calculated following equation (4).

2.4 Results and discussion

All pigs recovered well from the surgery, remained healthy throughout the entire study period, and consumed all meals during the feed adaptation and sample collection periods.

Nutrient analysis of the PD-BSFL meal was provided by Enviroflight (Maysville, KY, USA) and presented in Table 2.1. The CP of the PD-BSFL meal (49.3%, as-fed) was comparable to other published CP BSFL meal values (Matin et al., 2021; Crosbie et al., 2020; Renna et al., 2017). Additionally, the crude fat (15.1%, DM) and gross energy (5318 kcal/ kg, DM) were in close agreement with other published values for PD-BSFL meal (Matin et al., 2021; Renna et al., 2017). Meanwhile, the AA content was variable when compared to other published BSFL meal values. This is potentially due to the difference in the diet or rearing substrate for the larvae (i.e. variable protein: fat: carbohydrate ratios; Gligorescu et al., 2018), harvesting age (Do et al., 2020), analysis method, and other physiological attributes that might contribute to nutrient variations (Koutsos et al., 2019). The NDF value of the PD-BSFL meal used in this study was higher (32.9%, DM) compared to the other NDF values for defatted (14.2%, DM; Crosbie et al., 2020), partially-defatted (21.2 and 20.3%, DM; Matin et al., 2021; Biasato et al., 2019), or full fat BSFL meal (16.2%, DM; Crosbie et al., 2020). However, the NDF value is similar to a PD-BSFL meal (31.8%, DM) in a study by Kortelainen et al., (2014).

The ingredient composition and analysed nutrient composition of the NFD and test diet are presented in tables 2.2 and 2.3, respectively. The CP level of NFD and test diet were 0.00 and 18.51% respectively, which demonstrates that the NFD had 0% nitrogen, as expected.

Additionally, the indispensable and dispensable AA contents in the NFD were negligible when compared to the BSFL meal-containing test diet.

2.4.1 AID, SID, and DIAAS

The AID and SID results of the PD-BSFL meal are presented in table 2.4. SID values for CP and all AAs are overall 6-7% higher compared to the AID, except for threonine, cysteine, proline, and glycine (Table 2.4). The SID values of those AAs are 10-20% higher than the AID, which indicates that those AAs can be found at a higher level in the basal EAAL compared to other AAs. High concentrations of proline and glycine in basal EAAL have been reported in pigs fed NFD as feeding a nitrogen-deprived diet leads to catabolic state or a negative nitrogen balance (Jansman et al., 2002; de Lange et al., 1989). The animals will then catabolize primarily muscle body protein to supply AA for crucial metabolic functions and this breakdown leads to higher alanine and glutamine concentration (Rodwell, 1988). Most of the glutamine reaches the tissue of the GIT, and it is further metabolized to glutamate, ammonia, proline, and glycine into the lumen of the gut (Rodwell, 1988; Rogers and Phang, 1985). Thus, it is expected that, after correcting for basal EAAL, the SID of proline and glycine will be much greater than the AID values.

The SID coefficients for all indispensable AA of PD-BSFL meal were above 83%, with arginine and methionine having the highest SID value of 93.0 and 90.4%, respectively, and threonine having the lowest SID of 83.0%. For the dispensable AAs, the two lowest SID values were for cysteine (79.2%) and glycine (80.7%), while the highest were for proline (93.5%) and tyrosine (89.1%). The SID results of the PD-BSL meal for the crude protein, indispensable, and dispensable AAs are similar but overall lower when compared to the FF BSFL SID values determined previously (Crosbie et al., 2020). This could be due to the higher fat content in the FF BSFL meal (crude fat 32.5%, as fed), as higher fat tends to slow down gastric emptying; thus,

allowing more time for the proteolytic enzymes to breakdown dietary protein (Gentilcore et al., 2006). The SID coefficients of indispensable and dispensable AAs of the PD-BSFL meal are overall greater than BSF prepupae meal, which was also quantified using growing pigs (Tan et al., 2020). A possible explanation for this is that prepupae contains higher chitin content compared to the larval stage (Smets et al., 2020; Soetemans et al., 2020). Chitin, a non-protein nitrogen component, is considered an insoluble fiber as it shares structural similarity with cellulose (Finke, 2007). Furthermore, the presence of nitrogen has been detected in the insect-derived ADF fraction (Barker et al., 1998), which further confirms that fiber in insects is made up of mainly chitin. Inclusion of insoluble fiber has been shown to decrease the ileal digestibility of histidine, lysine, and tryptophan in a study with growing pigs (Wang et al., 2006). Additionally, another study also reported that the SID of histidine, lysine, serine, threonine, and tyrosine decreased as the level of fiber in the diet increased (Chen et al., 2015). Therefore, digestibility of some AAs decreases linearly with increasing concentrations of insoluble fiber.

The SID results of the PD-BSFL meal as determined in the current study were generally comparable to the commonly used feed protein ingredient, SBM, and overall higher compared to canola meal and fish meal (NRC, 2012; Table 2.5). The SID values of arginine, histidine, isoleucine, lysine, threonine, valine, and all dispensable AAs were slightly lower for PD-BSFL meal compared to SBM. When compared to canola meal, the SID of all indispensable and dispensable AAs are greater in PD-BSFL. Meanwhile, the SID values of all AAs are between 2-15% higher in PD-BSFL meal than fish meal. The SID coefficients of these ingredients were then further calculated by multiplying them to their corresponding AA concentration to produce SID AA content (%; Table 2.6). Standardized ileal digestible content is more useful for direct application by nutritionists for feed formulation. Fish meal has higher SID AA and CP content

compared to PD-BSFL meal, even though its SID coefficients are lower, because the AA concentration in fish meal is overall higher than that of PD-BSFL meal. The SID AA content of PD-BSFL meal is comparable but lower than SBM, while the trend is higher compared to canola meal (Table 2.6). This suggests that BSFL is a promising protein alternative to complement other protein ingredients in swine diet as its AA digestibility coefficient is comparable, if not higher, than the frequently-used protein rich ingredients. However, the cost effectiveness of incorporating BSFL in animal feed needs to be considered as well. BSFL meal has been reported to be competitive in price with fish meal, but not with other plant protein ingredients (i.e. SBM or canola meal (Veldkamp and Bosch, 2015). Fortunately, as the insect industry grows worldwide, the upscaling of production and optimization in processing will help insect meal prices to become more competitive with other protein ingredients in the future (Veldkamp and Bosch, 2015).

The SID coefficients of the indispensable AAs were then used to calculate DIAAS to express dietary protein quality of the PD-BSFL meal. The FAO classifies protein quality claims based on DIAAS value to be no protein quality claim (DIAAS < 75), high quality protein (DIAAS 75-99), and excellent quality protein (DIAAS ≥ 100; reviewed in Herreman et al., 2020). Based on equation (4) calculation, DIAA ratio was the lowest for total sulphur AA at 0.54, followed by tryptophan at 0.68, and lysine at 0.82. Therefore, when expressed as DIAAS using the reference AA pattern of growing children (6 months to 3 years), the dietary protein quality of PD-BSFL meal was found to be 54%, which means that protein quality claim cannot be made for this ingredient. As previously discussed, DIAAS is more commonly used in human nutrition due to dietary habits and abundant food options (Mansilla et al., 2020). Thus, the no protein quality claim based on DIAAS should not hinder the utilization of PD-BSFL meal in animal feed, but further investigation might be warranted for use in human food.

2.4.2 ATTD NDF, ADF, and GE

The apparent total tract digestibility (ATTD) results for GE, NDF, and ADF of PD-BSFL meal are presented in table 2.7. The GE ATTD for PD-BSFL meal was 77.46%, which is in agreement with the DF- and FF-BSFL meal (Crosbie et al., 2020). However, a limitation of calculating the ATTD GE using the difference (indirect) approach is that other energy-supplying feed ingredients in the diet are assumed to not interact with each other, which could alter the digestibility value (Adeola, 2001). The NDF and ADF ATTD values for the PD-BSFL meal were 45.25 and 44.68%, respectively, which were lower when compared to the DF- (NDF: 76.1%, ADF: 69.5%) and FF- (NDF: 86.1%, ADF: 91.0%) BSFL meal ATTD values (Table 2.7; Crosbie et al., 2020). A potential explanation to this is that the PD-BSFL meal in the current study has significantly greater NDF content, 31.3% (as-fed), compared to only 13.3 and 14.3% (as-fed) in the DF- and FF-BSFL meal, respectively (Crosbie et al., 2020). It has been shown that increased concentration of insoluble fiber in the diet resulted in lower digestibility of the fiber fraction (Hogberg and Lindberg, 2004; Degen et al., 2009). This was further confirmed with similar ATTD NDF ADF values found in conventional DDGS (ATTD NDF: 39.3%, ATTD ADF: 57.6%; Rho et al., 2017), which also had a higher NDF value of 28.9 % (as-fed). However, the digestibility of fiber is also dependent on multiple factors such as the physicochemical property of the fiber fraction and the animal's capacity to ferment fiber in the hind gut (Bach-Knudsen, 2001). Therefore, more investigation is warranted on the factors that could affect the total tract digestibility of fiber, which refers to chitin in the case of BSFL meal, in growing pigs.

2.5 Conclusions and implications

Results from this study suggest that PD-BSFL meal is an attractive and promising protein ingredient alternative in swine feed. It has high AA digestibility values that are comparable to

SBM, and higher than fish meal and canola meal; all three are frequently used in commercial animal feed. By providing the SID AA values of PD-BSFL meal, feed formulators can confidently use these values to formulate a more precise feed that will meet the nutrient requirements of the animal. Thus, over- or under-formulation can be prevented and PD-BSFL meal will more likely be incorporated in feed as their digestibility values are readily available. As previously mentioned, the usage of BSFL meal in animal feed will also rely heavily on its competitiveness in market price with other commonly-used protein ingredients. Thus, BSFL meal is more likely to be combined with other protein ingredients, instead of full replacement, in the meantime. The DIAAS value of PD-BSFL meal was also calculated and found to have no protein quality claim when growing child AA pattern was used as a reference. However, as previously discussed, DIAAS is a more valuable measure for human and not necessarily for animal nutrition.

2.6 Acknowledgements

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2.7 Tables

Table 2.1: Analyzed nutrient composition (%, as fed) of partially-defatted black soldier fly larvae (PD-BSFL) meal.

Item	PD-BSFL meal[1]
Dry matter, %	95.05
Crude protein, %	49.30
Gross energy, kcal/ kg	5057
Digestible energy, kcal/ kg	3590
Crude fat, %	14.40
Total starch, %	5.85
Crude fiber, %	6.40
Neutral detergent fiber (NDF), %	31.30
Acid detergent fiber (ADF), %	10.90
Ash, %	8.33
Calcium, %	1.67
Total phosphorus, %	1.10
Sodium, %	0.18
Potassium, %	1.49
Magnesium, %	0.39
Iron, ppm	205
Manganese, ppm	123
Copper, ppm	11.2
Zinc, ppm	106
Indispensable AA, %	
Arginine	2.32
Histidine	1.38
Isoleucine	1.90
Leucine	3.15
Lysine	2.64
Methionine	0.47

Phenylalanine	1.79
Threonine	1.26
Tryptophan	0.60
Valine	2.71
Dispensable AA, %	
Alanine	2.96
Aspartic Acid	3.97
Cystine	0.38
Glutamic Acid	5.64
Glycine	2.62
Proline	2.75
Serine	1.93
Fatty Acids, g/100 g	
Lauric (C12:0)	4.88
Arachidonic (C20:4)	<0.01
Eicosapentaenoic (C20:5)	<0.01
Docosahexaenoic (C22:6)	<0.01
Omega 3 fatty acids (total)	0.19
Omega 6 fatty acids (total)	2.59

[1]Analysed by Enviroflight, Maysville, KY, USA

Table 2.2: Ingredient composition (%, as-fed basis) of the nitrogen free diet (NFD) and BSFL meal-containing test diet

Ingredient Composition (%, as fed)	NFD	Test Diet
Partially defatted BSFL meal	-	36.50
Corn starch	84.80	51.52
Sucrose	6.17	3.75
Corn oil	2.06	1.25
Cellulose	2.06	2.06
Limestone	0.80	0.80
Monocalcium phosphate	2.30	2.30
NaCl	0.50	0.50
K_2CO_3	0.40	0.40
MgO	0.14	0.14
Vitamin and mineral premix[1]	0.60	0.60
Titanium dioxide	0.20	0.20

[1]Provided per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1,200 IU as cholecalciferol; vitamin E, 48 IU as dl-alpha-tocopherol acetate; vitamin K, 3 mg as menadione; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; vitamin B12, 0.03 mg; biotin, 0.24 mg; Cu, 18 mg from CuSO4×5H2O; Fe, 120 mg from FeSO4; Mn, 24 mg from MnSO4; Zn, 126 mg from ZnO; Se, 0.36 mg from Na2SeO3; and I, 0.6 mg from KI (DSM Nutritional Products Canada Inc., Ayr, ON, Canada).

Table 2.3: Analyzed nutrient composition (as-fed basis) of the nitrogen free diet (NFD) and BSFL meal-containing test diet.

Item	NFD	Test Diet
Proximate Analysis		
Dry matter, %	89.74	92.37
Gross energy, kcal/kg	3650	4142
Digestible energy, kcal/kg[1]	3025	3182
Crude protein, %	0.00	18.51
Crude fat, %	1.16	5.70
Crude fiber, %	1.90	4.96
NDF, %	2.12	11.80
ADF, %	1.51	4.18
Ash, %	3.64	6.89
Calcium, %	0.64	1.49
Phosphorus, %	0.41	0.88
Sodium, %	0.25	0.32
Potassium, %	0.20	0.32
Indispensable AA, %		
Arginine	0.01	0.79
Histidine	0.00	0.49
Isoleucine	0.01	0.60
Leucine	0.06	1.17
Lysine	0.01	0.94
Methionine	0.00	0.29
Phenylalanine	0.02	0.68
Threonine	0.01	0.68
Valine	0.02	0.88
Dispensable AA, %		
Alanine	0.03	1.15
Aspartate	0.03	1.67
Cysteine	0.00	0.16

Glutamate	0.08	2.24
Glycine	0.02	0.90
Proline	0.00	1.09
Serine	0.02	0.79

[1] The digestible energy of both diets was calculated following equation (4) and (5)

Table 2.4: Apparent and standardized ileal digestibility (AID, SID, %) of CP, indispensable and dispensable AA in partially defatted BSFL meal fed to growing pigs (n = 6).

Item	AID, %	SID, %	SD
Crude Protein	71.0	76.1	6.2
Indispensable AA, %			
Arginine	85.9	93.0	2.4
Histidine	77.4	83.4	4.7
Isoleucine	79.5	86.0	2.6
Leucine	81.6	88.8	3.3
Lysine	81.7	87.6	3.7
Methionine	83.1	90.4	3.9
Phenylalanine	81.7	89.2	3.6
Threonine	72.9	83.0	5.8
Valine	79.5	86.2	3.1
Dispensable AA, %			
Alanine	78.3	84.6	3.8
Aspartate	76.4	84.3	5.4
Cysteine	59.3	79.2	7.0
Glutamate	81.1	87.3	3.7
Glycine	66.4	80.7	6.6
Proline	75.7	93.5	3.7
Serine	72.4	83.8	7.5
Tyrosine	83.5	89.1	4.3

Table 2.5: Standardized ileal digestibility (SID, %) of CP, indispensable, and dispensable AA in partially defatted BSFL meal fed to growing pigs compared to commonly used protein ingredients, soybean meal (SBM), canola meal, and fish meal (FM)

Item	PD-BSFL	SBM[1]	Canola Meal[1]	FM[1]
Crude Protein	76.1	87.0	74.0	85.0
Indispensable AA, %				
Arginine	93.0	94.0	85.0	86.0
Histidine	83.4	90.0	78.0	84.0
Isoleucine	86.0	89.0	76.0	83.0
Leucine	88.8	88.0	78.0	83.0
Lysine	87.6	89.0	74.0	86.0
Methionine	90.4	90.0	85.0	87.0
Phenylalanine	89.2	88.0	77.0	82.0
Threonine	83.0	85.0	70.0	81.0
Valine	86.2	87.0	74.0	83.0
Dispensable AA, %				
Alanine	84.6	85.0	77.0	80.0
Aspartate	84.3	87.0	76.0	73.0
Cysteine	79.2	84.0	74.0	64.0
Glutamate	87.3	89.0	84.0	80.0
Glycine	80.7	84.0	78.0	75.0
Proline	93.5	113.0	92.0	86.0
Serine	83.8	89.0	75.0	75.0
Tyrosine	89.1	88.0	77.0	74.0

[1]SID for SBM – dehulled, solvent extracted; canola meal – solvent extracted; and FM – combined from different species (not listed) were obtained from the NRC 2012

Table 2.6: Standardized ileal digestibility content (SID Content, %) of CP, indispensable, and dispensable AA in partially defatted BSFL meal fed to growing pigs compared to commonly used protein ingredients, soybean meal (SBM), canola meal, and fish meal (FM)

Item	PD-BSFL	SBM[1]	Canola Meal[1]	FM[1]
Crude Protein	37.52	41.53	27.75	53.79
Indispensable AA, %				
Arginine	2.16	3.24	1.94	3.30
Histidine	1.15	1.15	0.83	1.21
Isoleucine	1.63	1.90	1.08	2.12
Leucine	2.80	3.19	1.91	3.71
Lysine	2.31	2.63	1.53	3.92
Methionine	0.42	0.59	0.60	1.51
Phenylalanine	1.60	2.11	1.14	2.03
Threonine	1.05	1.58	1.09	2.09
Valine	2.34	1.94	1.32	2.54
Dispensable AA, %				
Alanine	2.50	1.75	1.24	3.14
Aspartate	3.35	4.71	1.95	3.95
Cysteine	0.30	0.59	0.64	0.39
Glutamate	4.92	7.60	5.33	6.30
Glycine	2.11	1.67	1.40	3.53
Proline	2.57	2.86	1.86	2.49
Serine	1.62	2.10	1.12	1.82
Tyrosine	2.53	1.40	0.82	1.39

[1]SID content for SBM – dehulled, solvent extracted; canola meal – solvent extracted; and FM – combined from different species (not listed) were obtained from the NRC 2012

Table 2.7: Apparent total tract digestibility (ATTD, %) of NDF, ADF, and GE in partially defatted BSFL meal fed to growing pigs compared to defatted and full fat BSFL meal

	Partially-defatted BSFL meal		Defatted BSFL Meal (Crosbie et al., 2020)	Full Fat BSFL Meal (Crosbie et al., 2020)
	Mean	SD		
ATTD, %				
NDF	45.25	3.09	76.1	86.1
ADF	44.68	1.83	69.5	91.0
GE	77.46	7.62	74.6	76.0

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3 Chapter 3: Determination of the metabolic availability of methionine in black soldier fly larvae meal using the indicator amino acid oxidation (IAAO) method in growing pigs

3.1 Abstract

While ileal digestibility provides an estimate of amino acid (AA) bioavailability, the only true measurement of AA available for protein synthesis is metabolic availability (MA) measure. The objective of the present study was to determine the MA of methionine in black soldier fly larvae (BSFL) meal, an emerging alternative feed protein ingredient, using the indicator amino acid oxidation (IAAO) method. Eight Yorkshire barrows (18.77 ± 0.69 kg) were used in a 7×7 incomplete Latin square design. Each pig was randomly assigned to one of seven dietary treatments over seven 3-d experimental periods, where the first two days were diet adaptation and the last day was breath collection. Two diet types were used, reference and BSFL test diets, each supplying three graded intakes of methionine: 55, 65, and 75% of the estimated SID methionine requirement (NRC, 2012) and a basal diet, which was a common first point and supplied methionine at 45% of the estimated SID methionine requirement. DL-Methionine (DL-Met) was the sole source of methionine for the reference and basal diet, while a combination of BSFL meal and DL-Met was the source for the test diet. Indicator amino acid oxidation studies were performed where L-[1-13C]-Phenylalanine was provided in meals every 25-min and expired CO_2 was collected. The relative MA of methionine was calculated by comparing the slope of IAAO response following intake of graded methionine in BSFL test diets with slope of IAAO response to reference diet intake. The relative MA of methionine in BSFL meal was found to be 53.33% (APE) or 33.35% ($F^{13}CO_2$). The difference between the two MA values could potentially be due to the fluctuations in humidity during breath collection day and the rapid growth of the pigs that

affected the volume CO_2 (VCO_2) analysis, which was needed for the $F^{13}CO_2$ calculation. However, more investigations on what might cause this are warranted as MA results have been reported to be highly variable.

3.2 Introduction

Black soldier fly larvae (BSFL) meal is an attractive alternative protein ingredient in swine feed because it is sustainable, high in protein, and has functional health benefits (Makkar et al., 2014; Van Huis et al., 2013; Spranghers et al., 2018). A sustainable ingredient supply chain is key to achieve a 70% increase in global animal feed production as food demand grows in the coming years (FAO, 2012). However, before being incorporated into feed or food, it is important to characterize the protein quality of this novel protein ingredient. Protein quality refers to the ability of AA profile of a food source or a mixture of sources to fulfill the AA requirement of an individual (Nosworthy and House, 2017), which largely requires determining the bioavailability of the limiting AA. Three of the most predominant indispensable AAs in BSFL meal are leucine, valine, and lysine, while methionine is the first limiting AA (Enviroflight, Maysville, KY, USA; Yu et al., 2019; Crosbie et al., 2020). Methionine is also the second or third limiting AA in a commercial corn-soybean based swine diets (Moehn et al., 2008; NRC, 2012). Therefore, evaluation of the MA of methionine in BSFL meal is needed to better understand the amount of methionine that is actually available for the animals to use for protein synthesis or other metabolism purposes.

Application of the slope-ratio assay principle using the indicator amino acid oxidation (IAAO) method is an accurate and rapid approach to determine the MA of AA in feed ingredients for animals (Moehn et al., 2005; Moehn et al., 2007; Levesque et al., 2011) and humans (Rafii et al., 2020; Humayun et al., 2007; Prolla et al., 2013). The principle of the IAAO method to assess

bioavailability is that changes in the rate of the indicator AA oxidation can be observed as the AA of interest (i.e. test AA) becomes more or less bioavailable for protein synthesis (Moehn et al., 2005). Decreased oxidation of the indicator AA and increased protein synthesis occur as intake of the limiting AA increases (Holt et al., 1962; Pencharz and Ball, 2003). Thus, the change in the rate of oxidation of the indicator AA is inversely proportional to protein synthesis.

To determine the MA of AA using the IAAO method, some assumptions needed to be made. First, the test AA must be the first limiting AA in all diets. The purpose is to ensure that an increase in the test AA intake alone would drive changes in the oxidation of the indicator AA. Second, to achieve a linear response in the slope-ratio assay, the test AA must be supplied below the requirement (Littell et al., 1995). Third, the calculated MA is a relative and not absolute bioavailability. Thus, the reference diet with crystalline AA is assumed to be 100% bioavailable (Chung and Baker, 1992).

The objective of the current study was to use the IAAO method to determine the relative MA of methionine in BSFL compared to a reference, semi-purified diet fed to growing pigs. It was hypothesized that the MA of methionine from BSFL meal will be lower than the MA of methionine from a crystalline AA diet.

3.3 Materials and methods

The experimental protocol and study design were reviewed and approved by the University of Guelph Animal Care Committee (AUP# 4516). Handling and caring for the animals were in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009).

3.3.1 Animals and housing

Eight Yorkshire barrows with an average initial body weight (BW) of 18.77 ± 0.69 kg were obtained from Arkell Research Station, Guelph, ON. Barrows were moved to the research facilities in the Department of Animal Biosciences, University of Guelph, and for seven days, were pair-housed, fed ad libitum commercial diet, Pig Starter No.3 Ration (Floradale Feed Mill Limited, Floradale, ON), had access to ad libitum water, and each floor pen had sufficient age-appropriate toys (e.g., chains, balls, ropes). Subsequently, pigs underwent respiratory chamber adaptation for six days, where they were adapted to rest calmly inside the chamber in incremental number of hours, until they were calm to stay for 8-h. During this chamber adaptation period, pigs were fed the basal diet (45% SID methionine requirement; NRC, 2012) in multiple small meals every 25-min to acclimate them to the feeding regime on breath collection day later. Throughout the chamber adaptation until the end of the experimental period, pigs were individually housed in a temperature-controlled room (23 - 24^{o}C), had nose-to-nose access to neighboring pigs, ad libitum water access, and age-appropriate toys for enrichment. The total duration of the study, not including the chamber adaptation period, was 22 days. At the end of the IAAO study, all pigs were euthanized by captive bolt pistol and severing of the carotid artery.

3.3.2 Diet formulation, feeding, and nutrient analysis

The study design was a 7×7 incomplete Latin square (n=8), where all eight barrows were randomly assigned to one of the seven dietary treatments in each period and to ensure that all treatments were represented on each calorimetry day. There were seven, 3-day experimental periods, the first two days were diet adaptation and the third day was breath collection. The next day following each breath collection day, pigs received another diet treatment, adapted again for two days before subsequent breath collection was conducted with no wash-out period in between.

A similar approach has been used previously in chickens (Tabiri et al., 2002), dogs (Templeman et al., 2019; Mansilla et al., 2020), and humans (Humayun et al., 2007; Rafii et al., 2020). Two days diet adaptation has been demonstrated to be sufficient, as there was no significant different in IAAO response following 2- or 10-days adaptation in pigs (Moehn et al., 2004). Feed intake was restricted to 95 g/kg $BW^{0.75}$ (Shoveller et al., 2010), pigs were fasted for approximately 16 hours prior to the breath collection day, and body weight was recorded in the morning of each collection day to determine the feed allocation on sampling day and daily feeding allowance for the following period. Daily ration on non-sampling days was offered as a wet mash with feed-to-water ratio of 1:2 in two equal meals at 0830 and 1630 h. Meanwhile, on breath collection days, the AM ration was divided into 13 small meals, offered every 25 minutes and the PM ration was provided at 1630 h. All pigs finished their meals on adaptation days and had access to ad libitum water throughout all experimental periods.

Partially defatted black soldier fly larvae (BSFL) meal (CP: 49.3%; crude fat = 14.40%; as-fed) was supplied by Enviroflight, LLC (1118 Progress Way, Maysville, KY, USA). Two diet types were tested: a reference diet (REF) utilizing crystalline AA that was considered to be 100% bioavailable and a test diet (BSFL) in which the AA source was a combination of BSFL meal and crystalline AA. Both diet types were corn starch-based, with no other protein or AA sources used other than the crystalline AAs or the BSFL meal (Table 3.1). Metabolic availability of methionine from BSFL meal was calculated using the slope ratio assay principle, where the slope of IAAO response (i.e. $F^{13}CO_2$ or APE) following intake of graded levels of methionine in BSFL meal was compared to the slope of IAAO response following intake of graded levels of methionine in the reference diet. A corn starch-based basal diet with 1.3 g/kg diet of methionine, representing 45% of the estimated SID methionine requirement, served as the common first point for both the

reference and test diet slopes. Crystalline AA was the only source of protein or AA in the basal diet. Three methionine intake levels of 1.6, 1.9, and 2.2 g/kg diet supplied only by DL-Met (as-fed, 6% moisture), representing 55, 65, and 75% of the estimated SID methionine requirement for 25 – 50 kg pig (NRC, 2012) were used to measure the IAAO response to construct the reference slope. To construct the BSFL test diet slope, the same three levels of methionine were studied. In the BSFL test diets, a fixed amount of 1.32% inclusion of DL-Met was kept to supply 45% of the SID methionine requirement. Black soldier fly larvae meal was then added at the expense of cornstarch, corn oil, cellulose, and combination of crystalline AAs to achieve either 55, 65, or 75% of estimated SID methionine requirement.

All diets were formulated to be isonitrogenous, isoenergetic, and to have equal crude fiber with crystalline alanine (L-Alanine), corn oil, and Solka-Floc (pure cellulose) adjustment, respectively. Methionine was the first limiting AA in all diet treatments, with the highest being 75% of the estimated SID methionine requirement, and all other AAs were provided in excess (110%) of the estimated SID requirement (NRC, 2012). The concentrations of cysteine, phenylalanine and tyrosine were kept constant in all diets. The supply of tyrosine and cysteine was specifically important to ensure that no ^{13}C-phenylalanine and no methionine were directed to meet the demand for tyrosine and cysteine, respectively (Shiman and Gray, 1998). Phenylalanine intake was kept constant among all diets to ensure that any changes in the L-[1-^{13}C]-Phenylalanine (tracer isotope) oxidation were due to changes in the methionine bioavailability from the graded level of BSFL meal inclusion.

Dietary treatments were analyzed for DM, crude protein, and ash, while other proximate nutrient and AA analysis were calculated from the formulation spreadsheet (Microsoft Excel). Dry matter analysis followed Method 930.15 (AOAC, 2005), while ash analysis followed

Method 942.05 (AOAC, 1943), with 12 hours in the muffle furnace at 600^{O}C. Nitrogen content of dietary treatments were determined following Method 968.06 (AOAC, 2005) using a LECO analyzer (LECO Corporation, St. Joseph, MO). Crude protein was then calculated by multiplying the nitrogen content by a factor of 6.25. Analyzed and calculated nutrient analysis of the dietary treatments are presented in Table 3.2.

3.3.3 Tracer protocol, breath collection, and analysis

On breath sample collection days, all pigs received repeated 25-min oral doses of the stable isotope L-[1-^{13}C]-Phenylalanine (99%; Cambridge Isotope Laboratories, Inc. Saint-Laurent, QC), over a 4 h period. Isotope solution was made by mixing the L-[1-^{13}C]-Phenylalanine crystal isotope with Mili-Q water (Type 1 Ultrapure Water Systems, EMD MiliporeSigma) to a concentration of 6 and 7 mg/ml for the constant and prime doses, respectively. The two concentrations were decided based on how much of each solution was needed per kg of pig, to prevent the addition of too much water into the small meals later on. The isotope solution was added right before feeding in the chamber and it was mixed thoroughly within each small meal starting at meal six. A constant dose of 2 mg/kg BW (Levesque et al., 2010) was given orally every 25-min from meal 6 – 13. A priming dose of 1.75 × constant dose (3.5 mg/kg BW) was given once along with the constant dose at meal six (Levesque et al., 2010). Table 3.3 depicts the oral isotope administration, feeding, and breath collection schedule for the study. All pigs finished their small meals before the collection of each corresponding breath samples and prior to the next meal.

Four open circuit respiration chambers (63.50 cm width, 149.86 cm depth, and 93.98 cm height) were used on each sample collection day. The chambers were constructed of glass with tenderfoot flooring and a feeding bowl that was screwed on the flooring in each chamber. The

dimensions of the chambers allowed the pigs to lie down, stand, and turn around. Two back-to-back breath collection days occurred per period, since there were eight pigs but only four chambers. A 10-min gas equilibration period occurred once the pigs entered the chambers, to allow air in the chamber and the ventilating air stream to equilibrate. Following that was three, 25-min respiration calorimetry measurements to determine resting volume CO_2 and O_2 produced (VCO_2; VO_2) when the pigs were calm, not actively moving, and still being fasted. Calorimetry data was collected using the Qubit calorimetry software (Customized Gas Exchange System and Software for Animal Respirometry, Qubit System Inc., Kingston, ON). Three small meals without isotope were then given every 10-min to induce fed-state in the pigs in the first 30 min. Small meals were given every 25-min thereafter, with the last meal being given on minute 365, just before the last breath sample was collected. Three, 25-min intervals of background breath samples were collected prior to the first dose of isotope in meal six (Table 3.3). These background samples represent the amount of naturally-existing $^{13}CO_2$ in the exhaled breath that are required to calculate atom percent excess (APE).

The first breath sample in each sampling day was collected after the three 10-min interval feeding (induction of fed state). Breath samples continued to be collected every 25-min for each pig until the end of sampling period at 390 min (Table 3.3). A total of 11 breath samples were collected for each pig per period, including the three background breath samples. Air from the chambers were drawn by a rotary vane vacuum pump through a series of drierite-filled columns to the CO_2 analyzer (Qubit Model S155, Qubit Systems Inc.) and the gas switcher. From the gas switcher, expired air was pushed through midget bubblers, which contained 8 ml of 1 mol/L NaOH solution. The purpose of the NaOH solution was to trap any CO_2 released by the pigs from the

chambers for the subsequent CO_2 enrichment analysis (Shoveller et al., 2017). The sample was then stored in an air-tight serum tube and kept at room temperature until further analysis.

Analysis of ^{13}C enrichment in breath samples was done by the Environmental Isotope Laboratory, University of Waterloo (200 University Ave W, Waterloo, ON, Canada). Breath samples were analyzed with a Gasbench II interfaced with a Delta V Plus mass spectrometer (Thermo Scientific, Bremen, Germany).

3.3.4 Calculations

The $^{13}CO_2$ enrichment in expired breath at isotopic steady state was expressed as atom percent excess (APE, %) above background samples and was calculated according to equation 1:

(1) ECO_2 (APE, %) = APE of sample - $\bar{X}$ APE of background samples

where APE was the atom % ^{13}C obtained from the last three (minimum) breath analysis at isotopic steady state and $\bar{X}$ was the mean of APE of three background breath samples before the isotope was fed to the pigs. Isotopic steady state is defined as a state where the $^{13}CO_2$ enrichment reaches a plateau and no longer increasing or decreasing over a period of time.

The rate of $^{13}CO_2$ released from L-[1-^{13}C]-phenylalanine oxidation was calculated using the following equation (Matthews et al., 1980):

(2) $F^{13}CO_2$ (μmol/h) = $$\frac{FCO_2 \times ECO_2 \times 44.6 \times 60}{BW \times 0.82 \times 100}$$

where FCO_2 was the CO_2 production rate (ml/min), ECO_2 was the $^{13}CO_2$ enrichment in expired breath at isotopic steady state and expressed above background samples (APE). The constants 44.6 (μmol/ml) and 60 (min/h) were to convert the FCO_2 from ml/min to μmol/h. The factor 0.82 was

the correction for CO_2 retained in the body because of bicarbonate fixation. The factor 100 was to convert APE to a fraction.

The MA of methionine from the BSFL meal was calculated following this equation (Rafii et al., 2020; Littell et al., 1997):

(3) Metabolic availability = $\frac{\mathrm{bT}}{\mathrm{bR}}$

where bT and bR were the slopes for the IAAO response (i.e. $F^{13}CO_2$ or APE) following graded intake of BSFL test diets and reference crystalline AA diets, respectively.

Resting energy expenditure (REE) was calculated using the following equation:

(4) REE (kcal/d) = $\left(\left(\frac{3.94 \times \mathrm{O_2\ exchange}}{1000}\right) + \left(\frac{1.11 \times \mathrm{CO_2\ exchange}}{1000}\right)\right) \times 60 \times 24$

where O_2 and CO_2 exchange (ml/min) were obtained from the indirect calorimetry software. The constants 60 and 24 were to convert the gas exchange from min to hour to day.

3.3.5 Statistical Analysis

All statistical analysis was conducted using SAS version 9.3 (SAS Institute Inc). An α level of 0.05 was used to determine statistical significance in all analysis. Output data for body weight (BW), resting energy expenditure (REE), APE, and $F^{13}CO_2$ were tested for outliers (± 3.4 SE unit from the mean) using the PROC UNIVARIATE option for residual analysis. No outliers were identified. The effect of methionine levels, diet types, and the interaction of methionine level and diet type on BW and REE was tested using ANOVA, with pig and period as random effects. BW and REE results were expressed as least square means ± SEM (standard error of mean), and compared within its category based on the type of diet using the Tukey test. Methionine intake

was expressed as the % of methionine from the estimated SID methionine requirement (NRC, 2012). Regression within the analysis of variance using PROC GLIMMIX was used to construct the regression equation for each of the diet treatments, REF and BSFL test diet, to obtain the slopes of the two lines. Another PROC GLIMMIX procedure was done to test if there were any effects of methionine levels and adding methionine by either crystalline DL-Met or BSFL meal on the variations of APE and $F^{13}CO_2$, with pig and period as random variables. This procedure also tested whether the APE or $F^{13}CO_2$ slopes were significantly different from zero. APE and $F^{13}CO_2$ results were expressed as regression equation.

3.4 Results

All pigs remained healthy throughout the study period and consumed all meals during the feed adaptation period. All pigs, except one, finished their small meals prior to the corresponding breath sample collection on IAAO day. One pig would occasionally have some leftover feed and as such, data for some of his sampling periods were removed from the statistical analysis.

3.4.1 Body weight and resting energy expenditure

Methionine levels, diet types (i.e. BSFL test diet or REF), and the interaction of the two were not significant sources of variation for BW and REE ($p > 0.05$). The overall mean BW (± SEM) of pigs fed the BSFL and REF diets were not different and the mean were 30.77±1.34 and 31.09±1.34 kg, respectively ($p = 0.0919$; Table 3.4). The average REE of pigs fed the BSFL and REF diets were not different and the mean were 1924.39 ± 83.62 and 1848.58 ± 84.07, respectively ($p = 0.1690$; Table 3.4).

3.4.2 Linearity of response to increasing methionine intake

As methionine intake from the reference protein (i.e. DL-Met) increased from 45 – 75% estimated SID methionine requirement (NRC, 2012), the rate of ^{13}C phenylalanine oxidation ($F^{13}CO_2$) decreased linearly (Figure 3.1). Linear regression determined a negative slope of the best fit line of -0.044 ±0.01 ($p < 0.0001$) for the reference protein (Table 3.5). As the methionine from the common first point and BSFL meal increased from 45 – 75% estimated SID methionine requirement (NRC, 2012), the rate of ^{13}C phenylalanine oxidation remained the same and the slope (-0.015 ± 0.01) was not significantly different from zero ($p = 0.1149$; Table 3.5; Figure 3.1).

3.4.3 Metabolic availability of methionine in BSFL meal

Due to the non-significant slope of $F^{13}CO_2$ for the BSFL meal treatment, both APE and $F^{13}CO_2$ results are presented to calculate the relative MA of methionine. The increasing concentration of methionine and different diet types, but not the interaction of the two, were significant sources of variation for both APE and $F^{13}CO_2$ (p-value < 0.05). Increments of methionine intake from 45 – 75% estimated SID methionine requirement (NRC, 2012) from reference protein, DL-Met, significantly decreased the ^{13}C-Phe enrichment in expired breath at isotopic steady state (APE; slope = -0.00015 ± 0.000033, $p < 0.0001$; Figure 3.2; Table 3.6). Similarly, the increase of methionine intake from 55 – 75% estimated SID methionine requirement (NRC, 2012) from BSFL meal also significantly decreased the APE (slope = -0.00008 ± 0.000033, $p = 0.0197$; Figure 3.2; Table 3.6). The slope ratio of the APE response to additional methionine intake from BSFL meal compared with that of methionine from DL-Met was 0.533. Therefore, based on the APE measurement, the MA of methionine from BSFL meal was 53.33% (Table 3.6).

Similar to the APE response, methionine intake from the reference protein significantly decreased the rate of ^{13}C-phenylalanine oxidation ($F^{13}CO_2$; slope = -0.044 ± 0.01, $p < 0.0001$;

Table 3.5). However, the $F^{13}CO_2$ did not significantly decrease following the increasing intake of methionine from BSFL meal (slope = -0.015 ± 0.01, p = 0.1149; Table 3.5). When calculated, the slope ratio of $F^{13}CO_2$ response to additional methionine intake from BSFL meal compared with that of methionine from DL-Met was 0.3335. Therefore, based on the $F^{13}CO_2$ measurement, the MA of methionine from BSFL meal was 33.35% (Table 3.5).

3.5 Discussion

To our knowledge, this is the first study to determine the MA of methionine in BSFL meal relative to the crystalline DL-Met, using the IAAO method in growing pigs. Metabolic availability was calculated using the slope-ratio assay principle, where the slope of L-[1-^{13}C]-Phenylalanine oxidation following graded intake of protein-bound methionine from BSFL meal was compared to the slope of L-[1-^{13}C]-Phenylalanine oxidation following graded intakes of methionine from DL-Met, which was assumed to be 100% bioavailable. This same approach has been utilized in pigs on different ingredients and test AA, such as lysine in peas (Moehn et al., 2005) and threonine in corn and barley (Levesque et al., 2010). Additionally, the MA of AA in food has also been determined in humans using the same approach, for example, methionine bioavailability in cooked rice and peas (Rafii et al., 2020) and sulphur AA bioavailability in soy protein isolate (Humayun et al., 2007).

Application of the slope ratio assay assumes linearity in IAAO response to graded intake of test AA. To achieve this linear response, the highest concentration of the test AA in the diet must be at least 2 standard deviation below the population requirement (Moehn et al., 2005) and a minimum of three incremental levels of test AA are needed to obtain the regression line (Baker, 1986). The highest methionine concentration in this study was 75% of the estimated SID

methionine requirement (NRC, 2012) or 2.2 g/kg diet (as-fed). This level was used in the anticipation that the SID methionine requirement in NRC overestimates the true methionine requirement and to also anticipate if the NRC value does not account for population variability. All other indispensable AAs in the test and reference diets were set at 110% of estimated SID requirement, instead of 120% as seen in previous studies (Moehn et al., 2004; Moehn et al., 2008; Levesque et al., 2011), to have a better methionine to total protein ratio balance. If all other indispensable AAs are supplied far above the requirement (i.e. 120 – 150%), they will eventually be oxidized and lead to an increase in the nitrogen pool, which could potentially affect the oxidation of L-[1-^{13}C]-Phenylalanine. This is a problem since incremental intake of test AA should be the only factor that is driving indicator AA oxidation and not the oversupply of other AAs.

In the current study, the MA of methionine from BSFL meal was presented based on the slope comparison of two metrics, $F^{13}CO_2$ and APE. The slope of $F^{13}CO_2$ following intake of graded methionine from DL-Met in the reference diet was steeper (slope = -0.044 ± 0.01, p-value < 0.0001) than the graded intake of protein-bound methionine from BSFL meal (slope = -0.015 ± 0.01, p-value = 0.1149); however, a significant slope was not attained for $F^{13}CO_2$. The same trend was also seen for the APE, where the slope of the reference diet was steeper (slope = -0.00015 ± 0.000033, p-value < 0.0001) compared to the BSFL test diet (slope = -0.00008 ± 0.00003, p-value = 0.0197) and the APE was higher when equivalent amount of methionine in reference diet was compared with BSFL (Figure 3.2). Both slopes were significantly different from zero ($p < 0.05$), which confirmed that gradual intake of 10% increments of methionine significantly decreased the $^{13}CO_2$ and increased protein synthesis. A steeper reference diet slope indicates that the methionine from DL-met was more bioavailable for protein synthesis compared to methionine from BSFL meal, which was as expected.

Based on the current study, the relative MA of methionine from BSFL meal was 53.33 (APE metric) or 33.35% ($F^{13}CO_2$ metric). Typically, only the $F^{13}CO_2$ is presented as an outcome measurement in other published IAAO studies to determine MA (Moehn et al., 2005; Shoveller et al., 2010; Levesque et al., 2011; Rafii et al., 2020). However, the slope ratio of APE in response to the following intake of graded methionine was also presented in this study to account for the variation of resting volume CO_2 (VCO_2). Volume CO_2 was needed for the calculation of $F^{13}CO_2$ and was variable in the current study between pigs of similar BW within each period (Table 3.7). This demonstrated that BW alone was not a significant source of variation in VCO_2. Some potential factors that are believed to had contributed to the variations in the VCO_2 were fluctuations in the humidity and the rapid growth of the pigs. The study was conducted between the spring and summer months, where humidity level varies from one breath collection day to another. Changes in water vapor level in the air can lead to the dilution of CO_2 level and infrared light of the CO_2 analyzer being absorbed by particulate matter, which may cause an error in the CO_2 reading (Customized Gas Exchange System and Software for Animal Respirometry, Qubit System Inc.). In this study, the amount of water vapor was controlled by passing the gas through a series of drierite-filled columns, which were changed as the drierite became hydrated. However, this step might not be sufficient to tightly control for the humidity in the room. Another potential source of variation in VCO_2 was that the pigs were growing rapidly (average daily gain = 1-2 kg) throughout the study periods, even though their feed intake was restricted to 95 g/kg $BW^{0.75}$. Increase in BW should linearly correlate to an increase in expired VCO_2 and indeed, some pigs but not all increased VCO_2 as they grew (Table 3.7). However, the rapid growth of the pigs and substantial increase in expired CO_2 were believed to exceed the ability of the rotary vane vacuum pump to draw air from the chambers; thus, some VCO_2 readings might not be accurate towards the end of the study period

when the pigs were a lot heavier. The study was set up as a Latin square, so that each diet types and methionine levels were represented at all periods at different BW to construct the APE and $F^{13}CO_2$ slopes. Therefore, as previously stated, changes in the BW alone should not affect the variation in VCO_2, but the humidity and pump capacity might be the main reasons of the VCO_2 variations. Therefore, to account for all these potential variations, MA result in the APE metric are also presented.

Metabolic availability estimates have been shown to be comparable to the slope ratio growth assay, which is considered as a gold standard in estimating AA bioavailability (Lewis and Bayley, 1995; Van Barneveld, 1995; Moehn et al., 2005). However, there are also limitations in conducting MA studies and using MA values in animal nutrition. First, the estimate of MA of AA is variable from one study condition to another and has a considerable standard error (Batterham, 1992). This is due to the individual animal variation such as genotype, physiological stage, and other factors that can affect the retention of protein and AA in the body (Fan, 1994). Additionally, the AA balance in diet treatments, source of energy and protein in the diets, and the order in which AAs are absorbed at the tissue level can influence protein synthesis and retention in the body and therefore, contributing to the variation and high standard error in MA results (Fan, 1994). Large variation was even observed in the current IAAO study between the two different metrics that were calculated, which further confirms that MA results are highly variable. In the current study, the BSFL test diets were formulated to have a combination of BSFL meal and a fixed amount of DL-Met. This might also contribute to the variation and error in the result as intact protein such as BSFL meal has both free and protein-bound AA, while DL-Met only has free AA. Free AA can only be taken up by the individual AA transporter, while PEPT-1 transporter is for the uptake of protein-bound AA (Adibi, 1997). The main reason that a fixed amount of DL-Met was included

was to ensure practicality, as adding a higher level of BSFL meal to achieve 75% of estimated SID methionine requirement was perceived as non-practical in an industry setting. However, by using all BSFL meal in the test diets, this will help ensure that the potential issue of overcrowding the individual AA transporter by both DL-Met and free AA from BSFL meal would not affect the uptake capacity and therefore estimation of MA. The second limitation of MA measurement is that MA values are not additive between different ingredients (Fan, 1994). As stated previously, there are a lot of different dietary and individual animal factors that can affect and lead to variations in MA results. Thus, the determination of MA value of AA in a single ingredient is not additive when used to formulate a complete feed. Additivity is one of the most important factors when considering a protein quality method as the ultimate end goal is to use the protein quality value for feed formulation in commercial or research settings (Fan, 1994). Lastly, the IAAO method is expensive and laborious to conduct (Levesque et al., 2010).

In conclusion, the relative MA of methionine in BSFL meal was found to be 53.33% (APE metric) or 33.35% ($F^{13}CO_2$ metric). Despite the large variation and potential standard error, these MA values provide an insight to how much methionine from BSFL meal is actually bioavailable for protein synthesis in growing pigs. Future studies should also determine the MA of different AAs in the BSFL meal to provide a complete protein quality profile. Additionally, to minimize the variation of VCO_2 during the breath collection days, it would be beneficial to have the study in a humidity-controlled room and restrict the pigs feed intake to 90 g/kg $BW^{0.75}$ to further control their growth.

3.6 Acknowledgements

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3.7 Tables and Figures

Table 3.1: Ingredient composition (%, as-fed) of the basal diet with 45% estimated SID methionine requirement (BASAL), reference diet (REF), and BSFL test diet with 55, 65, and 75% estimated SID methionine requirement

Ingredient	BASAL	REF55	REF65	REF75	BSFL55	BSFL65	BSFL75
BSFL meal	-	-	-	-	6.30	12.50	18.50
Corn starch	65.22	65.24	65.21	65.23	64.49	63.77	62.98
Sucrose	8.80	8.80	8.80	8.80	8.80	8.80	8.80
Corn oil	3.90	3.90	3.90	3.90	2.80	1.75	0.80
Cellulose	4.32	4.32	4.32	4.32	3.55	2.79	2.06
Limestone	0.60	0.60	0.60	0.60	0.45	0.30	0.15
Monocalcium phosphate	2.70	2.70	2.70	2.70	2.40	2.05	1.75
NaCl	0.50	0.50	0.50	0.50	0.50	0.50	0.50
K_2CO_3	0.40	0.40	0.40	0.40	0.40	0.40	0.40
MgO	0.14	0.14	0.14	0.14	0.14	0.14	0.14
L-Arg	0.53	0.53	0.53	0.53	0.38	0.23	0.09
L-His	0.40	0.40	0.40	0.40	0.32	0.23	0.14
L-Ile	0.60	0.60	0.60	0.60	0.47	0.35	0.23
L-Leu	1.16	1.16	1.16	1.16	0.96	0.76	0.56
L-Lys HCl	1.43	1.43	1.43	1.43	1.22	1.01	0.81
DL-Met	0.13	0.16	0.19	0.22	0.13	0.13	0.13
L-Phe	0.68	0.68	0.68	0.68	0.57	0.46	0.35

L-Cys	0.32	0.32	0.32	0.31	0.29	0.27	0.24
L-Tyr	0.54	0.54	0.54	0.54	0.35	0.17	0.00
L-Thr	0.68	0.68	0.68	0.68	0.60	0.52	0.45
L-Ala	5.40	5.35	5.35	5.30	3.55	1.75	0.00
L-Trp	0.20	0.20	0.20	0.20	0.16	0.13	0.09
L-Val	0.76	0.76	0.76	0.76	0.58	0.41	0.24
Vitamin Mineral Premix[1]	0.60	0.60	0.60	0.60	0.60	0.60	0.60

[1]Vitamin mineral premix provided per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1,200 IU as cholecalciferol; vitamin E, 48 IU as dl-alpha-tocopherol acetate; vitamin K, 3 mg as menadione; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; vitamin B12, 0.03 mg; biotin, 0.24 mg; Cu, 18 mg from CuSO4×5H2O; Fe, 120 mg from FeSO4; Mn, 24 mg from MnSO4; Zn, 126 mg from ZnO; Se, 0.36 mg from Na2SeO3; and I, 0.6 mg from KI (DSM Nutritional Products Canada Inc., Ayr, ON, Canada).

Table 3.2: Analyzed and calculated proximate nutrient and amino acid (as-fed) of the basal diet with 45% estimated SID methionine requirement (BASAL), reference diet (REF), and BSFL test diet with 55, 65, and 75% estimated SID methionine requirement

Item	BASAL	REF55	REF65	REF75	BSFL55	BSFL65	BSFL75
Proximate Nutrient							
Dry matter[1], %	91.89	92.20	92.29	92.27	91.60	92.04	90.88
Calculated ME[2], kcal/kg	3620	3620	3620	3620	3613	3610	3609
Crude protein[1], %	12.60	11.70	11.67	12.12	11.71	11.57	11.76
Crude fat[3], %	3.91	3.91	3.91	3.91	3.73	3.59	3.52
Crude fiber[3], %	3.89	3.89	3.89	3.89	3.60	3.31	3.04
Ash[1], %	3.60	3.55	3.88	3.70	4.02	3.80	4.14
Calcium[3], %	0.68	0.68	0.68	0.68	0.68	0.68	0.68
Phosphorus[3], %	0.58	0.58	0.58	0.58	0.58	0.58	0.58
Indispensable AA[3], SID basis %							
Arginine	0.519	0.519	0.519	0.519	0.519	0.515	0.517
Histidine	0.392	0.392	0.392	0.392	0.396	0.393	0.394
Isoleucine	0.583	0.583	0.583	0.583	0.580	0.581	0.577
Leucine	1.137	1.137	1.137	1.137	1.134	1.134	1.132
Lysine	1.127	1.127	1.127	1.127	1.128	1.126	1.123
Methionine	0.131	0.160	0.190	0.220	0.160	0.189	0.218
Phenylalanine	0.673	0.673	0.673	0.673	0.675	0.674	0.673
Threonine	0.673	0.673	0.673	0.673	0.673	0.672	0.674
Valine	0.733	0.733	0.733	0.733	0.730	0.734	0.733

[1] Analyzed nutrients
[2] ME = metabolizable energy. Calculated using the Atwater coefficients of 9 for crude fat, 4 for crude protein, and 4 for carbohydrate (NFE, calculated) (Atwater and Bryant, 1900).
[3] Calculated nutrients

Table 3.3: Breath sample collection, oral isotope administration, and feeding schedule on the day of each IAAO sample collection

	Eq	VCO_2 Measurement			Induction to Fed State			Background Breath Collection			Breath Sample Collection							
Min	0-10	10-35	35-60	60-85	85-95	95-105	105-115	115-140	140-165	165-190	190-215	215-240	240-265	265-290	290-315	315-340	340-365	365-390
Feed	X	X	X	X	Meal 1	Meal 2	Meal 3	Meal 4	Meal 5	Meal 6	Meal 7	Meal 8	Meal 9	Meal 10	Meal 11	Meal 12	Meal 13	X
Breath	X	X	X	X	X	X	X	V	V	V	V	V	V	V	V	V	V	V
Isotope	X	X	X	X	X	X	X	X	X	P+C	C	C	C	C	C	C	C	C
VCO_2	X	V	V	V	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Min = minutes
Eq = gas chamber equilibration
X = no feed or isotope were given, no breath sample or VCO_2 record were collected
V = sample was collected
P+C = priming and constant L-[1-13C-Phenylalanine] isotope dose were given at 3.5 and 2 mg/kg BW, respectively
C = constant L-[1-13C-Phenylalanine] isotope dose of 2 mg/kg

Table 3.4: Body weight (BW; kg) and resting energy expenditure (REE; kcal/d) of growing pigs (n=8) in the IAAO study fed graded intake of methionine in the reference (REF) and BSFL meal test diets (BSFL)

Characteristic	REF Diets[1]	BSFL Diets[1]	p-value
BW (kg)	31.09 ± 1.34	30.77 ± 1.34	0.092
REE (kcal/d)	1849 ± 84.07	1924.39 ± 83.62	0.169

[1]Values are means ± SEMs

Table 3.5: Metabolic availability (MA, %) of methionine in BSFL meal based on the rate of L-[1-^{13}C-Phenylalanine] oxidation in response to graded intake of methionine in reference diets (REF) and BSFL test diets (BSFL meal)

Methionine Source	n	Slope equation	p-value	MA (%)
REF Diets	8	-0.044x + 5.35	<0.0001	100[1]
BSFL meal	8	-0.015x + 4.12	0.115	33.35

[1]Metabolic availability of methionine from DL-Met in REF diets was assumed to be 100%

Table 3.6: Metabolic availability (MA, %) of methionine in BSFL meal based on the ^{13}C enrichment in expired air (APE, %) in response to graded intake of methionine in reference diets (REF) and BSFL test diets (BSFL meal)

Methionine Source	n	Slope equation	p-value	MA (%)
REF Diets	8	-0.00015x + 0.01866	<0.0001	100[1]
BSFL meal	8	-0.00008x +0.01645	0.020	53.33

[1]Metabolic availability of methionine from DL-Met in REF diets was assumed to be 100%

Table 3.7: Average and standard deviation of body weight (BW; kg) and volume CO_2 (VCO_2; ml/min) of pigs (n=8) in different periods

Period	BW (kg)	SD	VCO_2 (ml/min)	SD
1	26.30	1.05	207.60	25.65
2	27.90	1.12	238.40	25.73
3	29.40	1.12	283.60	61.11
4	30.80	1.18	263.90	42.47
5	32.30	1.40	286.70	33.77
6	34.20	1.56	294.30	42.49
7	35.80	1.70	263.80	32.81

Figure 3.1: Linearity of the rate of L-[1-^{13}C-Phenylalanine] oxidation ($F^{13}CO_2$) in response to graded intake of methionine as free amino acid from DL-Met in reference diets and protein-bound methionine from BSFL meal

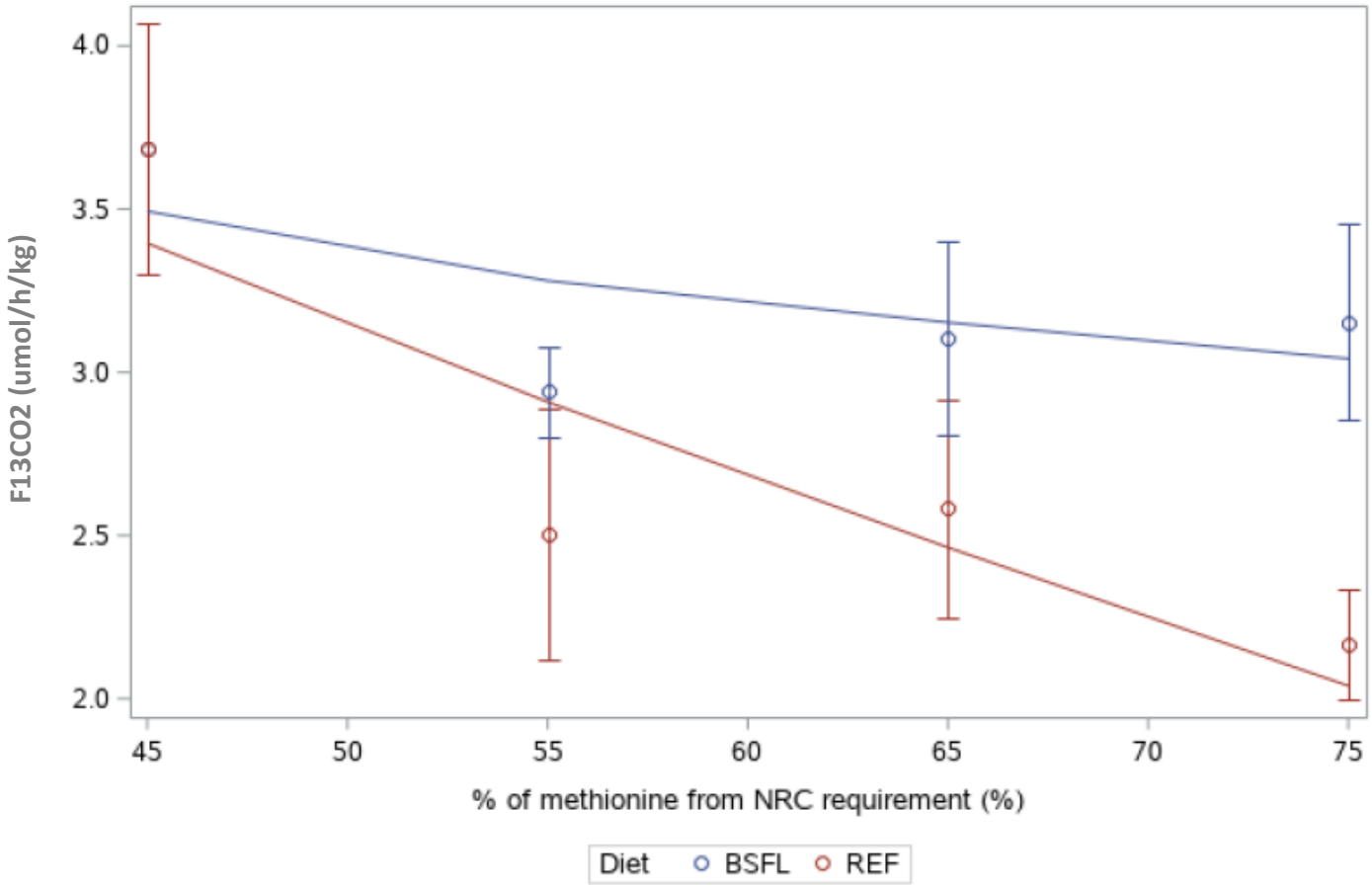
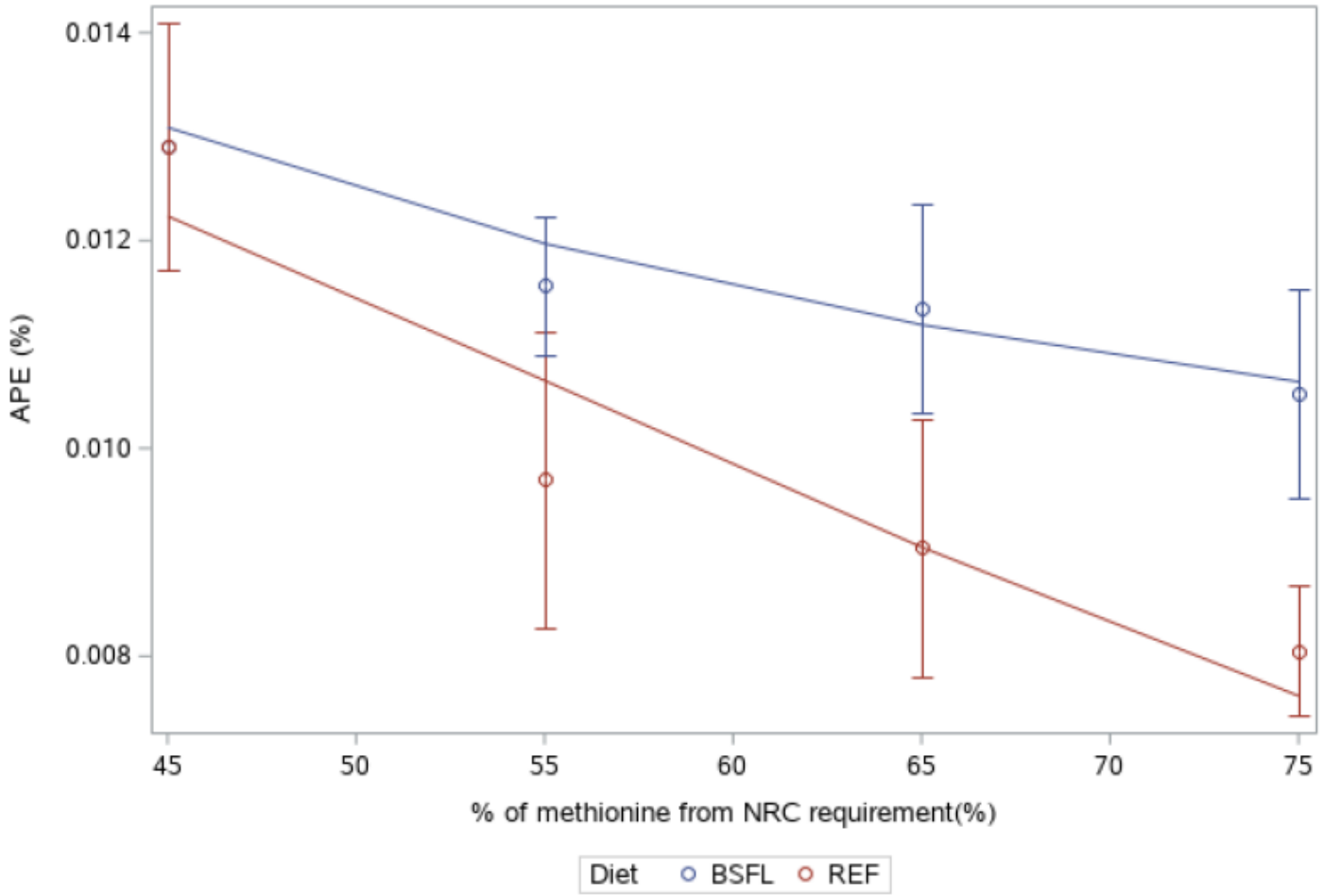


Figure 3.2: Linearity of the ^{13}C enrichment in expired air (APE, %) in response to graded intake of methionine as free amino acid from DL-Met in reference diets and protein-bound methionine from BSFL meal



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4 Chapter 4: General discussion

The continuous growth of the global population will require an increase in the food supply and emphasizes the urgency to screen alternative protein ingredients for use in feed and food. Black soldier fly larvae (BSFL) meal has gained interest for utilization in animal feed, mainly because it is perceived as a sustainable protein ingredient. Environmental sustainability concerns are increasing, as society becomes more conscious of the carbon footprint and environmental impacts contributed by agriculture and food production. Black soldier fly larvae meal is a unique protein ingredient because it can be reared on food waste or livestock manure to produce valuable protein biomass (Meneguz et al., 2018). However, due to health and safety concerns, currently in North America, BSFL are fed a standardized ration without using any organic waste. Available research studies focused mostly on the growth and/or productivity parameters of BSFL meal inclusion in swine (Biasato et al., 2019; Chia et al., 2019; Yu et al., 2019), poultry (Onsongo et al., 2018; Mwaniki et al., 2020), and aquaculture feed (St. Hilaire et al., 2007; Renna et al., 2017). Additionally, some studies also investigated the digestibility of BSFL meal in pigs (Crosbie et al., 2020), poultry (Mwaniki and Kiarie, 2019), and aquaculture species (Fisher et al., 2020). Since BSFL meal is a novel protein ingredient, it is equally important to characterize its protein quality, as availability of this data will allow better understanding to promote the utilization of BSFL meal in commercial feed formulation.

As stated in chapter 1, protein quality is a measure to determine the capacity of amino acid (AA) in a food source to meet the indispensable AA requirement of an individual (Nosworthy and House, 2017). In animal nutrition, protein quality is more commonly measured as AA digestibility due to its simpler procedure, but recent advances have also measured AA bioavailability as a parameter of protein quality. Throughout the chapters in this thesis, two methodologies,

standardized ileal digestibility (SID) and indicator amino acid oxidation (IAAO), were conducted to assess the protein quality of BSFL meal for pigs. The two methodologies were of interest because SID is considered as the gold standard in quantifying AA digestibility and has been used as an estimate of AA bioavailability. Moreover, SID AA data of ingredients are widely available, and routinely used in feed formulation (NRC, 2012; Stein et al., 2007). Meanwhile, IAAO measures metabolic availability (MA) of AA, which has been shown to be a more accurate representation of AA bioavailability to support tissue protein synthesis (Moehn et al., 2005). The global objective of this thesis was to compare the SID coefficient of methionine from BSFL meal with the MA value from the IAAO method to see if SID coefficient can be used as a reliable estimate of AA bioavailability. To the best of our knowledge, this thesis is the first to compare SID coefficient with MA result from IAAO on the same test ingredient, using the same species, animal source (genetics), body weight, and environment. Findings from this research can provide meaningful insights to understand how different SID coefficients are compared to MA values when used as an estimate of AA bioavailability. Ultimately, the results would provide quantitative value of SID coefficients and MA of BSFL meal for use by nutritionists and feed or food manufacturers.

In chapter 2, the SID coefficient of indispensable and dispensable AA of BSFL meal were determined in growing pigs. For indispensable AA, arginine (SID: 93.0%) and methionine (SID: 90.4%) were the most digestible, while threonine was the least (SID: 83.0%). Results from the current study were comparable to previous work on full fat BSFL meal (Crosbie et al., 2020), and higher compared to the BSF prepupae (Tan et al., 2020), mainly due to the higher chitin content in the prepupal stage that reduces digestibility (Chen et al., 2015; Smets et al., 2020). In chapter 2, the SID coefficient was then multiplied with the AA content of the BSFL meal to calculate the SID AA content, which is more applicable for nutritionists when formulating feed. Subsequently,

the SID AA content of BSFL meal was compared to the ones of soybean meal (SBM), canola meal, and fish meal (FM). The BSFL meal has lower SID AA contents compared to FM, but similar to SBM and canola meal (NRC, 2012). This result suggested that BSFL meal is a nutritionally attractive protein ingredient in swine feed as the AA is highly digestible and comparable to other commonly-used protein ingredients. However, prior to market application, other considerations, such as price, need to be well-thought-out.

In chapter 3, IAAO was conducted to determine the MA of methionine in BSFL meal relative to DL-Methionine (DL-Met), which was assumed to be 100% bioavailable. To the best of our knowledge, this is the first study that determined the MA of AA in BSFL meal in growing pigs using the IAAO with slope-ratio assay principle. Determination of the MA of methionine would benefit nutritionists to understand how much of the methionine content in BSFL meal is actually available for protein synthesis and to better understand the post absorptive utilization of AA. The MA of methionine in BSFL meal was found to be 33.35% ($F^{13}CO_2$) and/or 53.33% (APE). Although not commonly presented, the slope ratio of APE following intake of graded methionine was also presented to account for the random variation in VCO_2 contributed by the rapid growth of pigs and fluctuating humidity on breath collection days. No difference in BW and resting energy expenditure were observed between different diet treatments and methionine levels, which showed that the only outcome affected by the treatments was the protein synthesis parameter.

The MA of methionine in BSFL meal (53.33%, APE metric) was found to be 37% lower than the SID coefficient from our SID study (90.4%) and another recent estimate (90.2%; Crosbie et al., 2020). The lower MA compared to SID coefficient is expected. However, the difference between the two was surprising as the two studies were done using the same BSFL meal without any further processing, barrows of similar initial BW and age, and similar diet type (i.e. mash) and

ingredient composition. A potential explanation could be that during the processing of BSFL meal (i.e. oven drying from wet larvae to dry-powdered meal; personal communication with Enviroflight), methionine was exposed to heat and oxidized to methionine sulfoxide and methionine sulfone. Methionine sulfoxide and sulfone are able to be absorbed by the GIT through the same transport mechanism as methionine (Higuchi et al., 1982). Methionine sulfoxide has limited capacity to be utilized by the animals; however, the sulfone form is not nutritionally available (Anderson et al., 1976; Rutherfurd and Moughan, 2008). In turkeys, the relative availability of methionine sulfoxide was reported to be 50% (Parsons and Potter, 1981). When analyzing methionine content in the digesta samples in our SID study, performic acid oxidation followed by acid hydrolysis method was used (Method 994.12; AOAC, 2005). This particular approach has been reported to overestimate the methionine content, as the analysis would also account for both methionine sulfone and sulfoxide, which are not bioavailable to the animals (Wang and Parsons, 1998; Rutherfurd and Moughan, 2008). Batterham (1992) concluded that the SID coefficient of methionine in cottonseed meal was overestimated because methionine was being absorbed in a form that was unavailable for utilization. Another study by Wang and Parsons (1998) also found that ileal digestibility overestimates methionine bioavailability in meat and bone meal due to the methionine analysis by performic acid oxidation and acid hydrolysis. Therefore, it is likely that in our study, SID coefficient overestimates methionine bioavailability due to absorption of methionine in a form that is not bioavailable for the pigs. Meanwhile, the MA is a more accurate estimation of the true bioavailability as MA measures for all AA losses during digestion, absorption, and cellular metabolism (Elango et al., 2012).

Other published studies have also reported lower MA values compared to their corresponding SID coefficient as an estimate of bioavailability. Rafiii et al. (2020) reported that

the MA of methionine from chickpeas in human was 63%, while the SID coefficient in swine was 77%. Humayun et al. (2007) found that the MA of sulphur AA in soy protein isolate in humans was 72%, while when given to growing pigs, the SID coefficient was 86% (NRC, 2012). Though the comparison was done on different species, nutrition studies in pigs have been well-accepted as a model for humans (Hendriks et al., 2012). The MA of lysine in SBM in growing pigs using the IAAO method was reported to be 88%, which is similar to the reported SID coefficient of 90% (Moehn et al., 2007; NRC, 2012). Overall, it is expected that SID coefficient is higher compared to MA. This is because SID only measures AA losses during absorption and digestion, regardless of its form (Stein et al., 2007). However, not all AAs that are absorbed are bioavailable for protein synthesis in the animal. Thus, SID coefficient overestimates AA bioavailability.

Despite being more accurate in estimating AA bioavailability, there are also limitations associated with the IAAO method. First, MA of AA can only be determined one at a time, whereas SID coefficient can be determined for all AA at once. Second, IAAO is more expensive, laborious, and requires more sophisticated equipment compared to the SID method (Levesque et al., 2010). Due to the complexity of IAAO, the SID AA is currently acceptable to estimate AA bioavailability for most ingredients in swine feed. However, based on the comparison with the MA value in this thesis, caution needs to be taken when using SID values as they could overestimate bioavailability. Results from this thesis provide valuable SID coefficient of AA and MA value of methionine in BSFL meal. Methionine is an economically-important indispensable AA in swine nutrition as it is usually the second or third limiting AA in commercial corn-soybean based diets; thus, it is almost always supplemented (NRC, 2012). Availability of protein quality data will increase the likelihood of BSFL meal application in swine feed and therefore, expand protein ingredient choices for animal nutritionists. Subsequently, this will put less pressure on the protein ingredients shared with

the human food chain. Unfortunately, the current market price of BSFL meal is not competitive with SBM or canola meal, as it is still a very niche and small market compared to the two plant protein ingredients. Thus, in the meantime, BSFL meal is more suitable to be used as a complementary protein ingredient. In the coming years, as more commercial application research on BSFL meal and more manufacturers become available, the price is predicted to decrease gradually.

Future studies should determine the MA of other indispensable AAs in the BSFL meal and compare them with the SID coefficient, to further investigate or validate the theory that the significantly lower MA in methionine was due to oxidation of methionine to other forms that are not bioavailable. The MA of lysine should be determined next, as lysine is one of the most abundant AA concentrations in BSFL meal (Enviroflight), and it is the first limiting AA for pigs (NRC, 2012). To further promote commercial usage of BSFL meal, future studies should also investigate the economic feasibility of complete or partial substitution of current protein ingredients with BSFL meal in commercial feed.

Some potential modifications or additions that could have been done for the studies in this thesis are discussed. First, for the SID study, simultaneously comparing the SID coefficient of BSFL fed with feed-grade ingredients with the ones fed with livestock manure or food waste would be interesting, as this would help us to better understand how different feed source affects the AA digestibility of BSFL meal as a feed ingredient. Availability of this data along with the investigation of some health parameters on pigs fed BSFL meal reared on waste might steer the BSFL farming towards a more sustainable approach. Second, for the IAAO study, using BSFL meal solely in the test diets would help ensure that the free AA and intact protein transporters issue would not affect MA estimates as previously discussed in chapter 3. To minimize the variation of

VCO_2 during the breath collection days, it would be beneficial to have the study in a humidity-controlled room and restrict the pigs feed intake further. In conclusion, studies in this thesis provided insights of protein quality methodology comparison, which supported BSFL meal as a nutritionally promising protein ingredient in swine feed.

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