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# Asymptotic estimated digestibility, a new indicator of black soldier fly (*Hermetia illucens*) conversion efficiency in relation to larval density

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1 **Asymptotic estimated digestibility, a new indicator of black soldier fly (*Hermetia illucens*)**  
2 **conversion efficiency in relation to larval density**

3  
4 **Short title**

5 Asymptotic estimated digestibility, a new measure of conversion efficiency

6  
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23  
24 **Abstract**

25 Black soldier fly larvae (BSFL) are highly efficient at converting organic substrates into insect  
26 biomass suitable for livestock nutrition. BSFL conversion efficiency depends on the type and  
27 profile of their feed, and formulation approaches use macronutrient contents to reach a targeted  
28 zootechnical performance. However, digestibility varies between ingredients and BSFL  
29 digestibility data is lacking due to methods unsuitable for insects living in their feed. This study  
30 defined Estimated Digestibility (ED) as the difference between distributed feed and frass  
31 macronutrient weight, divided by macronutrient weight in distributed feed, and explored its  
32 variation with larval density. Using the same amount of chicken feed, same feeding time and 0  
33 to 29 larvae/cm<sup>2</sup>, ED of dry matter (DM), starch, nitrogen, ether extract (EE), neutral detergent  
34 fibre, acid detergent fibre, acid detergent lignin, ash and energy were evaluated, along with  
35 survival rate, feed conversion ratio (FCR) and larval individual weight gain on a fresh and dry  
36 basis. ED of DM, starch, nitrogen, EE and energy increased with larval density following an  
37 asymptotic trend. ED of ash increased between 0 and 11.4 larvae/cm<sup>2</sup> but decreased for higher  
38 densities. Using a logistic model, asymptotic ED of DM (80.3±1.3%), starch (99.0±2.3%),  
39 nitrogen (78.6±1.1%), EE (95.3±1.5%), ash (58.4±1.0%) and energy (80.6±1.2%) were  
40 determined. No effect of areal density on ED of fibre fractions was detected, presumably due  
41 to an inappropriate dosage method. Survival rate and individual weight gain peaked at densities  
42 below 5 larvae/cm<sup>2</sup>, FCR (dry to fresh) was improved with increasing density, while FCR (dry  
43 to dry) was optimised at 11.4 larvae/cm<sup>2</sup>. Asymptotic ED is a new indicator corresponding to  
44 the maximal fraction of macronutrients potentially digestible by BSFL. It should be used to  
45 develop a diet formulation approach based on digestible rather than crude macronutrient  
46 contents. Further studies should focus on evaluating asymptotic ED in different ingredients.

47  
48 **Keywords**

49 insect nutrition, formulation, macronutrient, alternative protein, Diptera

50

51 **Conflict of interest and funding statement**

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55 not interfere with the conclusions of the study.

## 1. Introduction

Black soldier fly larvae (BSFL; *Hermetia illucens* (L.); Diptera: Stratiomyidae) can quickly convert organic side-streams and biowaste into valuable proteins and lipids (Barragan-Fonseca *et al.*, 2017) which can be used in animal nutrition (Bosch and Swanson, 2021; Dörper *et al.*, 2021) and have been increasingly produced and studied by insect stakeholders. Both profitability and sustainability of BSFL production depend on their ability to convert feed provided to the insects into larval biomass. Conversion efficiency varies namely with feed, rearing conditions and genetics (Greenwood *et al.*, 2021). Significant variability of zootechnical performances (development time, prepupal weight, larval protein and lipid content, etc.) due to the type of feed and its macronutrient composition (mostly carbohydrates, proteins, lipids, fibres and ash) has been extensively documented (Gold *et al.*, 2018; Hopkins *et al.*, 2021). Barragan-Fonseca *et al.* used the Nutritional Geometry Framework to comprehensively map the impact of feed protein and carbohydrate content on BSFL performance. This work laid the ground for a conceptual diet formulation approach that would allow targeting the appropriate macronutrient profile to reach the expected zootechnical performance of BSFL. However, BSFL fed diets formulated with the same protein and carbohydrate contents but different ingredients showed bioconversion rates ranging from 14.5% to 31.8% (Gold *et al.*, 2020b). This variability is namely due to the use of crude macronutrient fractions for the formulation instead of the more appropriate digestible macronutrient contents. Indeed the digestibility of a macronutrient can significantly vary from one ingredient to another, as extensively documented in conventional livestock species such as pigs (Stein *et al.*, 2016) or poultry (Zaefarian *et al.*, 2015). Still, data on digestibility by BSFL are lacking.

Apparent Digestibility in conventional livestock species is defined on a dry weight basis as the amount of the considered macronutrient in ingesta subtracted by macronutrient in faeces, divided by macronutrient in ingesta (Barret *et al.*, 2005). Its calculation requires (1) estimating the amount of feed ingested (usually by comparing the weights of distributed and refused feed) and (2) collecting pure faeces (not contaminated with feed, urines, bedding, etc.) to perform macronutrient content analysis. BSFL feed in group on moist substrates that also constitute their living environment, and excreta cannot be separated from non-ingested feed, exuviae abandoned during moulting and possibly dead individuals (Seyedalmoosavi *et al.*, 2022). All these components are mingled together in a powdered material called frass. Therefore, both conditions to calculate Apparent Digestibility are challenging to meet. A possible partial solution is to attach an excreta collection device to the bottom end of a larva. Still, collected amounts of pure excreta are tiny and this method is impractical on many larvae (Gold *et al.*, 2020a). Furthermore, unlike mammals that produce faeces and urines separately, insects' metabolic wastes are released in the hindgut via Malpighian tubules (Weihrauch and O'Donnell, 2021). Hence their excreta consists of a mix of faeces (undigested feed material) and urines (metabolic wastes). Accordingly, (Waldbauer, 1968) highlighted the difference between Approximate Digestibility, which is calculated based on macronutrient weight in ingesta and in excreta, and Apparent Digestibility, which is similar but requires subtracting the weight of macronutrients in urines from total excreta to obtain the weight of macronutrients in faeces and thus be consistent with the definition of Apparent Digestibility in conventional livestock species. This refinement is suitable when insects' excreta can be separated from non-ingested feed, which is not the case in BSFL. Yet, some authors analysed frass to gain insight into BSFL nutrition but imprecisely referred to Approximate Digestibility (Klammsteiner *et al.*, 2021) (Permana and Ramadhani Eka Putra, 2018). This misuse is due to confusion between distributed and ingested feed and an unclear definition of BSFL excreta and frass (Bosch *et al.*,

2020). In the present paper, we consider excreta as a mix of faeces and urines, and we use the official definition of frass given by the European Commission: “the mixture of insect excrements with parts of dead insects and feeding substrate” (European Commission, 2021). Hence, we propose a new definition for a digestibility approximation specific to insects growing in their feed, like BSFL: Estimated Digestibility (ED) is calculated by dividing the difference between distributed feed and frass macronutrient weight by the amount of macronutrient in distributed feed.

Frass analyses are often performed in waste management studies to quantify “waste reduction” of dry matter (DM), also called “overall degradation”. These terms have the same definition as ED of DM but are imprecise and unsuitable in the field of animal nutrition. ED of DM ranges from 9 to 85% depending on the type of feed but also rearing conditions, such as feed moisture content, feeding duration or larval density (Lalander *et al.*, 2019; Rehman *et al.*, 2017). The latter’s effect on ED has only been indirectly investigated, with results suggesting that ED is positively associated with larval density (Brits, 2017; Permana and Ramadhani Eka Putra, 2018). This is supported by the hypothesis that a higher number of BSFL is more likely to consume a given amount of feed entirely. It can be assumed that a high density would ultimately result in reaching a maximal ED, corresponding to the full potential of the feed that BSFL can digest. However, this has never been documented. In contrast to ED of DM, values of ED of starch, protein, lipid, fibre and ash are scarce to non-existent.

Consequently, this study proposes a method to measure the maximal ED of a feed for BSFL. Null to extremely high larval densities were used, and an extensive analysis of remaining macronutrients in frass was performed to challenge the impact of density on the ED of each macronutrient. This approach aims to consider the maximal ED of a macronutrient as the digestibility coefficient of that macronutrient in that ingredient when developing a BSFL diet formulation tool.

## 2. Materials and methods

Two trials were performed with slight changes between Trial 1 and Trial 2.

### 2.1. Insects and feed used

BSF starter larvae were obtained from Agronutris (Toulouse, France). Before the trial period, neonate larvae were fed chicken feed (CF) (Maisadour - Sud-Ouest Aliment VT100 Poulet Jaune Démarrage, Haut-Mauco, France; Table 1) rehydrated to  $64 \pm 0.5\%$  moisture and were kept at around  $28^\circ\text{C}$  and 55% relative humidity (RH) for about seven days. They were sent to the laboratory, where they were successively sieved with 2.4 mm and 1.0 mm diameter sieves to standardise their size and remove most of the frass. The larvae were then gently homogenised by hand and three samples of about 1 g were taken and counted manually to measure the average weight of a starter larva (4.8 and 4.9 mg for Trial 1 and Trial 2, respectively) and to calculate the overall weight of larvae to use for each experimental density.

Table 1: Analytical chemical composition of the chicken feed used to feed black soldier fly larvae

	Starch	Nitrogen	Ether extract	Ash	NDF	ADF	ADL	Gross energy (kJ/g DM)
<b>Content (% DM)</b>	$42.1 \pm 1.9$	$3.4 \pm 0.2$	$2.8 \pm 0.1$	$6.1 \pm 0.2$	$14.4 \pm 0.9$	$5.7 \pm 0.7$	$1.3 \pm 0.9$	$15.8 \pm 0.5$

152 *Mean ± standard deviation. DM: dry matter; NDF: neutral detergent fibre; ADF: acid*  
 153 *detergent fibre; ADL: acid detergent lignin. Values were obtained on five samples for NDF,*  
 154 *ADF and ADL, four for starch and nitrogen and two for ether extract and ash.*

## 156 2.2. Experimental design

157  
 158 Experiments were conducted in 16.5×10.6×7 cm plastic containers (Pro-Jet BG110, Saint-  
 159 Ouen-l'Aumône, France) individually placed in a light pantyhose and closed with a lid with an  
 160 opening of 16×10 cm to prevent larvae from escaping while allowing air circulation. The feed  
 161 used was CF rehydrated to 68±0.5% moisture using hot tap water and mixed thoroughly  
 162 (Electrolux Professional Bermixer Pro 450 W, Saint-Denis, France; maximum power for 2:30  
 163 min) right before distribution. Each container received a unique input of feed for the whole  
 164 experimental period, consisting of a fresh weight (FW) of 420±1 g of CF, i.e. 134 g of dry  
 165 weight (DW), and a specified amount of starter larvae, covering an extensive range of larval  
 166 density (Table 2). Each larval density was replicated twice in Trial 1 and thrice in Trial 2.  
 167 Investigated densities were slightly changed from Trial 1 to Trial 2.

168  
 169 Table 2: Experimental larval densities

Number of larvae	Areal density (larvae/cm <sup>2</sup> )	Mass density (larvae/kg DM)	Feed availability (mg DM/larva)	Trial
0	0	0	-	1 & 2
400	2.29	2,976	336.0	1 & 2
600	3.43	4,464	224.0	1
700	4.00	5,208	192.0	2
800	4.57	5,952	168.0	1
1,000	5.71	7,440	134.4	1 & 2
1,500	8.57	11,161	89.6	1 & 2
2,000	11.43	14,881	67.2	1 & 2
3,000	17.14	22,321	44.8	1 & 2
5,000	28.57	37,202	26.9	1 & 2

170 *DM: dry matter*

171  
 172 Containers were placed in a climatic chamber at 28±0.5 °C and 75±2 % RH, with a L12:D12  
 173 photoperiod (Trial 1: Xylem Analytics WTW TS 1006-i, Weilheim, Germany; Trial 2: Percival  
 174 Scientific DR-36VL, Perry, USA). In both trials, larvae were kept in their container and  
 175 allowed to feed for seven days. Containers were relocated eight times to eliminate any effect  
 176 related to possible climatic heterogeneity within the chamber. No additional feed input,  
 177 sampling or mixing was performed during the trial period.

## 178 2.3. Sampling and analysis

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 180  
 181 At the end of the seven-day feeding period, all containers were removed from the chamber and  
 182 weighed. Larvae were manually separated from the frass. Depending on the stickiness of the  
 183 frass, they were either sieved or removed with forceps. They were rinsed with tap water in a  
 184 fine sieve, dried with paper towel and weighed collectively. The FW of frass was calculated as  
 185 the final weight of the container before removing the larvae, subtracted by the weight of the  
 186 cleaned larvae and the weight of the empty container.

187

188 Frass, including material sticking to the inner side of the container, was manually collected and  
 189 mixed with a spatula. DM content was determined on a 4 g sub-sample using a thermobalance  
 190 set at 120°C (Radwag MA 50.R, Radom, Poland). The chemical composition of frass and CF  
 191 was analysed on oven-dried (Mettler UE500, Schwabach, Germany; 60°C until constant  
 192 weight) and ground (Retsch ZM 1000, Haan, Germany; 1 mm grid) samples. Starch content  
 193 was determined by colourimetry (Roche Cobas Mira S, Basel, Switzerland; Randox GLUC-  
 194 PAP Kit, Crumlin, UK) after enzymatic hydrolysis (Merck, 10115-5G-F, Darmstadt, Germany)  
 195 according to Faisant *et al.*; nitrogen content was determined using the Dumas method  
 196 (Elementar EA Vario Micro Cube, Langensfeld, Germany); ether extract (EE) was  
 197 determined with the Soxhlet method using diethyl ether (Tecator Soxtec System HT6,  
 198 Höganäs, Sweden); ash content was determined gravimetrically by complete combustion at  
 199 550°C for eight hours (Nabertherm furnace, Lilienthal, Germany); neutral detergent fibre  
 200 (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using the  
 201 filter bag technique (Ankom 220 Fibre Analyzer, Macedon, NY, USA) according to the Van  
 202 Soest method, after treatment with acetone,  $\alpha$ -amylase and pronase to prevent interactions with  
 203 excess lipids, starch and proteins respectively (Godin *et al.*, 2010). The gross energy of each  
 204 sample was calculated using a coefficient of 17.17 kJ/g for starch and NDF, 23.45 kJ/g for  
 205 proteins (defined as  $N \times 6.25$ ) and 38.94 kJ/g for EE (Vaissaire, 2018). ED of DM, starch,  
 206 nitrogen, EE, ash, NDF, ADF, ADL and energy was calculated for each container using  
 207 Equation 1, where A stands for the considered macronutrient:  
 208

$$209 \text{ Estimated digestibility of } A = \frac{\text{Weight of } A \text{ in distributed feed} - \text{Weight of } A \text{ in frass}}{\text{Weight of } A \text{ in distributed feed}} \times 100 \quad (1)$$

210  
 211 For each container, a sample of 30 larvae was weighed on a precision balance (Sartorius  
 212 AZ153, Göttingen, Germany,  $\pm 0,001$  g) to calculate the mean individual fresh weight gain and  
 213 estimate the final number of larvae. The survival rate was calculated as the ratio between final  
 214 and initial numbers of BSFL. Feed conversion ratio (FCR dry to fresh) was calculated as the  
 215 ratio between DW of feed distributed and fresh larval weight gain. Larvae were killed by  
 216 freezing at -20°C. Due to improper sample management, no further analysis was performed on  
 217 Trial 1 larvae. Larvae from Trial 2 were oven-dried at 60°C until constant weight to calculate  
 218 their DM content. Mean individual dry weight gain and FCR on a dry basis (FCR dry to dry)  
 219 were also calculated for Trial 2.

#### 221 2.4. Statistical analysis

222  
 223 Collected data were analysed using R (version 4.1.2., R Core Team, 2021). The effect of areal  
 224 density and trial were assessed by ANCOVA and two-way ANOVA, with areal density  
 225 considered respectively as a quantitative or qualitative variable. For larval DM content, larval  
 226 DW gain and FCR (dry to dry), the effect of areal density was assessed by linear regression  
 227 and one-way ANOVA. The Bayesian Information Criterion (BIC) was used to select the best  
 228 model. As the effect of the trial cannot directly be related to a difference in treatment, it was  
 229 considered a random effect. Further treatments focused only on the impact of areal density and  
 230 considered all data together. Considering the pattern of scatter plots of ED of DM, starch,  
 231 nitrogen, EE, ash and energy, and of FCR (dry to fresh), the effect of areal density was  
 232 modelled for each variable using the nonlinear logistic model ‘logit’ from the ‘drc’ package.  
 233 The equation of the model is Equation 2, with  $-1/b$  the slope for  $X=0$ ,  $c$  the lower asymptote,  
 234  $d$  the upper asymptote and  $e$  the inflexion point:

$$235 Y = c + \frac{d-c}{1+\exp(b(X-e))} \quad (2)$$

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The logistic model was used to estimate the value of the asymptote and the areal densities required to reach 95% and 99% of the asymptotic performance (AD95 and AD99, respectively). Logistic models were also proposed for each trial separately.

### 3. Results

#### 3.1. Estimated digestibility

Figure 1 presents mean values and standard deviations of ED for each trial and areal density. For all macronutrients except NDF, ADF and ADL, two-way ANOVA provided lower BIC for ED than ANCOVA (Table 3). Areal density and interaction between density and trial had a significant effect ( $P < 0.05$ ) on ED of DM, starch, nitrogen, EE, ash and energy. In contrast, trial significantly impacted only the ED of DM, starch, ash and energy. The ANCOVA model resulted in the lowest BIC for ED of NDF and ADL, with a significant effect of trial for both, and of the interaction between trial and areal density for NDF only. Neither the ANCOVA nor the ANOVA model was significantly explanatory for ED of ADF.

In both trials, increasing areal density between 0 and 11.4 larvae/cm<sup>2</sup> positively affected ED of DM (from 3.0±2.0% to 79.7±1.8%), starch (from 24.6±7.8% to 98.7±1.2%), nitrogen (from -12.3±3.4% to 78.4±2.0%), EE (from 9.9±15.3% to 94.8±1.4%), ash (from -0.6±1.6% to 60.8±4.4%) and energy (from 11.0±2.3% to 80.3±1.2%). Higher densities showed similar ED values, resulting in an asymptotic trend, except for ED of ash that slightly decreased between 11.4 and 28.6 larvae/cm<sup>2</sup> from 60.8±4.4% down to 54.0±4.9%. Asymptotic ED determined by the logistic model were 80.3%, 99.0%, 78.7%, 95.3%, 58.4% and 80.6% for DM, starch, nitrogen, EE, ash and energy respectively (Table 4).

Areal density had no significant effect on ED of NDF, ADF and ADL. Therefore, no modelisation was proposed for these variables. The mean temperature recorded in the climatic chamber was 27.1°C for Trial 1 and 28.1°C for Trial 2.

Figure 1: (A) Mean estimated digestibility of dry matter, (B) starch, (C) nitrogen, (D) ether extract, (E) ash, (F) energy, (G) NDF (Neutral Detergent Fibre), (H) ADF (Acid Detergent Fibre) and (I) ADL (Acid Detergent Lignin) in black soldier fly larvae fed chicken feed and reared in seven days at varying densities in Trial 1 (n=2) and Trial 2 (n=3). Error bars: standard deviation.

Table 3: P-values of the linear models

Variable	Best model	P-value of the model	Areal density	Trial	Interaction
ED of dry matter	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	1.56E-04 ***	6.18E-03 **
ED of starch	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	4.86E-08 ***	5.02E-07 ***
ED of nitrogen	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	1.28E-01	2.92E-02 *
ED of ether extract	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	3.59E-01	4.13E-03 **
ED of ash	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	8.91E-05 ***	4.60E-02 *

ED of energy	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	9.43E-05 ***	1.54E-02 *
ED of NDF	ANCOVA	3.04E-03 **	4.95E-01	9.96E-04 ***	8.44E-02
ED of ADF	ANCOVA	1.81E-01	1.17E-01	9.32E-01	8.39E-01
ED of ADL	ANCOVA	7.46E-05 ***	1.47E-01	1.11E-04 ***	5.95E-03 **
Survival	ANCOVA	3.27E-06 ***	1.08E-02 *	7.39E-04 ***	5.46E-05 ***
Larval dry matter content	One-way ANOVA	9.35E-09 ***	9.35E-09 ***	-	-
FCR (dry to fresh)	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	1.40E-04 ***	1.60E-02 *
FCR (dry to dry)	One-way ANOVA	3.66E-10 ***	3.66E-10 ***	-	-
Individual fresh weight gain	Two-way ANOVA	< 2.2E-16 ***	< 2.2E-16 ***	1.61E-05 ***	3.35E-04 ***
Individual dry weight gain	One-way ANOVA	1.44E-10 ***	1.44E-10 ***	-	-

274 *Level of signification: P<0.001: \*\*\*; P<0.01: \*\*; P<0.05: \*. ED: Estimated Digestibility;*  
275 *NDF: Neutral Detergent Fibre; ADF: Acid Detergent Fibre; ADL: Acid Detergent Lignin;*  
276 *FCR: Feed Conversion Ratio.*

277

278 Table 4: Parameters (intercept and standard error) of the logistic model used to describe the  
279 effect of areal density on studied variables, and minimal estimated areal density to reach 95%  
280 and 99% of the asymptotic value (AD95 and AD99, respectively).

Variable	P-value of the model	b	c	d	e	AD95 (larvae/cm <sup>2</sup> )	AD99 (larvae/cm <sup>2</sup> )
ED of dry matter	0.00152	-0.509 (0.07) ***	-14.507 (9.076)	80.252 (1.268) ***	2.839 (0.46) ***	8.97	12.19
ED of starch	0.00117	-0.839 (0.213) ***	23.778 (5.149) ***	98.975 (2.344) ***	4.731 (0.287) ***	7.90	9.88
ED of nitrogen	0.06936	-0.611 (0.081) ***	-49.254 (17.129) **	78.601 (1.109) ***	1.461 (0.521) **	7.11	9.78
ED of ether extract	0.66621	-0.644 (0.115) ***	-14.538 (15.274)	95.259 (1.486) ***	1.93 (0.544) **	6.74	9.29
ED of ash	0.07319	-0.742 (0.099) ***	-7.784 (4.613)	58.386 (0.993) ***	2.801 (0.272) ***	6.95	9.17
ED of energy	0.00009	-0.591 (0.083) ***	0.956 (6.233)	80.576 (1.179) ***	3.171 (0.348) ***	8.13	10.92
FCR (dry to fresh)	0.03065	0.585 (0.064) ***	0.784 (0.019) ***	19.068 (32.653)	-2.012 (3.671)	8.48	11.23

281 *Level of signification: P<0.001: \*\*\*; P<0.01: \*\*; P<0.05: \*. ED: Estimated Digestibility;*  
282 *FCR: Feed Conversion Ratio.*

283

### 284 3.2. Life history traits and conversion efficiency

285

286 Figure 2 presents the results of life history traits and conversion efficiency. The ANCOVA  
287 model better explained survival rate. In contrast, the ANOVA model was more explanatory for

288 larval DM content, FCR (dry to fresh), FCR (dry to dry), individual FW gain and individual  
289 DW gain. For all these indicators, trial, areal density and the interaction of both had a  
290 significant effect, except for larval DM content, FCR (dry to dry) and individual DW gain,  
291 which were evaluated only in Trial 2.

292

293 Figure 2: (A) Survival rate, (B) larval dry matter content, (C) FCR (feed conversion ratio) on  
294 a fresh basis, (D) FCR on a dry basis, (E) individual fresh weight gain and (F) individual dry  
295 weight gain of black soldier fly larvae fed chicken feed and reared in seven days at varying  
296 densities in Trial 1 (n=2) and Trial 2 (n=3). Error bars: standard deviation.

297

298 The mean survival rate ranged between  $100.2 \pm 2.5\%$  and  $85.4 \pm 3.8\%$  and declined with  
299 increasing areal density. Values above 100% are a computational artefact due to a lack of  
300 accuracy in estimating the initial and final number of BSFL. FCR (dry to fresh) was  
301 continuously improved by increasing areal density, while FCR (dry to dry) only decreased  
302 between 2.29 and 11.43 larvae/cm<sup>2</sup> and worsened at higher densities. FCR (dry to fresh) was  
303 modelled with a logistic regression: lower asymptote, AD95 and AD99 were 0.78, 8.48  
304 larvae/cm<sup>2</sup> and 11.23 larvae/cm<sup>2</sup>, respectively. No converging logistic equation could be  
305 modelled for FCR (dry to dry). Larval DM content, individual FW gain and individual DW  
306 gain reached their maximal value at 4.00 larvae/cm<sup>2</sup> and decreased with rising areal density.

307

#### 308 4. Discussion

309

310 This study proposed a method based on frass analysis and variation of larval density to assess  
311 maximal ED in BSFL. The results showed that the ED of DM, starch, nitrogen, EE, ash and  
312 energy is positively affected by areal density, following a sigmoidal pattern that can be  
313 modelled with a logistic curve. High areal densities allowed more BSFL to collectively feed  
314 on the same amount of CF, leading to higher ED values, up to an asymptote that indicates the  
315 maximal fraction of feed BSFL can potentially digest. Indigestible macronutrients remaining  
316 in the frass were not biologically available for BSFL either because they are physically  
317 embedded in a resistant structure (such as proteins in a vegetal cell wall) or because larvae do  
318 not have the appropriate enzymatic equipment. In CF, BSFL were able to digest up to 80.3%,  
319 99.0%, 78.6%, 95.3%, 58.4% and 80.6% of DM, starch, nitrogen, EE, ash and energy  
320 respectively. ED of DM in CF in the literature ranged between 49.9 and 84.8% (Table 5). Brits  
321 explored the effect of density on ED of DM in kitchen waste and, although the experimental  
322 set-up did not allow for a clear asymptotic trend, a maximal value of 35.5% was obtained. This  
323 result suggests CF to be more digestible than kitchen waste. Values of ED of starch, nitrogen,  
324 EE, ash and energy in CF are unavailable in the literature, but some have been reported in other  
325 types of feed. ED of nitrogen ranged from 27% (in pig manure) to 72.4% (in soybean curd  
326 residue). In a mixture of yeast, wheat and potato by-products, ED of starch, nitrogen, lipids and  
327 energy were 55.8%, 37.9%, 72.9% and 46.8%, respectively (Table 5). Values of ED from the  
328 present study were the highest reported for these macronutrients. However, these results should  
329 be compared with caution due to a lack of standardisation in experimental conditions between  
330 studies, namely in terms of larval density and feeding time (Bosch *et al.*, 2020).

331

332 Table 5: Comparison of estimated digestibility in black soldier fly larvae (BSFL) for different types of feed and rearing conditions.

333 FW: fresh weight; DW: dry weight; DM: dry matter; CM: cattle manure; SBR: soybean curd residue.

Type of feed	Number of BSFL	FW of diet (g)	Moisture content	Larval density			Feeding time (day)	Number of feed supplies	Estimated Digestibility											Reference
				Areal density (larvae/cm <sup>2</sup> )	Mass density (larvae/kg DW)	Feed availability (mg DW/larva)			DM	Carbon	Nitrogen	Starch	Lipids	Energy	Phosphorus	Potassium	Cellulose	Hemicellulose	Lignin	
Chicken feed	600	1,300	61.5%	-	1,199	834	15	1	70.0%	-	-	-	-	-	-	-	-	-	-	(Gobbi <i>et al.</i> , 2013)
	200	310.7	58.8%	0.56	1,563	640	-	Several	84.8%	-	-	-	-	-	-	-	-	-	-	(Lalander <i>et al.</i> , 2019)
	80	48.0	60.0%	1.81	4,167	240	9	3	67.7%	-	-	-	-	-	-	-	-	-	-	(Gold <i>et al.</i> , 2020b)
	500	400	70.0%	2.40	4,167	240	9	4	49.9%	-	-	-	-	-	-	-	-	-	-	(Broeckx <i>et al.</i> , 2021)
	80	41.0	60.0%	2.80	4,878	205	9	1	54.7%	-	-	-	-	-	-	-	-	-	-	(Cohn <i>et al.</i> , 2022)
	80	32.8	60.0%	2.80	6,107	164	9	1	58.5%	-	-	-	-	-	-	-	-	-	-	
Kitchen waste	330	990	75.1%	3.35	1,340	746	15	1	25.8%	-	-	-	-	-	-	-	-	-	-	(Brits, 2017)
	440	990	75.1%	4.47	1,786	560	15	1	32.9%	-	-	-	-	-	-	-	-	-	-	
	528	990	75.1%	5.36	2,144	467	15	1	35.5%	-	-	-	-	-	-	-	-	-	-	
	660	990	75.1%	6.70	2,680	373	15	1	35.0%	-	-	-	-	-	-	-	-	-	-	
	880	990	75.1%	8.93	3,573	280	15	1	33.8%	-	-	-	-	-	-	-	-	-	-	
	1,320	990	75.1%	13.40	5,359	187	15	1	34.2%	-	-	-	-	-	-	-	-	-	-	
CM	1,000	1,000	78.4%	-	4,630	216	24.3	1	25.8%	52.0%	32.0%	-	-	-	38.7%	-	51.0%	51.7%	35.0%	(Rehman <i>et al.</i> , 2017)
80% CM + 20% SBR	1,000	1,000	79.2%	-	4,808	208	22.5	1	32.4%	60.0%	34.7%	-	-	-	41.3%	-	51.3%	43.3%	25.0%	

60% CM + 40% SBR	1,000	1,000	79.7%	-	4,926	203	20.6	1	46.5%	62.7%	48.0%	-	-	-	48.0%	-	55.0%	51.7%	32.3%	
40% CM + 60% SBR	1,000	1,000	80.7%	-	5,181	193	21.1	1	56.6%	65.3%	60.0%	-	-	-	50.7%	-	64.7%	63.3%	36.7%	
20% CM + 80% SBR	1,000	1,000	81.5%	-	5,405	185	21.1	1	68.3%	72.0%	72.0%	-	-	-	58.7%	-	69.7%	71.3%	33.3%	
SBR	1,000	1,000	82.3%	-	5,650	177	19.4	1	72.4%	70.7%	73.3%	-	-	-	68.0%	-	68.3%	81.7%	39.3%	
40% CM + 60% SBR	1,000	1,000	76.1%	-	4,179	239	20.6	1	42.0%	-	54.0%	-	47.7%	-	-	-	49.6%	55.1%	24.0%	(Rehman <i>et al.</i> , 2019)
Pig manure	22,500	18,830	76.1%	4.50	5,000	200	9	1	22.0%	27.0%	47.0%	-	-	30.0%	9.0%	6.0%	-	-	-	(Parodi <i>et al.</i> , 2021)
47% yeast concentrate + 47% wheat and potato by-product + 6% binding agent	10,000	4,000	71.7%	6.67	8,834	113	7	1	42.0%	45.8%	37.9%	55.8%	72.9%	46.8%	27.5%	12.6%	-	-	-	Adapted from Parodi <i>et al.</i> , 2020

334

335 Asymptotic ED in BSFL can also be compared to Apparent Digestibility and Approximate  
336 Digestibility in other insect species. Both vary significantly with feed, insect species, rearing  
337 conditions and larval instar. In mealworm larvae (*Tenebrio molitor*), wheat bran has an  
338 Approximate Digestibility of DM of 46% and an Apparent Digestibility of nitrogen, starch and  
339 lipids of 54%, 59% and 73%, respectively (Waldbauer, 1968). The Apparent Digestibility of  
340 wheat in growing pig and ruminant is 90% and 88% for organic matter, 84% and 70% for  
341 nitrogen, 24% and 63% for lipids, and 88% and 86% for energy, respectively (Sauvant *et al.*,  
342 2002). Hence BSFL show strong potential to digest high-quality feed such as CF, comparable  
343 to what can be observed in conventional livestock species.  
344

345 In contrast, ED of NDF, ADF and ADL were not affected by areal density. Mean ED values  
346 ( $22.6\pm 5.9\%$ ,  $6.6\pm 6.1\%$  and  $11.8\pm 15.6\%$ , respectively) were below those of other  
347 macronutrients, suggesting that BSFL are inefficient at digesting fibres. These results are  
348 consistent with reported digestive activity in BSFL, which are high for amylase, lipase, and  
349 protease but low for cellulase (Intayung *et al.*, 2021; Sun *et al.*, 2021). This also concurs with  
350 BSFL developing well on substrates rich in starch and proteins and having low growth  
351 performance on fibre-rich substrates (Gold *et al.*, 2018). However, these very low ED of fibres  
352 are inconsistent with results obtained on soybean curd residue, cattle manure and mixtures of  
353 both, where ED varied between 49.6% and 69.7% for cellulose, 43.3% and 81.7% for  
354 hemicellulose, 24.0% and 39.3% for lignin (Rehman *et al.*, 2017). This difference could  
355 indicate that fibre fractions are less digestible in CF than in cattle manure and soybean curd  
356 residue. However, low fibre ED from the present study could also result from an inappropriate  
357 dosage method. Indeed, it has been reported that the Van Soest method was not specific to  
358 vegetal fibre fractions and that ADF could be used to estimate chitin content in shellfish  
359 (Stelmock *et al.*, 1985). Although insect and shellfish chitin have structural differences  
360 (Brigode *et al.*, 2020), it can be assumed that at least part of the chitin from dead BSFL and  
361 exuviae in frass was quantified by the Van Soest method along with plant fibres. It remains  
362 unclear whether the Van Soest method is appropriate for estimating residual plant fibre content  
363 in frass. Other dosage methods should be explored, such as a pre-treatment of frass samples  
364 with chitinase. As chitin contains nitrogen, measuring frass chitin content would also allow a  
365 more accurate nitrogen ED determination.  
366

367 Although differences were detected between the results of both trials, presumably caused by a  
368 slightly higher temperature in Trial 2 ( $28.1^{\circ}\text{C}$ ) compared to Trial 1 ( $27.1^{\circ}\text{C}$ ), the overall pattern  
369 of the data and the values of asymptotic ED remained the same, except for ash. This supports  
370 the opinion that asymptotic ED is a robust indicator of the ability of BSFL to digest their feed,  
371 little to not impacted by temperature.  
372

373 With 0 larvae/cm<sup>2</sup>, ED was positive for DM ( $3.0\pm 2.0\%$ ), starch ( $24.6\pm 7.8\%$ ), EE ( $9.9\pm 15.3\%$ )  
374 and energy ( $11.0\pm 2.3\%$ ). This is likely due to the activity of microorganisms in the substrate  
375 that partly digested it to produce microbial biomass. BSFL activity modifies the microbial  
376 community and both affect each other in a process called “co-conversion” (Cifuentes *et al.*,  
377 2020; Xiao *et al.*, 2018), with BSFL consuming and digesting microbial biomass (Gold *et al.*,  
378 2018). Conversely, 0 larvae/cm<sup>2</sup> density resulted in negative ED for nitrogen ( $-12.3\pm 3.4\%$ ) and  
379 ash ( $-0.7\pm 1.6\%$ ), which indicated a negative balance between macronutrients removed and  
380 added to the substrate. Negative ED of nitrogen could result from diazotrophs in the feed that  
381 transformed atmospheric dinitrogen into microbial proteins or ammonia released in the  
382 substrate (Chan *et al.*, 1994). Without BSFL, the substrate kept a high moisture content:  
383 therefore, it can also be hypothesised that it absorbed the gaseous and hydrophilic ammonia  
384 produced by BSFL of other containers in the same climatic chamber. The increase in ash

385 concentration at null density is very likely due to the mineral content of tap water used for feed  
386 preparation. ED of ash reached its maximum at 11.4 larvae/cm<sup>2</sup> but decreased with higher  
387 densities, indicating that the substrate accumulated minerals. This might be an experimental  
388 artefact. As starter larvae were not completely free from frass, high-density treatments were  
389 also inoculated with more frass than those of lower areal density, hence receiving more  
390 undigested ash from pre-experimental feeding. Higher frass ash content at high larval density  
391 could also result from a higher number of exuviae shed by BSFL, which bioaccumulate  
392 minerals in the cuticle in the form of calcium carbonate (Rubio *et al.*, 2021).

393

394 Life history traits values obtained were consistent with data from the literature on chicken feed:  
395 (Liu *et al.*, 2019) reported survival rates between 40 and 93% and (Gold *et al.*, 2018) indicated  
396 that prepupal FW could range between 99 and 184 mg. Larval DM content varied in the range  
397 of 17.9% to 38.8% reported by Bosch *et al.* In the present study, high areal density caused  
398 stronger competition for feed resources, hence lower survival rate and individual FW and DW  
399 gain, as already observed (Brits, 2017; Jones *et al.*, 2019; Opare *et al.*, 2022). Dead BSFL could  
400 not be visually detected after seven days of feeding and were presumably consumed by other  
401 larvae or decomposed into the substrate. Similar adverse effects of density on survival or  
402 growth have been reported in other insect species such as *Drosophila melanogaster* (Henry *et*  
403 *al.*, 2020), *Musca domestica* (Kökdener and Kiper, 2020) or *Tenebrio molitor* (Deruytter and  
404 Coudron, 2021). Impaired larval DM content observed in the present work at high density  
405 suggests that limited access to feed caused BSFL to use their body energy resources by  
406 oxidising their fat reserve (Opare *et al.*, 2022). However, FCR (dry to fresh) was improved by  
407 increasing density, indicating that BSFL performance as a group outweighed each individual  
408 underperformance. The same occurred with FCR (dry to dry) but only up to 11.4 larvae/cm<sup>2</sup>.  
409 Interestingly, low densities ranging from 0.31 to 2.47 larvae/cm<sup>2</sup> are reported not to affect  
410 individual FW (Barragan-Fonseca *et al.*, 2018), presumably because the density was too low  
411 to induce competition. These contrasting findings suggest that, although increasing density  
412 results in competition and possible accumulation of toxic metabolic waste in the substrate, it  
413 also supports larval growth via mechanisms such as increased temperature and aeration of the  
414 substrate due to BSFL activity, cooperation through extra-oral digestion, or facilitated  
415 development of beneficial microbiota (Barragan-Fonseca *et al.*, 2018; Parra Paz *et al.*, 2015).

416

417 Differences between AD95 of ED of EE, ash, nitrogen, starch, energy and DM (6.74, 6.95,  
418 7.11, 7.90, 8.13 and 8.97 larvae/cm<sup>2</sup>, respectively) indicate that calculated optimal density to  
419 fully exploit the nutritional potential of a diet depends on the macronutrient considered. These  
420 differences could be related to the amount of each macronutrient in CF: it contained far less  
421 EE than starch and it might explain why EE requires fewer larvae to be optimally digested.  
422 Starch was the main component and the primary source of energy in CF. Therefore, AD95 of  
423 starch, DM and energy are relatively close. AD95 of FCR (dry to fresh) (8.48 larvae/cm<sup>2</sup>) is  
424 also close, which highlights that starch is an essential driver of BSFL weight gain (Barragan-  
425 Fonseca *et al.*, 2021).

426

427 Optimal areal density should be chosen based on the aim of the BSFL rearing facility (Myers  
428 *et al.*, 2008). FCR (dry to dry) should be the main driver for manufacturers producing insect  
429 proteins. According to present data, densities around 11.43 larvae/cm<sup>2</sup> would produce the best  
430 output. On the other hand, waste management stakeholders use BSFL to reduce the weight of  
431 treated substrate and should be driven by the ED of DM. Therefore, areal density around AD95  
432 of ED of DM (8.97 larvae/cm<sup>2</sup>) is to be used. However, optimal densities should also be based  
433 on economic considerations as starter BSFL can be costly to produce. Insect producers should

434 seek a trade-off between maximising the animal performance and minimising the cost of  
435 operations.

436

437 The results of this study were obtained on CF with a 68% moisture content and in specific  
438 containers. Further investigations should focus on evaluating asymptotic ED and AD95 for  
439 different types of feed, but also for different feeding conditions likely to affect BSFL digestion,  
440 such as moisture content (Dzepe *et al.*, 2020), temperature (Harnden and Tomberlin, 2016),  
441 shape and size of the container (Miranda *et al.*, 2021), particle size (Brits, 2017) and addition  
442 of beneficial microorganisms (Zhang *et al.*, 2021). The latter represents an excellent  
443 opportunity to improve fibre digestion by using cellulolytic bacteria or fungi (Peguero *et al.*,  
444 2022). ED of macronutrients of a standard diet could also be an insightful indicator when  
445 developing a genetic selection strategy for *H. illucens* (Eriksson and Picard, 2021), as Zhou *et al.*  
446 *al.* showed that strain had an impact on ED of DM.

447

448 High-density containers reached a plateau of ED corresponding to the maximal amount of of  
449 biologically available macronutrients. However, it remains unclear whether the remaining  
450 macronutrients were not digested because they were undigestible or because the substrate  
451 conditions evolved and inhibited further ingestion. Indeed, at the end of the seven-day feeding  
452 period, frass in high-density containers was dry and powdery. This texture is inconsistent with  
453 BSFL mouthparts, adapted to ingest semi-liquid feed (Bruno *et al.*, 2020). Further  
454 investigations on the effect of water supplementation during growth on asymptotic ED are  
455 needed. Similarly, as frass was moist and sticky in low-density containers, BSFL might have  
456 been able to digest it further with an extended feeding period, leading to an ED closer to its  
457 maximal value. This hypothesis is supported by the ED of DM of 67.7% reported on BSFL fed  
458 CF with a density of 1.81 larvae/cm<sup>2</sup> during nine days (Gold *et al.*, 2020b), far above the value  
459 of 29.7% in seven days predicted by the model of the present study.

460

461 ED calculation is based on chemical analysis of distributed feed and frass, which is a mixture  
462 of non-ingested feed, faeces, urines, dead individuals and exuviae. This indicator is suitable for  
463 insects living in their feed like BSFL. Yet a more accurate digestibility indicator could be  
464 developed based on what is done in other insect or livestock species. Using an indigestible  
465 marker (Barret *et al.*, 2005) and collecting pure excreta would allow for calculating ingesta and  
466 Approximate Digestibility (Equation 3). This could be done with an excreta collection device  
467 (Gold *et al.*, 2020a), by separating the top and bottom ends of BSFL by a perforated wall to  
468 allow them to feed from a compartment and release excreta in another one, or by putting clean  
469 BSFL in an empty container after a feeding period. However, all options lead to tiny excreta  
470 samples, are impractical on many larvae, and possibly stressful with an impact on digestion.  
471 Dissection of BSFL digestive tract to collect faeces should be avoided as the collected material  
472 may not be fully digested (Guillaume *et al.*, 1999). In insects producing excreta that can be  
473 separated from other frass constituents, uric acid can be dosed in excreta to calculate Apparent  
474 Digestibility (Equation 4), similarly to what is done in birds (Waldbauer, 1968). Yet the  
475 molecular form of nitrogen in metabolic waste excreted by Diptera remains unclear (Henry *et al.*,  
476 2020) and further work is needed on urinary wastes in frass and their degradation products,  
477 i.e. uric acid, allantoin, urea, ammonia, nitrite and nitrate (Barragan-Fonseca *et al.*, 2021). The  
478 material produced in the digestive tract, also called endogenous faeces (microbial biomass,  
479 enzymes and gut cells), could also be quantified to calculate True Digestibility (Equation 5)  
480 (Barret *et al.*, 2005). However, this indicator was developed on animals producing an important  
481 amount of pure easy-to-collect faeces, such as poultry or pigs and is complex to assess in small  
482 insects living in groups inside their feed (Figure 3). *In vitro* simulation of BSFL digestion is

483 limited by understanding the digestive tract's enzymatic and microbial activity (Gold *et al.*,  
484 2020c).

485

$$486 \text{ Approximate digestibility} = 1 - \frac{\text{Weight of excreta}}{\text{Weight of ingesta}} \quad (3)$$

487

$$488 \text{ Apparent digestibility} = 1 - \frac{\text{Weight of excreta} - \text{Weight of urines}}{\text{Weight of ingesta}} \quad (4)$$

489

$$490 \text{ Apparent digestibility} = 1 - \frac{\text{Weight of excreta} - \text{Weight of urines} - \text{Weight of endogenous faeces}}{\text{Weight of ingesta}} \quad (5)$$

491

492 Figure 3: Breakdown of feed distributed to black soldier fly larvae during digestion

493

## 494 5. Conclusion

495

496 The present work proposes a method to evaluate BSFL feed efficiency via asymptotic ED, a  
497 new indicator defined as the maximal fraction of initial macronutrients that are not in the frass  
498 at the end of the feeding period. ED of major macronutrients are assessed on a standard diet  
499 with a focus on the effect of larval areal density. Asymptotic ED corresponds to the maximal  
500 proportion of each macronutrient that BSFL can potentially digest. Therefore, when  
501 formulating a diet for BSFL based on several ingredients, crude macronutrient content  
502 multiplied by the asymptotic ED of that macronutrient should be considered for each  
503 ingredient, rather than the sole crude macronutrient content. Further studies should focus on  
504 determining asymptotic ED of macronutrients in different types of ingredients that could be  
505 used as BSFL feed (such as agricultural side-streams, food waste or animal manure) and  
506 evaluate the effect of rearing conditions on asymptotic ED.

507

## 508 Author contributions

509

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517

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525

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527

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