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IMPACT OF ANALYTICAL METHOD ON THE *in vitro* DIGESTIBILITY OF BLACK SOLDIER FLY MEAL (*Hermetia illucens*)

IMPACTO DO MÉTODO ANALÍTICO SOBRE A DIGESTIBILIDADE *in vitro* DA FARINHA DE MOSCA SOLDADO-NEGRA (*Hermetia illucens*)

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ABSTRACT

The use of Black Soldier Fly Meal (BSFM) as a protein source in animal nutrition has increased in recent years. *In vitro* assays provide a rapid and strategic approach for evaluating the nutritional quality of feed ingredients. However, the diversity of available methodologies complicates the selection of the appropriate protocol. This study aimed to assess the influence of two laboratory devices—a circulating water bath and an orbital shaker—on the *in vitro* protein digestibility of defatted BSFM (BSFMd) and poultry viscera meal (PVM), using a modified gastric digestion protocol for pet food. Samples were incubated with a 0.02% pepsin–acid solution for 2 h at 39 °C, followed by digestion with pancreatin for 4 h at 39 °C and pH 6.8, under either equipment. BSFMd (54.6% crude protein) exhibited the highest digestibility (96.4%) when processed in a circulating water bath. In contrast, PVM (59.8% crude protein) showed low digestibility in both systems (30.35% and 30.00%, respectively). Compared to the modified AOAC method (73.14%), the current approach was effective for

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BSFMd, however, the shorter digestion time may limit its applicability to PVM. The protein source and heat transfer method significantly affected *in vitro* digestibility. BSFMd demonstrated high digestibility and suitability for rapid evaluation using simplified *in vitro* methods. These findings support the potential of BSFMd as a sustainable and digestible protein source for animal feed and highlight the need for specific methodologies based on the physicochemical properties of each ingredient.

Keywords: alternative proteins; circulating water bath; dogs; orbital shaker.

RESUMO

O uso de Farinha de Mosca Soldado Negra (BSF) como fonte de proteína na nutrição animal aumentou nos últimos anos. Ensaios *in vitro* fornecem uma abordagem rápida e estratégica para avaliar a qualidade nutricional dos ingredientes da ração. No entanto, a diversidade de metodologias disponíveis complica a seleção de protocolos apropriados. Este estudo teve como objetivo avaliar a influência de dois dispositivos de laboratório — um banho de água circulante e um agitador orbital — na digestibilidade proteica *in vitro* de farinha de BSF desengordurada (BSFMd) e farinha de vísceras de aves (PVM), usando um protocolo de digestão gástrica modificado para alimentos para animais de estimação. As amostras foram incubadas com uma solução de pepsina-ácida a 0,02% por 2 h a 39 °C, seguida de digestão com pancreatina por 4 h a 39 °C e pH 6,8, em qualquer um dos equipamentos. A BSFMd (54,6% de proteína bruta) exibiu a maior digestibilidade (96,4%) quando processada no banho de água circulante. Em contraste, o PVM (59,8% de proteína bruta) apresentou baixa digestibilidade em ambos os sistemas (30,35% e 30,00%, respectivamente). Comparada ao método AOAC modificado (73,14%), a abordagem atual foi eficaz para BSFMd; no entanto, o menor tempo de digestão pode limitar sua aplicabilidade ao PVM. A fonte de proteína e o método de transferência de calor afetam significativamente a digestibilidade *in vitro*. O BSFMd demonstrou alta digestibilidade e adequação para avaliação rápida usando métodos *in vitro* simplificados. Essas descobertas corroboram o potencial do BSFMd como uma fonte de proteína sustentável e digestível para ração animal e destacam a necessidade de metodologias específicas baseadas nas propriedades físico-químicas de cada ingrediente.

Palavras-chave: agitador orbital; banho-maria circulante; cães; proteínas alternativas.

1 INTRODUCTION

Population growth and the rising demand for protein in recent decades have intensified competition among different sectors for resources meant for food production. Industries such as pet food, livestock, and aquaculture compete for raw materials traditionally allocated to human consumption, highlighting the need to identify alternative, sustainable ingredients with high nutritional value. In this context, Black Soldier Fly Meal (BSFM, *Hermetia illucens* L.) has gained interest due to its high protein content (30–50% of dry matter, reaching up to 561 g/kg in larval meals) and its environmental and economic viability (ČALOUDOVÁ *et al.*, 2025; KRÖGER; HEIDE; ZENTEK, 2020; MAPA (MINISTÉRIO DA AGRICULTURA, 2020), aiming to replace animal proteins such as poultry viscera meal and meat and bone meal.

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Although already approved for use in animal nutrition in several countries, including Brazil, BSFM still lacks studies that deepen the understanding of its digestibility and the actual availability of its nutrients (FERRUA; SINGH, 2010). Assessing digestibility is essential for determining the quality of an ingredient and ensuring its applicability in commercial formulations. In this context, *in vitro* assays have been established as valuable tools, as they allow digestibility to be estimated in a faster, more accurate, and economically feasible way compared to *in vivo* trials, which require a longer time, higher costs, and the use of experimental animals (BOSCH; VERVOORT; HENDRIKS, 2016; HERVERA *et al.*, 2009). Based on two enzymatic steps that simulate the relationship between organic matter and energy digestibility, as well as the accuracy of digestibility prediction from organic matter disappearance, *in vitro* assays serve as an initial screening tool to identify promising ingredients before conducting more complex and costly *in vivo* trials (HERVERA *et al.*, 2009).

The literature highlights significant variability in *in vitro* protocols for pet, with differences in pepsin concentration, incubation time, and temperature, as well as the type of agitation employed (magnetic, orbital, or water bath). This methodological diversity compromises the comparability of studies, hindering the standardization of results and the validation of data that support the inclusion of BSFM in commercial diets (BOSCH *et al.*, 2014; HERVERA *et al.*, 2007, 2009; ZANOTTO; BELLAVER, 2005). Enzymatic digestion protocols generally use pepsin and pancreatic enzymes. Pepsin, in particular, is widely applied in laboratory analyses to evaluate the protein solubility of animal-origin meals and has also been described for determining BSFM digestibility (BIAGI *et al.*, 2016; BOSCH; VERVOORT; HENDRIKS, 2016; HERVERA *et al.*, 2009). Standard protocols for animal crude protein determination are typically conducted by specialized laboratories, following the guidelines of the Association of Official Analytical Chemists (AOAC, 1995), which recommend a 0.2% (m/v) pepsin solution with an enzymatic activity of 1:10000 in a single gastric stage. However, alternative approaches described in the literature propose adjustments in pepsin concentration (ranging from 0.2% to 0.0002% m/v) to evaluate protein quality. For pet feed, processing time, digestibility in two steps (gastric and intestinal phases), and incubation temperature are also factors evaluated to better simulate protein digestibility in *in vitro* assays (HERVERA *et al.*, 2007, 2009; ZANOTTO; BELLAVER, 2005). Equipment selection also plays a determining role, as differences in thermal contact and sample homogenization may directly affect digestibility outcomes (BOSCH; VERVOORT; HENDRIKS, 2016; LIU *et al.*, 2023). In Brazil, according to Normative Instruction No. 110 of November 24, 2020, BSFM is officially classified as an “ingredient of animal origin,” which

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allows the application of AOAC enzymatic protocols. Nevertheless, this guideline prescribes sample–pepsin agitation for 16 hours at 45 °C, whereas the gastric phase described in several studies for pets is shorter (2 hours at 39 °C) (HERVERA *et al.*, 2007, 2009). Furthermore, insect proteins are more easily digested than animal proteins, making it essential to use a specific protocol for this.

The temperature and type of contact between the enzyme and the sample bath also influence the digestibility of the meal, and this information is often described in the literature. One of the heat contact methods described *in vitro* digestibility is an orbital shaker or magnetic stirring (BOSCH; VERVOORT; HENDRIKS, 2016), while in another, the samples are incubated in a water bath shaker (LIU *et al.*, 2023). A common type of agitator used *in vitro* digestibility is water bath stirring equipment or the Wagner type agitator, which is placed inside an incubator with temperature control (ZANOTTO; BELLAVER, 2005). However, they are a specific type of equipment and are not available in most pet food industries. Magnetic agitation, orbital agitation, and agitation without information are some methodologies for *in vitro* digestibility described in the literature (BOSCH; VERVOORT; HENDRIKS, 2016; HERVERA *et al.*, 2009). The lack of methodological standardization compromises the accuracy of inter-study comparisons and hinders the selection of appropriate methods. The influence of the equipment and type of contact beyond the standardization of the gastric phase on BSFM and PVM digestibility is fundamental to validating the methodology and confidence of the data. Therefore, this study aimed to evaluate the influence of two different laboratory devices, a circulating water bath and an orbital shaker, on the digestibility of two commercial meals, Black Soldier Fly (BSF) and poultry viscera, used for pet feed, applying *in vitro* assays with modifications in the gastric phase.

2 MATERIAL AND METHODS

2.1 Substrates

A commercial BSFM, Nutro Bugs (200 g, batch 15122023), was acquired in its defatted form (BSFMd) from a local store and stored in its original packet under refrigeration (-20ºC) until future analyses. The poultry viscera meal (PVM) was donated by a Brazilian pet food company and stored in its original packaging, under refrigeration (-20ºC) until future analysis.

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2.2 Moisture, a_w, and pH

Moisture content, pH, and water activity were measured for BSFMd and PVM. All the analytical determinations were carried out in triplicate. The moisture content was determined according to method 930.15 (AOAC, 2006), which consists of drying the samples at 105°C overnight (for at least 8 hours), and was calculated as the difference between the initial weight (wet mass) and the final weight (dry mass), divided by the wet mass (Equation 1).

$$U(\%) = \frac{weight\ initial - weight\ end}{weight\ initial} * 100 \qquad \text{Eq. 1}$$

Water activity was measured with the Aqualab® device (Decagon, USA), and the pH was determined by stirring 1 g of the medium in 10 mL of distilled water in a vortex mixer at maximum speed and measuring the pH of the supernatant with a pH meter (Tecnal, Brazil) (COSTA *et al.*, 2017).

2.3 Chitin content

The extraction and chitin content of BSFMd were evaluated according to (SOETEMANS; UYTTEBROEK; BASTIAENS, 2020) using demineralization, deproteinization (7 times), and washed with demineralized water. The values were expressed as the percentage of chitin content in the dry mass.

2.4 *In vitro* digestibility

The BSFMd and PVM were digested using an *in vitro* modified method in two digestive steps – gastric digestion and small intestinal phase (HERVERA *et al.*, 2009; SEO *et al.*, 2024), according to Figure 1.

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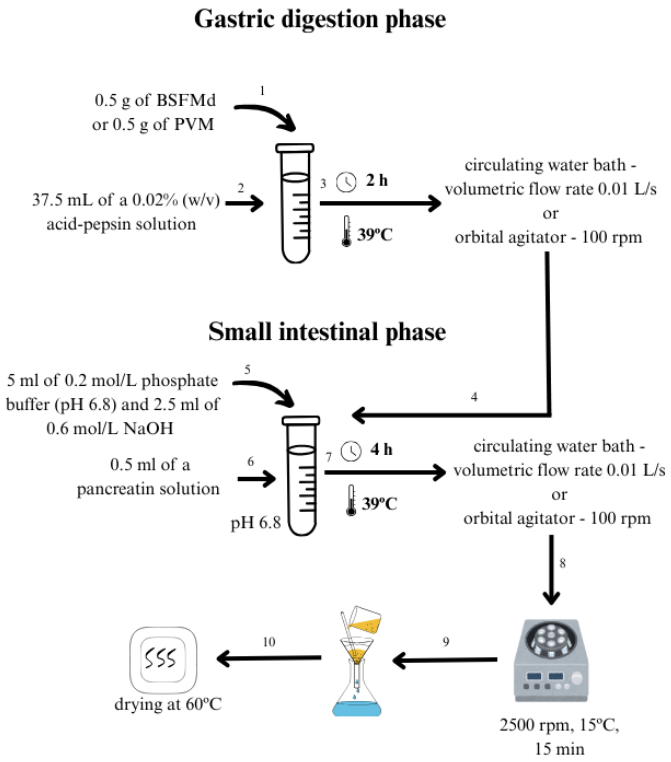
Figure 1 – Diagram of the *in vitro* digestibility methodology of BSFMd and PVM



Source: The authors (2025)

For the gastric digestion phase, a 0.5 ± 0.01 g BSFM defatted or PVM sample was weighed and added to Falcon tubes (50 ml) containing 37.5 ml of a 0.02% (w/v) acid-pepsin solution (pepsin 1:10000; Dinâmica dissolved in HCl 0.0744 N) prepared according to the adapted method described by (ZANOTTO; BELLAVER, 2005). The samples were incubated for: i) 2 h in a circulating water bath (MARCONI MA470) at 39ºC, volumetric flow rate 0.01 L/s, ii) for 2 h in an orbital agitator (100 rpm) at 39°C. For the small intestinal phase, the pH of the samples was adjusted to 6.8 with 1 mol/L NaOH or 1 mol/L HCl, and 5 ml of 0.2 mol/L phosphate buffer (pH 6.8) and 2.5 ml of 0.6 mol/L NaOH were added and mixed on a vortex mixer (HERVERA *et al.*, 2007, 2009). Then, 0.5 ml of a pancreatin solution (100 mg/ml) (Êxodo Científica: protease 25 U/mg; amylase 25 U/mg; lipase 2 U/mg) was added (HERVERA *et al.*, 2007, 2009) and the Falcon tubes were incubated in a circulating bath for 4 h at 39ºC or in an orbital agitator (100 rpm) for 4 h at 39ºC. The samples were centrifuged (2500

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rpm, 15ºC, 15 minutes) and filtered (ZANOTTO; BELLAVER, 2005). The precipitate was dried at 60 °C until a constant mass was obtained. The assays were performed in triplicate. The crude protein levels and digestibility were measured in the indigested fraction from the *in vitro* simulation of digestion and calculated (Equation 2) according to (SEO *et al.*, 2024).

$$in\ vitro\ CP\ (\%) = \frac{Food\ CP\ (g) - Undigested\ fraction\ CP\ (g)}{Food\ CP\ (g)} * 100 \qquad \text{Eq. 2}$$

The final digestibility was calculated according to Equation 3.

$$digestibility\ (\%) = \frac{in\ vitro\ CP\ (\%)}{Original\ sample\ CP} * 100 \qquad \text{Eq. 3}$$

The crude protein content of BSFMd and PVM were determined using the Kjeldahl method (INSTITUTO ADOLFO LUTZ, 2008). The determination of protein solubility of BSFMd was evaluated by AOAC and modified according to (ZANOTTO; BELLAVER, 2005).

2.5 Statistical analysis

The digestibility results were represented as mean ± standard error. Data were first evaluated for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) before statistical analysis. Digestibility data showed a normal distribution but heteroscedastic behavior; therefore, a one-way analysis of variance using the Kruskal–Wallis test was performed, and when significant differences were detected, Welch's test was applied, adopting a significance level of 5% ($p < 0.05$).In contrast, moisture, water activity (aw), and pH data met the assumptions of normality and homoscedasticity and were thus subjected to a one-way ANOVA, followed by Tukey's test for mean comparisons. All statistical analyses were conducted using the Jamovi® software.

3 RESULTS AND DISCUSSION

3.1 Moisture, a_w, and pH of the BSFMd and PVM

Moisture, a_w, and pH (Table 1) are important parameters of meal quality. Moisture is one of the most influential parameters in assessing the stability of a product, and can affect its storage,

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packaging, and processing. The ideal moisture content for animal meals is estimated to be between 4 and 6%, and cannot exceed 10%, according to the legislation that recommends the maximum value for flours, of up to 15.0% (MINISTÉRIO DA AGRICULTURA E PECUÁRIA, 2020). The moisture contents of BSFMd (5.88%) and PVM (4.09%) differed significantly ($p \leq 0.05$), but complied with the ideal parameters required by Brazilian legislation.

Table 1 – Values of moisture, aw, and pH of black soldier fly meal defatted (BSFMd) and poultry viscera meal (PVM; mean ± standard error)

Sample	Moisture (%)	a_w	pH
BSFMd	$5.88^a \pm 0.07$	$0.45^a \pm 0.02$	$8.86^a \pm 0.04$
PVM	$4.09^b \pm 0.09$	$0.42^a \pm 0.01$	$5.86^b \pm 0.02$

Source: The authors (2025)

In dry foods, such as meals, moisture levels should be low, since they are normally stored at room temperature, a condition that favors the occurrence of many chemical and biological reactions (SANT'ANNA *et al.*, 2019). However, excessively low moisture content may indicate overprocessing, such as overcooking or overdrying. This condition can lead to amino acid degradation, thereby reducing the nutritional quality and digestibility of proteins. Additionally, under certain conditions, overprocessing may promote the formation of compounds with toxic potential or adverse health effects. It is noteworthy that BSF whole, freshly harvested larvae exhibit a high moisture content, typically around 70%. Various drying technologies can be employed to achieve the desired low moisture levels, including hot-air, microwave, freeze, spray, and infrared drying.

The average water activity (*aw*) values recorded for BSFMd and PVM (Table 1) showed no significant difference ($p \leq 0.05$), indicating good stability of the resulting meal, consistently remaining below 0.60. This range is sufficient to prevent microbial growth and effectively suppress degradative processes such as enzymatic reactions, browning, and lipid oxidation (KAREL, 1989). The *aw* levels observed were also below the minimum thresholds required for the development of most microorganisms, including bacteria (>0.90), yeasts (>0.80), molds (>0.60), halophilic bacteria (>0.65), and osmophilic yeasts (>0.62), as reported by (FERREIRA NETO; FIGUEIRÊDO; QUEIROZ, 2005). Although the values fall within the ideal range for meal or flour preservation (<0.60), the observed *aw* of 0.45 and 0.42 suggests a slight degree of overprocessing, as the target

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was a value closer to 0.60. This indicates that slightly more energy may have been used than was strictly necessary. However, it is important to note that after a certain point in the dehydration process, even small reductions in moisture content can cause a sharp decrease in water activity. As a result, the margin between achieving the desired *aw* and overprocessing becomes very narrow. Therefore, implementing this drying technique on an industrial scale requires accurate monitoring and precise control of processing conditions, along with tailored adjustments for each raw material (VANDEWEYER *et al.*, 2024). Furthermore, the *aw* not only influences microbial stability but also affects other deteriorative mechanisms, such as lipid oxidation and non-enzymatic browning. Research has shown that lipid oxidation in low-moisture foods tends to be lowest at *aw* values between 0.20 and 0.30 (VU *et al.*, 2020). Consequently, for specific applications, including those involving dried insect-based and animal products, targeting final *aw* values below 0.60 may be beneficial for preventing additional quality degradation, even if this results in longer processing times. Similar PVM values for *aw* were observed by (VOLPATO *et al.*, 2023).

PVM showed an acidic pH (5.86) (Table 1) which was significantly different ($p \leq 0.05$) from that of BSFMd, and their levels were lower than the values described in the literature (COSTA; ROMANELLI; TRABUCO, 2008). The alkaline pH (8.86) of BSFMd (Table 1) resembles the data obtained by (OLIVEIRA, 2025). Previous studies have indicated that this value directly reflects the characteristics of the environment and substrate used for larval rearing. Even when the initial pH of the substrate is acidic or neutral, there is a tendency toward alkalinization throughout the process, which is attributed to the release of ammonia and other alkaline compounds by larvae (CATTANEO *et al.*, 2024). It is also noteworthy that the method used to kill BSF larvae can significantly influence their final pH (ZHEN *et al.*, 2020).

3.2 *In vitro* digestibility

The methodological adaptation implemented in the present study, which involved the reduction in the duration of the gastric phase and insertion of the small intestinal phase, and the comparative evaluation between the orbital shaker and a circulating water bath in the *in vitro* protein digestibility assay (Figure 2), resulted in significantly higher digestibility values for BSFMd in the circulating water bath, reaching 96.4%, while for PVM, this system (30.35%) did not show a significant difference from the orbital shaker (30.00%).

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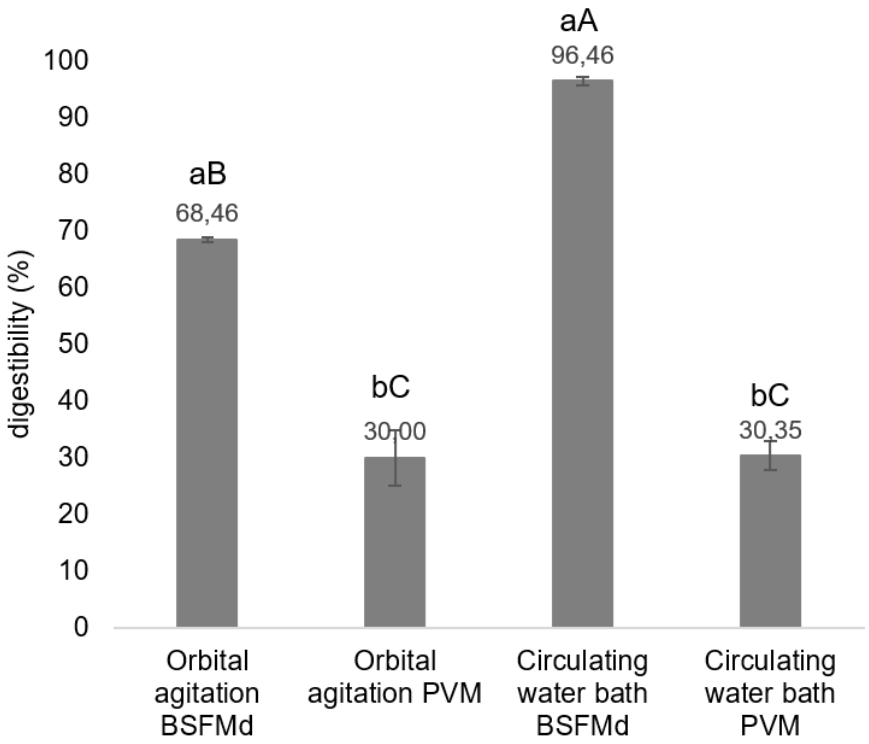
Figure 2 – *In vitro* digestibility of defatted black soldier fly meal and poultry viscera meal in two laboratory devices: circulating water bath and orbital shaker.



Equal capital letters indicate no significant differences between the samples and equipment at the 5% significance level. Equal lowercase letters within the same column indicate no significant differences at the 5 % significance level. Source: The authors (2025)

When evaluating the *in vitro* digestibility of the meals in the same equipment (Figure 2), BSFMd and PVM showed a significant difference ($p \leq 0.05$). The *in vitro* digestibility of BSFMd was higher (29% higher) in a circulating bath (Figure 2) and differed significantly ($p \leq 0.05$) from that obtained by orbital agitation. However, the *in vitro* digestibility of PVM was not significantly different ($p \leq 0.05$) from the circulating water bath and orbital agitation. PVM showed lower digestibility values, independent of the equipment used, and was significantly different ($p \leq 0.05$) from BSFMd in all assays evaluated. Poultry byproduct meal is one of the most commonly used animal-protein sources because of its high protein content and digestibility (FAGBENRO; FASAKIN, 1996). However, the reduction in the test time may not have been sufficient to digest animal protein, resulting in a

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reduction in its digestibility. Despite being a source of amino acids, animal-based meals require a longer digestion time (COSTA; ROMANELLI; TRABUCO, 2008).

Although the use of the orbital shaker demonstrated lower efficiency for BSFMd, the values obtained are consistent with those reported by (PENAZZI *et al.*, 2021), who evaluated the *in vivo* digestibility of *Hermetia illucens* (BSF) in dogs. Conversely, the high digestibility values observed with the circulating water bath are comparable to those reported for the *in vitro* digestibility of proteins from other freeze-dried and ground insects, such as the housefly (*Musca domestica*, 93.3%) and *Tenebrio molitor* (92.5%) (BOSCH; VERVOORT; HENDRIKS, 2016). In the same study, the *in vivo* digestibility of BSF was reported to be 87.7%, representing an intermediate value compared to those observed in the present study. Regarding methodological differences, it is noteworthy that, unlike the procedure adopted in the present study, the *in vitro* digestibility assay conducted by (BOSCH; VERVOORT; HENDRIKS, 2016) was performed under constant magnetic stirring, whereas in the present study, an orbital shaker or a circulating water bath was used.

The total protein content of defatted Black Soldier Fly meal was 54.6% whereas that PVM was 59.8%. The *in vitro* digestibility values reported in this study (Figure 2) refer specifically to the digestibility of the protein fraction relative to the total protein content in the sample, without considering the chitin content. The chitin content in the BSFMd dry matter (5.73 ± 0.32 %) is in line with the range cited (between 2 and 11%) in the literature for BSF (SOETEMANS; UYTTEBROEK; BASTIAENS, 2020). This linear polymer of -(1–4) N-acetylglucosamine is specifically described in BSF flies and larvae (BOSCH; VERVOORT; HENDRIKS, 2016; SOETEMANS; UYTTEBROEK; BASTIAENS, 2020), and the degradation of chitin in the gastrointestinal tract of dogs is described as low. This may make chitin a non-fermentable fiber, due to the low microbial chitinolytic activity observed in dogs (BOSCH; VERVOORT; HENDRIKS, 2016). When applying the N fraction from chitin to total N for this meal, assuming a 0% digestibility of chitin (BOSCH; VERVOORT; HENDRIKS, 2016), and considering BSF constituted 6.22% N (CHEN *et al.*, 2025), the *in vitro* digestibility would be 69.15 ± 0.38% for orbital agitation and 97.14 ± 0.66% for circulating water bath, maintaining the significant difference ($p \leq 0.05$) for the sample in different equipment.

Although (BONOMINI; PRANDI; CALIGIANI, 2024) pointed out that lipids and chitin can influence the protein digestibility of *Hermetia illucens* (BSF) larvae, only defatted larvae were used for comparison in the present study (Table 2). It is known that the digestibility of insect protein can

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be influenced by its lipid fraction. According to (BONOMINI; PRANDI; CALIGIANI, 2024), lipid oxidation can promote protein aggregation, thereby reducing protein digestibility by limiting the accessibility of digestive enzymes. The absence of fat in BSFMd may have contributed to the protein digestibility values observed in this study (Figure 2).

Table 2. Comparison of the *in vitro* digestibility of Black Soldier Fly larva meal under different conditions and laboratory equipment

Equipment	**Temperature**	**Time**	**Protein Digestibility *in vitro***	**Reference**
Orbital Shaker-Incubator	37ºC	5h	64,3%	(BONOMINI; PRANDI; CALIGIANI, 2024)
Water bath shaker with constant agitation	37ºC	15min + 3h + 3h	90%	(RODRÍGUEZ-RODRÍGUEZ *et al.*, 2023)
Only describe digestion	37ºC	4h	78,74%	(XU *et al.*, 2023)
Thermal shaker	37ºC	6h	75%	(TRAKSELE *et al.*, 2021)
Continuous stirring	38ºC	24h + 96h	66 – 68,7%	(MARONO *et al.*, 2015)
Constant magnetic stirring	39ºC	6h	87,7%	(BOSCH; VERVOORT; HENDRIKS, 2016)
Constant magnetic stirring	39ºC	6h	89,7	(BOSCH *et al.*, 2014)

Source: The authors (2025)

Although numerous studies have described *in vitro* digestibility assays in the literature (HERVERA *et al.*, 2007, 2009; SEO *et al.*, 2024), a standardized protocol specifically designed for dogs remains unavailable, making method selection challenging. The AOAC protocols for general protein digestibility recommend a single-stage methodology (pepsin solution, 16 hours at 45°C) for analysis in meals; however, studies in the literature, for dogs, describe a two-stage digestion model, in which the gastric phase involves incubation with a pepsin solution for 2 hours at 39°C (HERVERA *et al.*, 2007, 2009). The duration of this gastric phase (2 hours), as applied in the present study, is

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based on protocols originally developed for human digestion models (BRODKORB *et al.*, 2019). Several studies have employed human digestion protocols to evaluate the digestibility of *Black Soldier Fly* meal for both human and animal nutrition purposes, often incorporating an additional step (BONOMINI; PRANDI; CALIGIANI, 2024; TRAKSELE *et al.*, 2021). The temperature used in the present study (39°C) is consistent with *in vitro* assays designed for companion animals (SEO *et al.*, 2024) and reflects an adaptation to the body temperature range of adult dogs (37.6–39.4°C) following physical activity (SIQUEIRA *et al.*, 2005; VILLANOVA JUNIOR *et al.*, 2020).

Gastric digestion involves a series of peristaltic contractions of the stomach wall that serve to mix and break down food particles (LI; FORTNER; KONG, 2019). This peristaltic motion is among the most challenging physiological processes to replicate using standard laboratory equipment. However, the literature indicates that the canine stomach exhibits gastric motility and physiological emptying patterns that are relatively similar to those of the human small intestine (LEE *et al.*, 2017). Reported peristaltic velocities range from approximately 1.5 to 5.0 mm/s, depending on stomach positioning and measurement methodologies (LI; FORTNER; KONG, 2019). As shown in Figure 2, the use of a circulating water bath system resulted in a higher *in vitro* digestibility value (96.46%) for BSFMd, while for PVM, this system (30.35%) did not have a significant difference ($p \leq 0.05$) from the orbital shaker (30.00%). The relatively low water flow rate (0.01 L/s) within the apparatus may have enhanced heat transfer to the sample, contributing to stable enzyme activity and improved digestion efficiency. This type of equipment is particularly suitable for applications requiring uniform and consistent temperature control, such as enzymatic reactions (BALOGUN *et al.*, 2020). However, some amino acids are more difficult to digest. In addition to the influence of raw material composition, amino acid digestibility can be affected by the thermal processing of PVM. Insufficient processing of PVM may result in incomplete hydrolysis, resulting in lower nutrient digestibility values. The Maillard reaction during thermal processing reduces lysine digestibility, while reactions between the carboxyl groups of aspartic and glutamic acids and the α-amino group of lysine can also occur during thermal processing. The resulting products of these reactions contain bonds that may be resistant to enzymatic hydrolysis (EYNG *et al.*, 2010), which may have contributed to the lower enzymatic digestibility levels of PVM in this study.

To compare the effectiveness of the proposed methodology, the *in vitro* digestibility of BSFMd was evaluated using the AOAC method and modified (ZANOTTO; BELLAVER, 2005). In this

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method, 73.14% of the protein was digested using a 0.02% pepsin solution in an orbital shaker (45°C, 16h, 15 rpm), while for PVM, the AOAC method and modified (pepsin 1:10000, 0.02% in HCl (0.075 $M.L^{-1}$) showed 60% of digestibility (COSTA; ROMANELLI; TRABUCO, 2008). The results indicate that animal-based meals require extended degradation times due to their compositional characteristics, and that assays with shorter digestion periods may underestimate their digestibility. Under the conditions applied in this study, BSFMd showed effective digestibility in both equipment. These results highlight the importance of aligning assay protocols with the physicochemical properties of the tested meals.

Orbital shakers are among the most commonly used devices in *in vitro* digestibility assays. Their simple and functional design enables cost-effective, high-throughput testing (SHIUE *et al.*, 2020). During operation, the enzyme-substrate mixture rotates at the angular velocity of the shaker platform, generating multiaxial shear forces at the center and uniaxial shear forces at the edges of the Erlenmeyer flask (WARBOYS; GHIM; WEINBERG, 2019). Depending on the agitation speed, this dynamic can mimic the peristaltic movements of the gastrointestinal tract. Notably, the agitation speed reported in previous studies shows considerable variability, ranging from the absence of specification (described only as continuous agitation) to values around 100 rpm, and up to 150 rpm (BOSCH; VERVOORT; HENDRIKS, 2016; XU *et al.*, 2023; ZANOTTO; BELLAVER, 2005). This inconsistency prompted the standardization of the agitation speed to 100 rpm in the present study. However, further research is needed to investigate the specific influence of shaker speed on protein digestibility.

3 CONCLUSIONS

The results of this study demonstrated that the method and the type of equipment employed directly influence protein digestibility outcomes. Although there is limited information regarding direct comparisons of heat transfer efficiency between water baths and orbital shakers, our data suggest that water baths provide more efficient heat transfer for meals with easy digestion, probably due to the direct contact of the vessel with heated water. In contrast, orbital shakers, despite their widespread use in laboratory settings, may present thermal limitations, as they rely on air as the main medium for heat conduction, and higher assay time. The proposed adjustments to the gastric

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phase, when integrated with the intestinal phase, proved to be a practical refinement in low-complexity meals, reducing assay time while maintaining reliability. Thus, these observations highlight the importance of advancing methodological refinements and encourage further efforts to establish patterns for digestibility tests.

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