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Evaluation of standardized ileal digestibility of amino acids and metabolic availability of methionine, using the indicator amino acid oxidation method, in black soldier fly larvae (*Hermetia illucens*) meal fed to growing pigs

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Abstract

Standardized ileal digestibility (SID, %) of crude protein (CP) and amino acid (AA) and the metabolic availability (MA) of Met using the indicator amino acid oxidation (IAAO) method, in partially defatted black soldier fly larvae (PD-BSFL) meal were determined in growing pigs in 2 experiments. The Met SID value was then compared numerically with the Met MA to understand how different SID is compared with its MA value. In Exp. 1, 6 ileal-cannulated barrows (initial body weight [BW] = 18.03 ± 0.34 kg) were used in a 2-period switch back design and fed either a nitrogen-free diet (NFD) or test diet, with PD-BSFL meal as the sole source of AA, over two 11-d experimental periods, at a feeding level of 2.8 × estimated maintenance digestible energy requirement. Barrows were adapted for 9-d to the diet, followed by continuous 8-h ileal digesta collection on day 10 and 11. Digesta were pooled per pig within period. The SID of CP and Met of PD-BSFL meal were 76.1 ± 6.2% and 90.4 ± 3.9%, respectively. In Exp. 2, 7 barrows (initial BW = 18.77 ± 0.69 kg) were used in a 7 × 7 Latin square design with L-[1-^{13}C]-Phe as the indicator AA. Each pig was randomly assigned to 1 of 7 dietary treatments over seven 3-d experimental periods. Two diet types were studied including reference (crystalline AA) and PD-BSFL test diets, each supplying graded intakes of Met at 55, 65, and 75% of the estimated SID requirement (NRC, 2012). The MA of Met was determined by comparing the IAAO response between the reference and PD-BSFL test diet using the slope-ratio method. Linear regression determined a negative slope of the best fit line for both the reference and test diets ($P < 0.05$). The MA of Met in PD-BSFL meal was 53.3%, which is as expected lower than the SID value. While it is generally appreciated that MA will be less than SID, the use of SID is more practical. In cases where SID cannot explain physiological outcomes of feeding a novel ingredient, IAAO may provide additional insight into whether MA should be explored.

Lay Summary

The interest in black soldier fly larvae (BSFL) meal as a protein ingredient in swine feed has grown in the past years. As a novel protein ingredient, it is beneficial to evaluate the amino acid (AA) digestibility and metabolic availability (MA) of the limiting AA, Met, in pigs, in BSFL meal prior to incorporation in feed for a more precise formulation. Two different methodologies were used to determine the AA digestibility and MA, standardized ileal digestibility (SID) and indicator amino acid oxidation (IAAO) method, respectively. Based on the SID values, the AA digestibility of BSFL meal, for some, but not all AA, is comparable to other commonly used protein ingredients in commercial swine feed. When compared with the MA result of Met, the Met SID value is much lower. This indicates that not all digested Met is available for protein synthesis or other metabolic processes in the animal.

Key words: black soldier fly larvae meal, indicator amino acid oxidation, Met, metabolic availability, standardized ileal digestibility, swine

Abbreviations: AA, amino acid; APE, atom percent excess; BSFL, black soldier fly larvae; BW, body weight; CP, crude protein; ECO_2, enrichment of $^{13}CO_2$; IAAO, indicator amino acid oxidation; MA, metabolic availability; NDF, neutral detergent fiber; NFD, nitrogen free diet; PD-BSFL, partially defatted black soldier fly larvae; REE, resting energy expenditure; SBM, soybean meal; SID, standardized ileal digestibility; VCO_2, volume carbon dioxide (CO_2)

Introduction

Black soldier fly larvae (BSFL; *Hermetia illucens*) has become a growing interest as a protein ingredient alternative for animal feed that has 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; Meneguz et al., 2018). In regard to nutrient profile, the His, Leu, Trp, and Val content of BSFL meal are comparable to that of soybean meal (SBM), canola meal, and meat and bone meal (NRC, 2012; Enviroflight, Maysville, KY). The BSFL is rich in lauric acid and chitin, which have antimicrobial activity

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(Skrivanova et al., 2006) and prebiotic properties (Selenius et al., 2018), respectively. Therefore, BSFL may also be considered as a potential alternative to in-feed antibiotic (Spranghers et al., 2018).

Prior to incorporating new protein sources into animal feed, it is imperative to assess AA digestibility and/or bioavailability to enable a more accurate diet formulation (Adeola et al., 2016) as nutrient-deficient diets can lead to reduced growth and productivity (Pomar et al., 2011); whereas, excess AA can lead to increase in feed cost and nitrogen excretion into the environment (Hauschild et al., 2010; Pomar et al., 2011). Standardized ileal digestibility (SID) is a method to measure the disappearance of AA in the small intestine, as a proxy for AA digestibility (Stein et al., 2007). Values of SID AA are widely used in swine feed formulation because of the additivity among ingredients in mixed diets (Stein et al., 2005; NRC, 2012) and SID values are also commonly used as an estimate of AA bioavailability (Mosenthin et al., 2000; Columbus and de Lange, 2012). However, SID values can overestimate AA bioavailability, particularly for AA that are damaged during processing and although absorbed, are unavailable for protein synthesis (Finot and Magnenat, 1981; Moughan and Rutherfurd, 1996). Therefore, it is advantageous to also measure the metabolic availability (MA) of AA in novel protein ingredients.

The MA of AA can be determined using the indicator amino acid oxidation (IAAO) combined slope-ratio method. This technique has been validated and used to determine the MA of AA in feed ingredients for animals (Moehn et al., 2005, 2007) and humans (Humayun et al., 2007; Rafii et al., 2020). The principle of the IAAO method to assess MA is that changes in the rate of oxidation of the indicator AA can be observed as the test AA becomes more or less bioavailable for protein synthesis (Moehn et al., 2005). To drive the oxidation of the indicator AA, the test AA must be the first limiting in the experimental diets. Methionine was the test AA in this study as it is usually the second or third limiting AA in commercial swine diets (Moehn et al., 2008; NRC, 2012) and Met has the lowest indispensable AA content in the BSFL meal used in this study. Therefore, evaluation of the MA of Met in BSFL meal is beneficial to contextualize how much of dietary Met is available for protein synthesis or other metabolic purposes.

Despite being frequently used as an estimate of AA bioavailability, to the best of our knowledge, there is currently no published study that directly compares the SID value and MA of AA using the same test ingredient and animal population (i.e., age and genetics). Comparison between the two can provide insight to how different SID are compared with MA values when used as an estimate of AA bioavailability. Additionally, whereas SID of the AA content of BSFL meal has been studied in pigs (Crosbie et al., 2020; Tan et al., 2020), the MA of its AA, has yet to be determined. Therefore, the objectives of the current studies were: 1) to determine the SID values of crude protein (CP) and AA of partially defatted BSFL (PD-BSFL) meal fed to growing pigs, 2) to quantify the MA of Met from PD-BSFL meal using the IAAO method, and 3) to compare the SID value with the MA value of Met in PD-BSFL meal in growing pigs. We predicted that the SID value of Met obtained from the SID method will be greater than the MA result obtained from the IAAO method.

Materials and Methods

The experimental protocol and study design were reviewed and approved by the University of Guelph Animal Care Committee (Exp. 1: AUP# 4439 and Exp. 2: AUP# 4516). Handling and caring for the animals were in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009).

Black soldier fly larvae meal

Partially defatted BSFL meal was supplied for both experiments by Enviroflight, LLC (1118 Progress Way, Maysville, KY). Chemical analysis of the PD-BSFL meal is presented in Table 1. The analysis of dry matter, CP, crude fat, crude fiber, neutral detergent fiber (NDF), acid detergent fiber, ash, AA, and fatty acids content was provided by Enviroflight, following the AOAC 930.15 (2005), AOAC 990.03 (2005), AOAC 954.02 (2005), AOCS Ba 6a-05, ANKOM A200, ANKOM A2001, AOAC 942.05 (2005), AOAC 994.12 (2005), and AOAC 996.06 (1996) methods, respectively. The AOAC 990.03 (2005) method to measure CP uses 6.25 as the nitrogen to protein conversion factor and would overestimate the CP in BSFL meal due to its chitin content (Diener et al., 2009; Jonas-Levi and Martinez, 2017). To address the overestimation of CP due to chitin, lower nitrogen to protein conversion factors for BSFL meal have been proposed (Janssen et al., 2017; Nery et al., 2018; Smets et al., 2021). Macro and microminerals were analyzed by SGS Canada Inc (Guelph, ON) following the atomic absorption spectrophotometric method (Method 968.08; AOAC 1996). The gross energy was analyzed at the Department of Animal Biosciences, University of Guelph (Guelph, ON) using the bomb calorimeter (IKA Calorimeter System C 5000; IKA Works Inc., Wilmington, NC). The digestible energy value was calculated by multiplying the gross energy value with the digestibility %, where the digestibility followed the index method (Adeola, 2001).

Experiment 1: standardized ileal digestibility

Animals, housing, and ileal cannulation surgery

Six Yorkshire barrows with an average initial body weight (BW) of 18.03 ± 0.34 kg were obtained from the Arkell Swine Research Station (Guelph, ON). During the first 7-d adaptation, the barrows were pair-housed in plexi-glass pens with tenderfoot flooring, in a temperature-controlled room (23 to 24 °C) and were provided with ad libitum access to a commercial starter ration and water, and toys as environmental enrichment (e.g., chains and rubber balls). After the adaptation period, all pigs were fasted for 15 h prior to the ileal cannulation surgery, which followed the procedure described by Sauer et al. (1983). A 6-d recovery period followed the surgery. During this time and throughout the rest of the study period, pigs were housed individually with ad libitum access to water and toys. The surgical site was cleaned twice daily with warm water and zinc oxide cream was applied to prevent skin irritation around the cannula.

Experimental design

The study was a 2-period switch back design, where all 6 barrows were randomly assigned to either a nitrogen-free diet (NFD; Rho et al., 2017) or a diet containing 36.5% PD-BSFL meal as the sole source of AA (Table 2) in each period. Both diets were supplemented with vitamins and minerals to meet the estimated requirements (NRC, 2012). Titanium dioxide was added at 0.20% as an indigestible marker to determine

Table 1. Analyzed chemical composition (%, as-fed) of PD-BSFL meal

Item	PD-BSFL meal
Dry matter,[1] %	95.05
Crude protein,[1] %	49.30
Gross energy,[2] kcal/ kg	5057
Digestible energy,[2,3] kcal/ kg	3594
Crude fat,[1] %	14.40
Crude fiber,[1] %	6.40
Neutral detergent fiber,[1] %	31.30
Acid detergent fiber,[1] %	10.90
Ash,[1] %	8.33
Calcium,[4] %	1.84
Phosphorus (total),[4] %	1.05
Sodium,[4] %	0.22
Copper,[4] ppm	13.55
Zinc,[4] ppm	109.11
Manganese,[4] ppm	132.42
Chitin,[5] %	2.41
Indispensable AA,[1] %	
Arg	2.32
His	1.38
Ile	1.90
Leu	3.15
Lys	2.64
Met	0.47
Phe	1.79
Thr	1.26
Trp	0.60
Val	2.71
Dispensable AA,[1] %	
Ala	2.96
Asp	3.97
Cys	0.38
Glu	5.64
Gly	2.62
Pro	2.75
Ser	1.93
Tyr	2.84
Fatty acids,[1] %	
Lauric (C12:0)	4.88
Omega 3 fatty acids (total)	0.19
Omega 6 fatty acids (total)	2.59

[1]Analyzed by Enviroflight, LLC (1118 Progress Way, Maysville, KY).
[2]Analyzed and/or calculated by the researcher at the Department of Animal Biosciences, University of Guelph, ON.
[3]The digestible energy was calculated by multiplying the gross energy content, determined by bomb calorimeter, with the digestibility calculation by index method (Adeola, 2001).
[4]Analyzed by SGS Canada Inc, Guelph, ON.
[5]Average chitin level (range 2.17%–2.66%, as fed), not based on acid detergent fiber assumption.

component digestibility. Diets were provided as a wet mash (feed-to-water ratio of 1:2) in 2 equal meals per day at 0830 and 1630 h to supply 2.8 × estimated maintenance digestible energy requirement (NRC, 2012). Each experimental period lasted 11 d, with 9 d adaptation period, followed by 2

Table 2. Ingredient composition (%, as-fed) of the NFD and PD-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
Calculated nutrient content,[2] %		
Crude protein	0.02	18.13

[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 Inc., Ayr, ON, Canada).
[2]Calculated based on the NRC (2012) and USDA (US Department of Agriculture) Food Data Central ingredient nutrient values. Nutrient values of PD-BSFL meal were provided by Enviroflight, LLC (1118 Progress Way, Maysville, KY).

d of continuous ileal digesta collection for 8-h after the pigs finished their allocated amount of morning meal. For ileal digesta collection, 10 mL of 10% formic acid was added to a collection bag and then attached to the cannula using an elastic band and was replaced as needed. Digesta were stored in the refrigerator (~4 °C) during collection and pooled per pig and period. Ileal digesta samples were then stored at –20 °C until analyses.

Sample preparation and chemical analysis

Prior to chemical analysis, ileal digesta samples were freeze dried, finely ground, and mixed thoroughly. Diets and ileal digesta samples were analyzed for dry matter using the AOAC method 930.15 (2005). Nitrogen content of samples was determined using a LECO analyser (LECO Corporation, St. Joseph, MO), utilizing the combustion method (Method 968.06; AOAC, 2005). Crude protein was calculated by multiplying the nitrogen content by a factor of 6.25. Titanium dioxide content of samples was analyzed following Myers et al. (2004) with some adjustments. Specifically, anhydrous sodium sulphate (Na_2SO_4) was added to each ash sample instead of the catalysts potassium sulphate (K_2SO_4) and copper II sulphate ($CuSO_4$). Samples were then digested with sulphuric acid (H_2SO_4) at 120 °C for at least 20 h instead of at 420 °C for 2 h (Myers et al., 2004; Crosbie et al., 2020). The titanium analysis was repeated 3 times and each time, a sample was read in triplicates on the UV spectrophotometer. Additionally, diets and ileal digesta samples were analyzed for AA contents using ultra performance liquid chromatography (UPLC; Waters Corporation, Milford, MA). All AA except Met and Cys

Table 3. Analyzed chemical composition (as-fed) of the NFD and BSFL meal-containing test diet

Item	NFD	Test diet
Dry matter, %	89.74	92.37
Gross energy, kcal/kg	3,650	4,142
Digestible energy,[1] kcal/kg	3,025	3,182
Crude protein, %	0.00	18.51
Crude fat,[2] %	1.16	5.70
Crude fiber,[2] %	1.90	4.96
Neutral detergent fiber, %	2.12	11.80
Acid detergent fiber, %	1.51	4.18
Ash, %	3.64	6.89
Calcium,[2] %	0.64	1.49
Phosphorus,[2] %	0.41	0.88
Sodium,[2] %	0.25	0.32
Indispensable AA, %		
Arg	0.01	0.79
His	0.00	0.49
Ile	0.01	0.60
Leu	0.06	1.17
Lys	0.01	0.94
Met	0.00	0.29
Phe	0.02	0.68
Thr	0.01	0.68
Val	0.02	0.88
Dispensable AA, %		
Ala	0.03	1.15
Asp	0.03	1.67
Cys	0.00	0.16
Glu	0.08	2.24
Gly	0.02	0.90
Pro	0.00	1.09
Ser	0.02	0.79
Titanium dioxide, %	0.14	0.15

[1]The digestible energy of both diets was calculated by multiplying the gross energy content, determined by bomb calorimeter, with the digestibility calculation by index method (Adeola, 2001).
[2]Analyzed by SGS Canada Inc, Guelph, ON.

were analyzed following the acid hydrolysis method, while the performic acid oxidation with acid hydrolysis – sodium metabisulfite method was used for the determination of Met and Cys (Method 994.12; AOAC, 2005). Diets were analyzed for ash following AOAC 942.05 (2005), neutral and acid detergent fiber, following the filter bag technique ANKOM A200 and A2001, respectively, and gross energy was determined using a bomb calorimeter (IKA Calorimeter System C 5000; IKA Works Inc., Wilmington, NC). Further, diets were also analyzed for crude fiber, crude fat, and macrominerals by SGS Canada Inc (Guelph, ON). The crude fiber analysis followed the fritted glass crucible (Method 978.10; AOAC, 1996). Crude fat analysis followed the petroleum ether extraction method (AOCS Am 5-04; ANKOM Technology, 2009). Lastly, the macromineral analysis followed the atomic absorption spectrophotometric method (Method 968.08; AOAC 1996). Analyzed chemical composition of the diets is presented in Table 3.

Calculations

Basal endogenous AA losses was calculated after the pigs were fed the NFD, following the equation in Stein et al. (2007). The SID values of CP and AA contents were calculated by correcting the apparent ileal digestibility for the basal endogenous AA losses, according to the Stein et al. (2007) equation. Data were considered as outliers when they fell outside the upper and lower limit, which were calculated using the following equation:

$$\begin{aligned} \text{Lower range limit} &= \text{Q1} \quad (1.5 \times \text{IQR}) \\ \text{Upper range limit} &= \text{Q3} + (1.5 \times \text{IQR}) \end{aligned} \qquad (1)$$

where Q1 was quartile 1, Q3 was quartile 3, and IQR was interquartile range

Experiment 2: indicator amino acid oxidation

Animals and housing

Seven Yorkshire barrows with an average initial BW of 18.77 ± 0.69 kg were obtained from Arkell Swine Research Station (Guelph, ON). A 7-d adaptation period followed, where barrows were pair-housed, fed ad libitum commercial starter diet, had access to ad libitum water, and object enrichment. Subsequently, the pigs underwent respiratory chamber adaptation for 6 d, where they were adapted inside the chamber in incremental number of hours, until they were comfortable (i.e., calm, not moving too much, not panting) to stay for 8-h. During this chamber adaptation period, pigs were fed a basal diet (contained 45% of the SID Met requirement; NRC, 2012) in multiple small meals every 25-min to acclimate them to the feeding regime on breath collection days. For example, in a 1-h adaptation period, pigs would receive 2 meals; in a 2-h period, they would receive 4 meals, and so on. Throughout the chamber adaptation until the end of the experimental period, pigs were individually housed in a temperature-controlled room (23 to 24 °C), had nose-to-nose access to neighboring pigs, ad libitum water access, and enrichment.

Diet formulation, feeding, and nutrient analysis

The study design was a complete 7 × 7 Latin square (n = 7), where all 7 barrows were randomly assigned to 1 of the 7 dietary treatments in each period, ensuring that all treatments were represented on each calorimetry day per period. There were seven 3-day experimental periods; the first 2 d were diet adaptation, followed by breath collection on day 3. Feed intake was restricted to 95 g/kg $BW^{0.75}$ (Shoveller et al., 2010). Daily rations on nonsampling days were offered as a wet mash with feed-to-water ratio of 1:2 at 0830 and 1630 h.

Metabolic availability of Met from PD-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 Met in PD-BSFL meal was compared with the reference diet. A total of 7 corn starch-based diets were tested and the ingredient composition is presented in Table 4. All diets were first limiting in Met, with the highest Met content formulated to 75% of the estimated SID Met requirement (NRC, 2012). Total Phe + Tyr intake in all diets was 1.20% as fed basis. All other indispensable AA were provided in excess (110%) of the estimated SID requirement (NRC, 2012). A basal diet with 1.4 g/kg diet of Met, representing 45% of the estimated SID Met requirement, served as the

Table 4. Ingredient composition (%, as-fed) of the basal diet with 45% estimated SID Met requirement (BASAL), reference diet (REF), and BSFL test diet with 55, 65, and 75% estimated SID Met requirement

Ingredient	BASAL	REF55	REF65	REF75	BSFL55	BSFL65	BSFL75
PD-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).

common first point for both the reference and test diet slopes. Crystalline AA, which are considered to be 100% bioavailable, were the only source of AA in the basal diet. Three reference diets (REF55, REF65, and REF75) were also formulated with crystalline AA as the sole source of AA. Three Met intake levels, aimed to be at 1.6, 1.9, and 2.2 g/kg diet supplied only by DL-Met (as-fed), representing 55, 65, and 75% of the estimated SID Met requirement for 25–50 kg pig (NRC, 2012) were used to measure the IAAO response to construct the reference slope. The AA sources of the 3 test diets (BSFL55, BSFL65, and BSFL75) were a combination of PD-BSFL meal and crystalline AA. To construct the BSFL test diet slope, the same 3 levels (i.e., 55, 65, and 75%) of estimated SID Met requirement were studied using the PD-BSFL SID value obtained in Exp. 1. In the test diets, a fixed amount of 0.13% inclusion of DL-Met was kept to supply 45% of the SID Met requirement. Black soldier fly larvae meal was then added at the expense of corn starch, corn oil, cellulose, and a combination of crystalline AA. All 7 diets were formulated to be isonitrogenous, isoenergetic, and to have equal crude fiber concentrations with crystalline Ala (L-Ala), corn oil, and Solka-Floc (pure cellulose) adjustments, respectively.

Analyzed and calculated nutrient contents of dietary treatments are reported in Table 5. Dietary treatments were analyzed for dry matter, CP, AA, and ash following the same methods as in Exp.1. Amino acid analysis was performed by Ajinomoto Health and Nutrition North America, Inc (Itasca, IL), following the total AA method 994.12 (AOAC, 2005).

Tracer protocol, breath collection, and analysis

On each IAAO study day, the morning ration was divided into 13 small meals and the afternoon ration was provided once breath collection was complete at 1630 h. Prior to pigs entering the chambers on breath collection day, BW was recorded after a 16-h overnight fasting period. Four open circuit respiration chambers (63.50 cm width, 149.86 cm depth, and 93.98 cm height) were used. The chambers were constructed of glass with tenderfoot flooring and feeding bowls that were secured to the flooring in each chamber. 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. Subsequently, three 25-min respiration calorimetry measurements were conducted to determine resting CO_2 and O_2 volume produced (VCO_2; VO_2; respectively) when the pigs were calm, not actively moving, and in a fasted state. Volume CO_2 was measured during fasted state because of the increased variability associated with fed state-VCO_2 in free-living animals. Calorimetry data were collected using the Qubit calorimetry software (Qubit System Inc., Kingston, ON).

After collection of the fasted resting breath samples, 3 small meals without isotope were offered every 10-min to induce

Table 5. Analyzed and calculated nutrient and amino acid (as-fed) of the basal diet with 45% of the estimated SID Met requirement (BASAL), reference diet (REF), and BSFL test diet with 55, 65, and 75% of the estimated SID Met requirement

Item	BASAL	REF55	REF65	REF75	BSFL55	BSFL65	BSFL75
Analyzed nutrient, %							
Dry matter	91.89	92.20	92.29	92.27	91.60	92.04	90.88
Crude protein	11.59	11.70	12.30	12.71	13.57	12.68	12.12
Ash	3.60	3.55	3.88	3.70	4.02	3.80	4.14
Total indispensable AA, %							
Arg	0.492	0.520	0.501	0.503	0.556	0.543	0.510
His	0.320	0.345	0.363	0.268	0.353	0.365	0.360
Ile	0.553	0.550	0.560	0.606	0.599	0.691	0.685
Leu	0.984	1.179	1.127	1.269	1.319	1.240	1.132
Lys	1.253	1.358	1.123	1.060	1.139	1.163	1.008
Met	0.141	0.188	0.194	0.212	0.200	0.212	0.240
Phe	0.672	0.723	0.681	0.720	0.745	0.714	0.681
Thr	0.696	1.504	0.442	0.195	0.325	0.809	0.695
Val	0.842	0.602	1.207	1.236	0.578	0.799	0.695
Total dispensable AA, %							
Cys	0.169	0.172	0.152	0.152	0.183	0.180	0.191
Calculated crude protein,[1] %	12.64	12.62	12.65	12.62	12.60	12.59	12.57
Calculated ME,[2] kcal/kg	3,620	3,620	3,620	3,620	3,613	3,610	3,609
Calculated total indispensable AA,[1] %							
Arg	0.519	0.519	0.519	0.519	0.519	0.515	0.517
His	0.392	0.392	0.392	0.392	0.396	0.393	0.394
Ile	0.583	0.583	0.583	0.583	0.580	0.581	0.577
Leu	1.137	1.137	1.137	1.137	1.134	1.134	1.132
Lys	1.127	1.127	1.127	1.127	1.128	1.126	1.123
Met	0.131	0.160	0.190	0.220	0.160	0.189	0.218
Phe	0.673	0.673	0.673	0.673	0.675	0.674	0.673
Thr	0.673	0.673	0.673	0.673	0.673	0.672	0.674
Val	0.733	0.733	0.733	0.733	0.730	0.734	0.733
Calculated total dispensable AA,[1] %							
Cys	0.155	0.155	0.155	0.152	0.154	0.154	0.153

[1]Calculated using an excel-based formulation software developed by Dr. Anna Kate Shoveller, using nutrient ingredient values from NRC, 2012 and PD-BSFL meal ingredient values from Enviroflight, LLC.
[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).

fed-state in the pigs. A priming dose of 1.75 × constant dose (3.5 mg/kg BW) was given once along with the constant dose at meal 6 (Moehn et al., 2003). A constant dose of 2 mg/kg BW (Levesque et al., 2011) of the stable isotope L-[1-^{13}C]-Phe (99%; Cambridge Isotope Laboratories, Inc. Saint-Laurent, QC) was given orally every 25-min between meals 6 and 13, inclusive. All pigs consumed their meals prior to the collection of the corresponding breath samples. The mechanism of breath collection was that air from the chambers was drawn by a rotary vane vacuum pump through a series of drierite-filled columns to the CO_2 analyser (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 sodium hydroxide (NaOH) solution. The purpose of the NaOH solution was to trap any CO_2 released by the pigs from the chambers for the subsequent $^{13}CO_2$ enrichment analysis (Shoveller et al., 2017). Breath samples were 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 conducted at the Environmental Isotope Laboratory, University of Waterloo (200 University Ave W, Waterloo, ON). Breath samples were analyzed with a Gasbench II interfaced with a Delta V Plus mass spectrometer (Thermo Scientific, Bremen, Germany).

Calculations and statistical analysis

The $^{13}CO_2$ enrichment (ECO_2) in expired breath at isotopic steady state was expressed as atom percent excess (APE, %) above background samples and was calculated as follows:

$$ECO_2\ (\text{APE}, \%) = \text{APE of sample} - \overline{X}\ \text{APE of background samples} \quad (2)$$

where APE was the atom % ^{13}C obtained from a minimum of last 3 breath analysis at isotopic steady state and $\overline{X}$ was the mean of APE of 3 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]-Phe oxidation ($F^{13}CO_2$) was calculated following the equation by Matthews et al., 1980:

$$F^{13}CO_2\ (\mu mol/h/kg) = \frac{FCO_2 \times ECO_2 \times 44.6 \times 60}{BW \times 0.82 \times 100} \quad (3)$$

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 average of fasted FCO_2/BW from all breath collection days within each pig was used as a constant in the $F^{13}CO_2$ calculation for all dietary treatments for each pig. The FCO_2/BW values that were outside the upper and lower limit were considered outliers and thus, were not included in the average FCO_2/ BW calculation for that pig. The upper and lower limit were calculated following Eq. 1.

The MA of Met from the PD-BSFL meal was calculated following the equation by Littell et al., 1997:

$$\text{Metabolic availability} = \frac{bT}{bR} \quad (4)$$

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:

$$REE\ (kcal/d) = \left(\left(\frac{3.94 \times O_2\ exchange}{1000} \right) + \left(\frac{1.11 \times CO_2\ exchange}{1000} \right) \right) \times 60 \times 24 \quad (5)$$

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.

Statistical analyses were conducted using SAS version 9.3 (SAS Institute Inc). Output data for BW, 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. The effects of Met inclusion, diet type, and the interaction of the two on BW and REE were tested using a mixed model, with pig and period as random effects. Body weight and REE results were expressed as least square means ± SEM and means were separated using the Tukey post-hoc test. Methionine intake was expressed as the % of Met from the estimated SID Met 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 2 lines. The effect of Met inclusion and the addition of Met via crystalline DL-Met or BSFL meal inclusion on the variations of APE and $F^{13}CO_2$ were tested using PROC GLIMMIX, 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. A *P*-value of < 0.05 was used to determine statistical significance.

Results

All pigs remained healthy throughout the study periods in Exp. 1 and 2. In Exp. 2, all pigs finished the small meals prior to the corresponding breath sample collection.

Experiment 1: standardized ileal digestibility

The CP level of NFD and test diet were 0.00 and 18.51%, as fed, respectively (Table 3). The indispensable and dispensable AA contents in the NFD were negligible when compared with the PD-BSFL meal test diet. The SID results of the PD-BSFL meal are presented in Table 6. The SID values were then further calculated by multiplying them to their corresponding AA concentration in PD-BSFL meal to produce SID content (%; Table 6).

Experiment 2: indicator amino acid oxidation

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 sources of variation for BW and REE ($P > 0.05$). The overall mean BW and REE of

Table 6. Standardized ileal digestibility (SID, %) and SID content (%) of CP, indispensable and dispensable AA in PD-BSFL meal fed to growing pigs ($n = 6$)

Item	SID, %	SEM	SID content, %
Crude protein	76.1	3.08	37.5
Indispensable AA, %			
Arg	93.0	0.99	2.2
His	83.4	1.91	1.2
Ile	86.0	1.08	1.6
Leu	88.8	1.36	2.8
Lys	87.6	1.52	2.3
Met	90.4	1.60	0.4
Phe	89.3	1.48	1.6
Thr	83.0	2.40	1.1
Val	86.2	1.29	2.3
Dispensable AA, %			
Ala	84.6	1.57	2.5
Asp	84.3	2.19	3.4
Cys	77.5	3.35	0.3
Glu	87.3	1.50	4.9
Gly	80.7	2.96	2.1
Pro	94.6	1.84	2.6
Ser	83.8	3.06	1.6
Tyr	89.2	1.76	2.5

pigs fed the BSFL and REF diets were not different ($P = 0.092$ and $P = 0.132$, respectively; Table 7).

Metabolic availability of Met in PD-BSFL meal

The increasing concentration of Met and different diet types, but not the interaction of the two, were sources of variation for both $F^{13}CO_2$ and APE ($P < 0.05$). As Met intake from the reference protein (i.e., DL-Met) increased from 45% to 75% estimated SID Met requirement (NRC, 2012), the rate of ^{13}C-Phe oxidation ($F^{13}CO_2$) decreased linearly (Figure 1). Linear regression determined a negative slope of the best fit line of –0.040 ± 0.01 ($P < 0.0001$) for the reference protein (Table 8). As Met from the common first point and PD-BSFL meal increased from 45 to 75% of the estimated SID Met requirement (NRC, 2012), the rate of ^{13}C-Phe oxidation decreased linearly and the slope (–0.021 ± 0.01) was different from zero ($P = 0.02$; Figure 1; Table 8). When calculated, the slope ratio of $F^{13}CO_2$ response to additional Met intake from PD-BSFL meal compared with that of Met from DL-Met was 0.525. Therefore, based on the $F^{13}CO_2$ measurement, the MA of Met from the PD-BSFL meal was 52.5%.

Increments of Met intake from 45% to 75% of the estimated SID Met requirement (NRC, 2012) from reference protein, DL-Met, decreased the ^{13}C-Phe enrichment in expired breath at isotopic steady state (APE; slope = –0.00015 ± 0.000033, $P = 0.0001$; Figure 2; Table 8). Similarly, the increase of Met intake from the common first point to 55%–75% estimated SID Met requirement (NRC, 2012) from PD-BSFL meal also decreased the APE (slope = –0.00008 ± 0.000033, $P = 0.020$; Figure 2; Table 8). The slope ratio of the APE response to additional Met intake from PD-BSFL meal compared with that of Met from DL-Met was 0.533. Therefore, based on the APE, the MA of Met from PD-BSFL meal was 53.3%.

Table 7. Body weight (BW, kg) and resting energy expenditure (REE, kcal/d) of growing pigs ($n = 7$) in the IAAO study fed graded intake of Met 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	1,840.69 ± 82.39	1,921.97 ± 81.96	0.132

[1]Values are means ± SEMs.

Discussion

To the best of our knowledge, this is the first study to compare SID value with MA of AA result on the same test ingredient, using the same species, animal source (genetics), initial BW and housing environment. The SID value of CP in PD-BSFL meal was low (76.1%), even though the SID values of all indispensable AA were above 83.0%. This is likely due to the high chitin content of the PD-BSFL meal. Chitin, a non-protein nitrogen polysaccharide, is the main component of the insect exoskeleton and is considered as the main component that makes up fiber in insects (Newton et al., 1977; Finke, 1984, 2002). High chitin content can negatively influence the protein digestibility of an ingredient (Sanchez-Muros et al., 2014) and overestimate the CP in BSFL meal (Diener et al., 2009). Therefore, the CP of the PD-BSFL meal in this study can appear greater, but the digestibility is lower as a proportion from the nitrogen is chitin. Furthermore, the presence of chitin causes an overlap in the analyses of CP and NDF as chitin would be included in the nitrogen and fiber analyses. Insoluble ash has also been shown to interfere with the measurement of NDF in feedstuffs and ash-free NDF values are lower compared with the uncorrected value (Crocker et al., 1998). When compared with the commonly used swine feed protein ingredients, the SID values of Arg, Leu, Met, Phe, Val, and Ala of the PD-BSFL meal were comparable to SBM (dehulled,

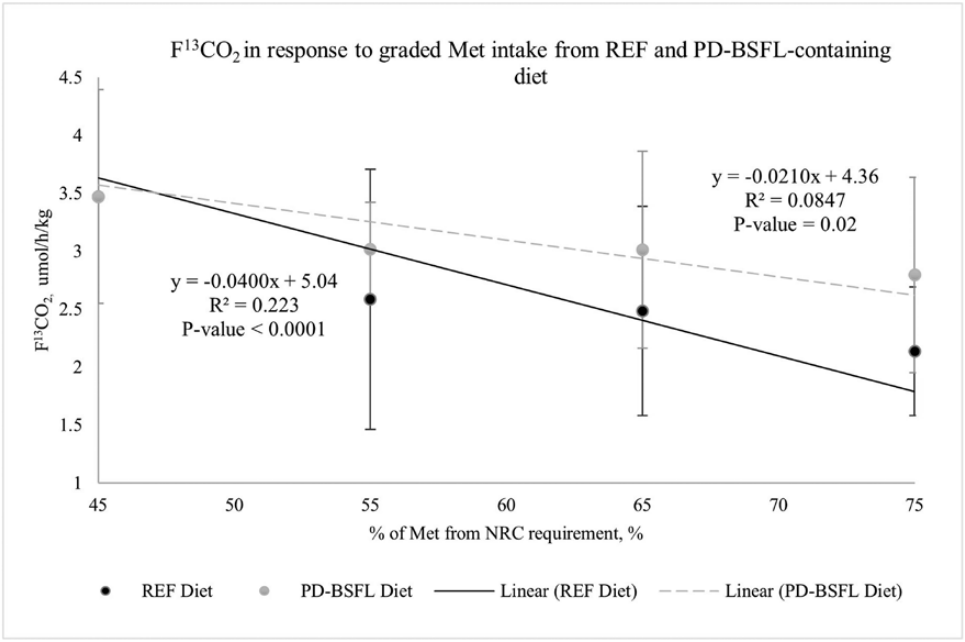

Figure 1. Linearity of the rate of L-[1-^{13}C]-Phe oxidation ($F^{13}CO_2$) in response to graded intake of Met as free amino acid from DL-Met in reference (REF) diets and protein-bound Met from partially defatted black soldier fly larvae (PD-BSFL) meal in growing pigs ($n = 7$).

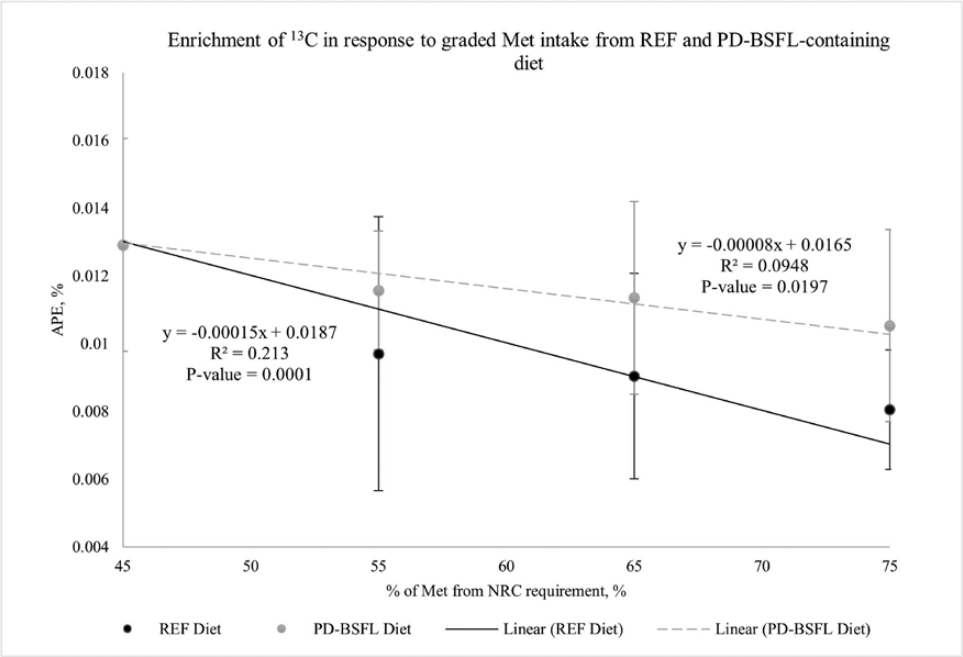

Figure 2. Linearity of the ^{13}C enrichment in expired air (atom percent excess; APE, %) in response to graded intake of Met as free amino acid from DL-Met in reference (REF) diets and protein-bound Met from partially defatted black soldier fly larvae (PD-BSFL) meal in growing pigs (n = 7).

Table 8. Metabolic availability (MA, %) of Met in PD-BSFL meal based on the rate of L-[1-^{13}C]-Phe oxidation and the ^{13}C enrichment in expired air (APE, %) in response to graded intake of Met in reference diets (REF) and BSFL test diets (BSFL meal)

Methionine source	*n*	Slope equation	Standard error	*P*-value	MA, %
L-[1-^{13}C]-Phe oxidation[1]					
REF diets	7	−0.0400x + 5.04	0.00871	<0.0001	100[2]
BSFL meal	7	−0.0210x + 4.36	0.00870	0.02	52.5
^{13}C enrichment (APE, %)[1]					
REF diets	7	−0.000150x + 0.0187	0.000033	0.0001	100[2]
BSFL meal	7	−0.0000800x +0.0165	0.000033	0.0197	53.3

[1]IAAO response variable used to calculate MA of Met.
[2]Metabolic availability of Met from DL-Met in REF diets was assumed to be 100%.

solvent extracted; CP: 47.73%; NRC, 2012), and overall, above that reported for canola meal (solvent extracted; CP: 37.50%; NRC, 2012). For Met, the SID value of PD-BSFL meal was 0.4 and 13.6 percentage units greater than SBM and canola meal, respectively. In addition, SID AA content was also calculated as it is considered to be more useful for direct application by nutritionists. For indispensable AA, SBM is on average 0.3 percentage units greater, while canola meal is 1.0 percentage unit lower (NRC, 2012) compared with the PD-BSFL meal. This indicates that PD-BSFL meal is a protein alternative to complement other protein ingredients in swine diets.

The SID values of AA do not truly quantify bioavailability because not all digested AA are absorbed in a form that will allow the animals to metabolically utilize them (NRC, 2012). Therefore, the second objective of our study was to determine the MA of Met in PD-BSFL meal. Application of the slope ratio assay assumes linearity in IAAO response to graded intake of test AA by formulating the highest concentration of the test AA in the diet to be at least 2 standard deviations below the population requirement (Moehn et al., 2005). As the test AA, Met was the first limiting AA in all diets and the highest Met concentration in this study was 75% of the estimated SID Met requirement (NRC, 2012). All other indispensable AA 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; Levesque et al., 2011), to have a better Met to total protein ratio balance. If all other indispensable AA are supplied far above the highest Met level, 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]-Phe. This is a problem since incremental intake of the test AA should be the only factor affecting the indicator AA oxidation and not the oversupply of other AA. The concentrations of Cys, Phe, and Tyr were kept constant in all diets. Supply of Tyr and Cys beyond the requirement was important to ensure that no Phe and no Met were directed to meet the demand for Tyr and Cys, respectively (Shiman and Gray, 1998). Phenylalanine concentration was consistent among all diets to ensure that any changes in the L-[1-^{13}C]-Phe oxidation were due to changes in the Met bioavailability from the graded level of PD-BSFL meal. Furthermore, no difference in BW and REE were observed among different dietary treatments and as such, only the dietary supply of Met affected L-[1-^{13}C]-Phe oxidation as an indicator of rates of protein synthesis.

In the current study, the MA of Met from the PD-BSFL meal was presented based on the slope comparison of 2 IAAO responses, $F^{13}CO_2$ and APE. The MA of Met was found to be 52.5 ($F^{13}CO_2$) and/or 53.3% (APE). Typically, only the $F^{13}CO_2$ is presented as an outcome measure 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 was also presented in this study as

the calculation of APE is based on raw data and the enrichment of $^{13}CO_2$ at background and steady state are the only components used to calculated it. On the other hand, $F^{13}CO_2$ calculation involves multiple different components, and each component, especially the VCO_2, add variability towards the end result. Additionally, to overcome the high day-to-day variability of fasting VCO_2 within pigs, the average FCO_2/BW was used in the $F^{13}CO_2$ calculation. The variability of VCO_2 expired in the fasted state depends on the physical condition and behavior of the pigs on each breath collection day. Therefore, to account for all these potential variations, MA value based on the APE response is likely to be more accurate and is proposed as the default MA value in IAAO studies where highly active animals are used.

In comparison with the SID value of Met from Exp. 1, the MA of Met in the PD-BSFL meal was found to be 37.1 percentage units lower (SID = 90.4% versus MA = 53.3%). The lower MA compared with SID value is expected. However, the magnitude between the two was quite surprising as the two studies were conducted using the same PD-BSFL meal without any further processing, barrows of similar initial BW, age, genetics, and similar ingredient composition in the experimental diets. A potential explanation could be that during the processing of the PD-BSFL meal (i.e., oven drying from wet larvae to dry-powdered meal), Met was exposed to heat and oxidized to Met sulfoxide and Met sulfone. These Met forms can be absorbed by the gastrointestinal tract through the same transport mechanism as Met (Higuchi et al., 1982), but they are nutritionally unavailable for the animals (Anderson et al., 1976; Rutherfurd and Moughan, 2008). When analyzing Met content in the digesta samples in Exp. 1, performic acid oxidation followed by the acid hydrolysis method was used. This approach has been reported to overestimate Met content, as the analysis would also account for Met sulfone and sulfoxide (Batterham, 1992; Wang and Parsons, 1998; Rutherfurd and Moughan, 2008). Therefore, it is likely that in Exp. 1, SID value overestimates Met bioavailability due to absorption of unavailable forms of Met.

Aside from the heat processing of BSFL meal that could lead to a greater Met sulfoxide and sulfone content, another potential explanation is that naturally, BSFL meal has a greater concentration of these oxidized forms of Met. Kar et al. (2021) reported that the plasma Met sulfoxide concentrations in pigs fed BSFL-containing diet was greater than those fed a SBM-based diet. This was further confirmed by the closer values between the MA of Met from chickpeas and soy protein isolate with their corresponding SID values (Humayun et al., 2007; NRC, 2012; Rafii et al., 2020). Therefore, a substantial difference between SID and MA value of Met may be expected when insects are the test ingredient.

Metabolic availability quantification using the IAAO method is regarded as a more accurate approach to estimate AA bioavailability because it accounts for all losses during digestion, absorption, and utilization of the test AA (Elango et al., 2008). Standardized ileal digestibility only measures AA losses or disappearance during digestion and absorption, regardless of its form (Stein et al., 2007). However, there are also limitations associated with the IAAO method. First, the estimate of MA of AA is variable from one study condition to another and has a considerable standard error (Batterham, 1992) due to the individual animal variation and other factors that can affect the retention of protein and AA in the body (Fan, 1994). Second, determination of MA value of AA in a single ingredient is not additive when used to formulate a complete feed (Fan, 1994). Lastly, IAAO method is more expensive, laborious, and requires more sophisticated equipment compared with the SID method (Levesque et al., 2010). Despite of these limitations, estimation of the MA of the limiting AA would provide a useful estimate of the protein quality of an ingredient to guide feed formulation as limiting AA determines protein quality.

In conclusion, due to the complexity of the IAAO method, SID AA is widely acceptable to estimate AA bioavailability for most ingredients in swine feed. However, caution should be taken when using SID values for this purpose as they could overestimate AA bioavailability. Results from these 2 studies provide SID value of AA and MA value of Met in PD-BSFL meal that makes it possible to incorporate BSFL meal in pig diets, but the role of other forms of methionine needs to be considered before BSFL is used in high inclusion rates. Due to the non-competitive cost, BSFL meal is currently more suitable to be used as a complementary protein ingredient (Veldkamp and Bosch, 2015). Future studies are needed to determine the economic feasibility of complete or partial substitution of current protein ingredients with BSFL meal in commercial feed.

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Author's Contributions

F.T., L.A.H., G.C.M., C.L.L. and A.K.S designed the experiment. F.T. conducted research of Exp.1 and 2. F.T. analyzed the data and all authors contributed to writing of the manuscript. A.K.S. had primary responsibility of the final content.

Conflict of Interest Statement

A.K.S. is the Champion Petfoods Chair in Canine and Feline Nutrition, Physiology and Metabolism, was previously employed by P&G and Mars Pet Care, serves on the Scientific Advisory Board for Trouw Nutrition, is a consultant for Champion Petfoods, and has received honoraria and research funding from various commodity groups, pet food manufacturers, and ingredient suppliers. All other authors have no conflicts of interest to declare.

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