Defatted black soldier fly larvae meal as an alternative to soybean meal for broiler chickens

by Chobanova, S., Karkelanov, N., Mansbridge, S.C., Whiting, I.M., Simic, A., Rose, S.P. and Pirgozliev, V.R.

Chobanova, S., Karkelanov, N., Mansbridge, S.C., Whiting, I.M., Simic, A., Rose, S.P. and Pirgozliev, V.R. (2023) 'Defatted black soldier fly larvae meal as an alternative to soybean meal for broiler chickens', *Poultry*, *2*(3), pp.430-441.

poultry

MDPI

Article

Defatted Black Soldier Fly Larvae Meal as an Alternative to Soybean Meal for Broiler Chickens

Sashka Chobanova [1], Nikolay Karkelanov [1], Stephen Charles Mansbridge [2], Isobel Margaret Whiting [2], Antonija Simic [2], Stephen Paul Rose [2] and Vasil Radoslavov Pirgozliev [2,*]

[1] Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria; sira@abv.bg (S.C.); niki_kyrkelanov@abv.bg (N.K.)
[2] National Institute of Poultry Husbandry, Harper Adams University, Shropshire TF10 8NB, UK; smansbridge@harper-adams.ac.uk (S.C.M.); iwhiting@harper-adams.ac.uk (I.M.W.); asimic@harper-adams.ac.uk (A.S.); sprose@harper-adams.ac.uk (S.P.R.)
* Correspondence: vpirgozliev@harper-adams.ac.uk

Abstract: The production of soybean meal (SBM) has a substantial impact on the environment and reducing its inclusion in poultry diets by using alternative protein sources, such as insect meal, is an important challenge for nutritionists. This study aimed to compare the productive performance of broiler chickens fed one of two isonitrogenic (195 g/kg CP) and isocaloric (12.91 MJ/kg) diets. The first diet contained SBM as the main protein source, whereas SBM was completely replaced by defatted meal from Black Soldier Fly larvae (*Hermetia illucens* L.; BSFL) in the second diet. Compared to the BSFL diet, the final body weight (BW) and weight gain (WG) of birds fed the SBM diet was ~17% greater and feed was utilised 19% more efficiently ($p < 0.05$). The differences in WG and FCR were supported by improved energy metabolism metrics, fat digestibility and digestibility of acid detergent fibres (ADFD) ($p < 0.05$). The present study shows that a complete replacement of dietary SBM with BSFL meal must be achieved with care, ensuring that all other factors (e.g., insect processing technology, feed additive supplementation, non-protein nitrogen accounting, mineral balance, fatty acid profile, amino acid supplementation) have been considered.

Keywords: black soldier fly larvae; insect meal; broilers; metabolisable energy; chitin digestibility; leg health

check for updates

Citation: Chobanova, S.; Karkelanov, N.; Mansbridge, S.C.; Whiting, I.M.; Simic, A.; Rose, S.P.; Pirgozliev, V.R. Defatted Black Soldier Fly Larvae Meal as an Alternative to Soybean Meal for Broiler Chickens. *Poultry* **2023**, *2*, 430–441. https://doi.org/10.3390/poultry2030032

Academic Editors: Ilias Giannenas, Michael Hess and Douglas E Cosby

Received: 19 July 2023
Revised: 13 September 2023
Accepted: 19 September 2023
Published: 20 September 2023

1. Introduction

To meet the growing global demand for poultry meat, production has increased from 9 million tonnes in 1961 to 133 million tonnes in 2020 [1]. Overall, poultry meat represented almost 40 percent of global meat production in 2020. Intensively reared meat birds require over 20% of high-quality dietary protein, including the indispensable amino acid lysine, which is usually the first limiting amino acid [2]. Soybeans contain high levels of crude protein comprising of amino acids, including lysine and other important nutrients, making them valuable for the poultry feed sector. However, due to growing global demand for soybean meal (SBM), the price is continuously increasing, thus making SBM one of the most expensive components in typical non-ruminant diets [3]. In addition, soybean production is increasingly associated with change of land use (e.g., deforestation), resulting in a negative impact on the environment [4]. Although research has been carried out on the use of various European produced plant protein sources including field beans [5], distilled dried grains with solubles [6], sunflower meal [7] and rapeseed meal [8], there are still limits on the suitability of their amino acid profiles, nutrient availability and yield. Thus, there is a requirement for new, sustainable and easy-to-produce alternative protein sources for the modern poultry industry. Terrestrial invertebrates, e.g., insects, are considered a promising high-quality sustainable source of dietary fat and protein for poultry [9–11]. However, the feeding value of insects for poultry is variable, particularly due to processing methods (e.g., heating, drying) [12]. Regardless, insect-based feed materials may be essential for decreasing the dependence of the European poultry industry on imported SBM [13,14]. In addition, insects can be part of a circular economy whereby they do not compete with humans for resources, utilising organic waste to grow [15]. However, the optimum amount of insect meal that can be incorporated in poultry diets to guarantee optimal animal productive performance still needs to be established. For example, insect meal has been reported to replace 100% of SBM in some studies [16]; however, others observed reduced nutrient digestibility and performance when replacing over 25% of SBM with insect meal [17–20]. Thus, it has been hypothesised that a complete replacement of SBM with insect meal in well-balanced diets will bring the same growth performance results when fed to broilers.

The aim of this study was to compare the production performance of broiler chickens, including feed intake (FI), weight gain (WG) and feed conversion ratio (FCR), when fed one of two isonitrogenic (195 g/kg CP) and isocaloric (12.91 MJ/kg) diets. The first diet contained SBM as the main protein source, whereas SBM was completely replaced by defatted meal from Black Soldier Fly larvae (*Hermetia illucens* L.; BSFL) in the second diet. The impact of these diets on N-corrected apparent metabolisable energy (AMEn), nutrient digestibility and litter quality was also studied.

2. Materials and Methods

2.1. Insect Meal Sample and Experimental Diets

The insect meal sample used in this experiment was from larvae (no older than 17 days) of the Black Soldier Fly (*Hermetia illucens* L.; Diptera: Stratiomyidae) and was purchased from HexaflyTM (Navan, Co., Meath, Ireland) (Table 1). The production process followed EC regulations [21], whereby the product was pre-washed and then oven-cooked for between 15 and 20 min at 120 °C. Following this step, the product was transferred to a 60 °C oven for conditioning for 40 min before fat separation. The dry product was processed through a screw press (OLEXA®, Arras, France) to separate the dry meal (defatted BSFLM) from the fat component. Two isocaloric and isonitrogenic diets were produced containing 12.91 MJ/kg metabolisable energy (ME) and 195 g/kg crude protein (CP) (Table 2). One of the diets was formulated with the main component being 317 g/kg maize, 280 g/kg wheat, 230 g/kg soybean meal (SBM diet). The other diet was formulated with 290 g/kg maize, 428 g/kg wheat and 160 g/kg BSF (BSF diet) without any inclusion of SBM. Both diets contained 80 g/kg sunflower meal. The diets were formulated to approximate breeders' recommendations for the growing phase of this genotype (Aviagen Ltd., Edinburgh, UK). The insect mela was finely milled from the producer. Cereals were milled by hammer mill and 6 mm mesh. All diets were supplied with 5 g/kg of TiO_2 as an indigestible feed marker.

2.2. Birds, Management and Sample Collection

The experiment was conducted at the poultry research facility of Trakia University (Stara Zagora 6000, Bulgaria) and approved by the University Research Ethics Committee. The study was designed in compliance with the guidelines of the European and Bulgarian legislation regarding the protection of animals used for experimental and other scientific purposes [22] put into law in Bulgaria with Regulation 20/2012. A total of 80 day-old female broiler (Ross 308) chicks, obtained from a commercial hatchery (Martivo—Rumen Kirchev Ltd., Sliven, Bulgaria), were raised in a single-floor pen until 10 d and fed a commercial broiler starter diet (230 g/kg CP and 12.56 MJ/kg AME). At 10 d age, 72 broilers, excluding light or heavy birds, were allocated to 24 pens, each holding 3 birds. The pens had a wire-mesh floor and were equipped with individual feeders and drinkers. Birds were raised under standard rearing conditions for broiler chickens (Aviagen Ltd., Edinburgh, UK). Each diet was fed to 12 pens following randomisation. Birds and feed were weighed at the start (at 10 days old) and at the end of the study (at 28 days old), at which point FI, WG and FCR were determined. Excreta were collected for three days from 25 to 28 d of age, oven-dried at 60 °C, milled and used for the determination of dietary AMEn, total tract dry matter and nutrient digestibility coefficients. At the end of the study, all birds were humanely killed via cervical dislocation and then the ileal contents, from vitelline diverticulum to ileo-caecal junction, were collected and pooled by pen. Pooled samples of digesta were then mixed, oven-dried at 55 °C, milled and used for determination of the ileal nitrogen digestibility coefficient (NDi).

Table 1. Proximate, carbohydrate, mineral and amino acid composition of Black Soldier Fly larvae meal.

Proximate and Carbohydrate Composition (g/kg)		**Indispensable Amino Acids (g/kg) [a]**	
Dry matter	963	Arginine	25.3
Gross energy (MJ/kg)	22.04	Histidine	19.9
Crude protein (N × 6.25)	512.0	Isoleucine	22.5
Crude fat	171.0	Leucine	33.6
Crude ash	85.0	Lysine	32.6
Acid detergent fibre	82.0	Methionine	9.4
Neutral detergent fibre	319.0	Phenylalanine	20.5
Lignin	26.7	Threonine	20.8
Chitin	55.3	Valine	31.5
Minerals		**Dispensable amino acids (g/kg) [a]**	
Calcium (g/kg)	9.0	Alanine	32.0
Phosphorus	6.9	Aspartic acid	48.1
Available phosphorus (g/kg)	5.2	Cystine	4.1
Heavy metals [a]		Glycine	27.7
Arsenic, mg/kg	<0.1	Glutamic acid	59.9
Cadmium, mg/kg	0.08	Proline	28.2
Lead, mg/kg	0.07	Serine	24.1
Mercury, mg/kg	<0.01	Tyrosine	36.0

[a] Obtained from manufacturing company.

Table 2. Composition of broiler chicken diets (g/kg, as fed) used in the experiment.

Ingredients (g/kg)	**Control**	**Insect Meal**
Maize	317.00	290.00
Wheat	280.00	400.85
Sunflower meal (36% CP)	80.00	80.00
Soybean meal (46% CP)	230.00	0.00
Insect meal	0.00	160.00
Lysine	2.90	3.60
Methionine	1.40	1.10
Threonine	0.85	1.10
NaCL	2.50	2.50
Sodium bicarbonate	2.00	2.00
Vitamin and mineral premix [a]	2.00	2.00
Choline chloride	1.85	1.85
Monocalcium phosphate	5.50	6.00
Calcium carbonate	15.00	14.00
Vegetable oil	54.00	30.00
Titanium dioxide	5.00	5.00
	100	100
Calculated analysis (as fed):		
Crude protein g/kg	195	195
Metabolisable energy MJ/kg	12.05	12.05
Crude fat g/kg	75.7	35.4
Ca g/kg	8.5	8.2
Available P g/kg	5.5	5.7
Na g/kg	2.0	2.0
K g/kg	8.4	4.0

Table 2. *Cont.*

Ingredients (g/kg)	Control	Insect Meal
Available lysine g/kg	10.7	10.7
Available methionine g/kg	4.3	4.3
Analysed composition (as fed):		
Gross energy (MJ/kg)	17.06	16.66
Dry matter (g/kg)	920	917
Crude protein (g/kg)	181	196
Fat (g/kg)	66	31
Acid detergent fibre (g/kg)	76.0	69.0
Neutral detergent fibre (g/kg)	157.5	189.0
Acid detergent lignin (g/kg)	36.1	25.8

[a] The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by NRC [2]. All the experimental diets were designed to be low in P. The premix provided (units/kg diet) retinol 3600 µg, cholecalciferol 125 µg, α-tocopherol 34 mg, menadione 3 mg, thiamine 2 mg, riboflavin 7 mg, pyridoxine 5 mg, cobalamin 15 µg, nicotinic acid 50 mg, pantotenic acid 15 mg, folic acid 1 mg, biotin 200 µg, iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 0.5 mg, zinc 80 mg, iodine 1 mg, selenium 0.2 mg and molybdenum 0.5 mg.

2.3. Laboratory Analysis

Dry matter (DM) of feed, BSFL and excreta samples was determined by drying of samples in a forced-draft oven at 105 °C to a constant weight [23]. Crude protein (CP; 6.25 × N) in feed, BSFL, digesta and excreta samples was determined by the combustion method [24] using a LECO FP-528 N (Leco Corp., St. Joseph, MI, USA). Oil (as ether extract) was extracted with petroleum ether by the ether extraction method [25] using a Soxtec system (Foss Ltd., Warrington, UK). The gross energy (GE) values of the feed, BSFL and excreta samples was determined in a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL, USA) as previously reported [26]. Acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents in the excreta, feed and BSFL, plus acid detergent lignin (ADL) content in feed and BSFL, were determined as described by Van Soest et al. [27]. Crude ash, individual minerals and amino acids (AA) contents in BSFL were analysed following standard techniques as previously defined [28,29]. Titanium dioxide in feed and digesta, to calculate NDi, was determined by the adapted procedure of Leone [30].

2.4. Calculations

The content of chitin in BSFL was determined as described by Hanh et al. [31].

$$\text{Chitin} = \text{ADF} - \text{ADL}$$

where ADF (g) = acid detergent fibre; ADL (g) = acid detergent lignin.

The AMEn value of the experimental diets was determined following the method of Hill and Anderson [32].

$$\text{AMEn} = ((\text{GE Intake} - \text{GE Output} - 34.39 \times \text{N Retained}))/(\text{Feed intake})$$

where AMEn (MJ/kg) = N-corrected apparent metabolisable energy content of the diet; GE = gross energy of the diet and excreta, respectively; GE Intake = GE of diet multiplied by feed intake; GE Output = GE of excreta multiplied by excreta voided; 34.39 (MJ/kg) = energy value of uric acid. The retained N was calculated as

$$\text{N Retained} = \text{N Intake} - \text{N Output}$$

where N Intake = N in diet multiplied by feed intake; N Output = N in excreta multiplied by excreta voided.

The energy conversion ratio (ECR) was also determined as the AMEn ingested to achieve the weight gain over the period of 7 to 21 d of age.

$$\text{ECR} = (\text{AMEn Intake})/(\text{Weight gain})$$

where AMEn Intake = dietary AMEn multiplied by average daily feed intake; Weight gain = average daily weight gain. The ECR describes the relative efficiency of the use of metabolisable energy for growth, implicit that a more efficient energy use towards growth is related to a lower ratio.

Total tract dry matter digestibility (DMD) coefficient was calculated using the following equation:

$$\text{DMD} = (\text{Dry matter intake} - \text{Dry mater output})/(\text{Dry matter intake})$$

where Dry matter intake = dry matter of diet multiplied by feed intake; Dry matter output = dry matter of excreta multiplied by dry excreta voided.

Total tract dietary nutrient digestibility coefficients were calculated using the following equation:

$$\text{Total tract nutrient digestibility} = (\text{Nutrient intake} \times \text{Nutrient output})/(\text{Nutrient intake})$$

where Nutrient intake = feed intake multiplied by respective nutrient; Nutrient output = dry excreta voided multiplied by the respective nutrient.

Ileal nitrogen digestibility coefficient (NDi) was calculated using the following equation:

$$\text{NDi} = ((\text{N}/\text{Ti})\text{Diet} - (\text{N}/\text{Ti})\text{Digesta})/((\text{N}/\text{Ti})\text{Diet})$$

where (N/Ti) Diet = ratio of the nitrogen to titanium in diet, and (N/Ti) Digesta = ratio of the nitrogen to titanium in ileal digesta.

2.5. Statistical Analysis

Performance data were analysed using Genstat (22nd edition) statistical software (IACR Rothamsted, Hertfordshire, UK). Comparisons among the studied variables were performed using one-way ANOVA. Data were checked for homogeneity and normality prior to ANOVA. Results were considered significant at $p < 0.05$. Data are expressed as means and their pooled standard errors of means (SEM).

3. Results

The analysed chemical composition (g/kg) of the BSFL meal is presented in Table 1. The BSFL had 963 g/kg DM and contained 22.04 MJ/kg GE. Crude protein was the main constituent in the BSFL meal followed by crude fat. The BSFL meal contained 512 g/kg CP, 171 g/kg CF, 85 g/kg ash, 319 g/kg NDF, 82 g/kg ADF, 26.7 g/kg lignin, 55.3 g/kg chitin, 9.0 g/kg calcium and 6.9 g/kg phosphorus, from which 5.2 g/kg was available. Among the indispensable AAs, the contents of leucine, lysine and valine were the highest, and the lowest content was determined for methionine. Glutamic and aspartic acids were the main dispensable AAs, with cystine having the lowest content.

The proximate and carbohydrate analyses of the two experimental diets are presented in Table 2. The SBM diet had 0.4 MJ/kg greater GE, contained 35 g/kg more crude fat but had 15 g/kg less CP compared to the BSFL diet. The SBM diet also contained more ADF and ADL, but less NDF than the BSFL diet.

Table 3 presents information on growth performance variables, AMEn and the efficiency of energy utilisation in female broilers (10–28 d). There were no differences ($p > 0.05$) in initial bird BW. Final BW and WG of birds fed SBM diet was about 17% higher and they utilised feed 19% more efficiently compared to BSFL-fed birds ($p < 0.05$). However, there were no differences in daily FI ($p > 0.05$), although the intake of AMEn tended ($p < 0.1$) to be higher in birds fed the SBM diet. The differences in WG and FCR were supported by increased dietary AMEn ($p < 0.001$), AMEn:GE ratio and the ECR ($p < 0.05$).

Table 3. Effect of experimental diets on growth performance of broiler chickens and dietary energy metabolism.

Item Treatment	BW (g/b 10 d)	BW (g/b 28 d)	FI (g/b/d)	WG (g/b/d)	FCR (g:g)	AMEn (MJ/kg)	AMEn Intake (MJ/b/d)	AMEn:GE	ECR
SBM	175	929	69.3	39.9	1.760	13.27	0.92	0.778	24.10
BSFL	179	775	68.1	33.1	2.094	12.47	0.85	0.748	27.13
SEM	9.0	34.5	2.31	1.86	0.0707	0.118	0.027	0.0070	0.978
Probabilities									
p-value	0.779	0.005	0.710	0.018	0.003	<0.001	0.078	0.007	0.039

BW: body weight; FI: daily feed intake; WG: weight gain; FCR: feed conversion ratio corrected for mortality; AMEn: dietary N-corrected apparent metabolizable energy; ECR: energy conversion ratio; SBM: soybean-based diet; BSFL: black soldier meal-based diet.

Results of nutrient digestibility coefficients and excreta variables are shown in Table 4 at study end point (28 d). Coefficients of DMD and total tract fat digestibility (FD) were lower for the BSFL diet ($p < 0.05$ and <0.001, respectively) compared to SBM. There were no statistically significant differences ($p > 0.05$) between diets for NDi and total tract nitrogen digestibility (NDt) coefficients. There was no difference in NDF digestibility (NDFD) between the two diets ($p > 0.05$). However, the ADF digestibility (ADFD) of the SBM diet was approximately 44% greater ($p < 0.001$) compared to BSFL diet. The moisture of fresh excreta was 6% higher in birds fed the SBM diet ($p < 0.001$). Birds fed SBM diet voided 23% more fresh excreta ($p < 0.05$) comparted to BSFL fed birds. No difference was observed between dry excreta voided ($p > 0.05$).

Table 4. Effect of experimental diets on nutrient digestibility coefficients and excreta moisture at study end point.

Item Treatment	NDi	NDt	DMD	FD	ADFD	NDFD	EM	EW	ED
SBM	0.642	0.679	0.779	0.970	0.454	0.499	0.802	157.8	30.1
BSFL	0.699	0.711	0.750	0.917	0.255	0.470	0.753	121.1	29.8
SEM	0.0258	0.0203	0.0090	0.0075	0.0251	0.0181	0.0091	10.14	1.67
Probabilities									
p-value	0.133	0.280	0.031	<0.001	<0.001	0.275	<0.001	0.018	0.893

NDi: ileal nitrogen digestibility coefficient; NDt: total tract nitrogen digestibility coefficient; DMD: total tract dry matter digestibility coefficient; FD: total tract fat digestibility coefficient; ADFD: total tract acid detergent fibres digestibility coefficient; NDFD: total tract neutral detergent fibres digestibility coefficient; EM: moisture of fresh excreta, kg/kg; EW: daily voided wet excreta per bird during the days of excreta collection, g/b/d; ED: daily voided dry excreta per bird during the days of excreta collection, g/b/d; SBM: soybean meal diet; BSFL: black soldier fly larvae meal diet.

4. Discussion

The observed minor differences in protein and fat contents of the diets between analysed and calculated values are likely explained by differences in the nutrient composition of the actual ingredients that were used and the values used in the software when formulating the diets. The analysed composition of BSFL was within expected values [19,33,34]. However, variations in fat, protein and chitin content of BSFL are recognised and mostly depend on larvae age and rearing substrate [15,35]. The ADF content of BSFL is closely associated with the chitin content of insects. Typically, ADF is further adjusted for amino acid [36] or ADL, (Hahn et al. [31] content, to increase the accuracy of chitin determination. Thus, variation in chitin content may be expected depending on the method of calculation selected.

Birds remained healthy during the study with two cases of unexplained mortality, one on each diet. It should be noted that the BW of the birds was lower than Ross 308 female broiler target weight, possibly due to being fed mash rather than pelleted feed and being kept in small groups [36–38]. However, this was not considered to be detrimental to the experimental objectives. Mash diets may be more susceptible to selective feeding of dietary ingredients, however this is likely to be limited in practice and no measurements are available for the present study.

In an experiment with quail, Cullere et al. [33] reported no difference in FI, WG and FCR between birds fed isocaloric and isonitrogenic diets containing 0%, 10% and 15% of insect meal at the expense of SBM. Feeding 10% BSFL to broilers, Lalev et al. [39] reported greater overall growth performance variables including FI, WG and feed efficiency, compared to the control fed birds. The same research group (Lalev et al. [40]) also reported positive growth performance in response to 10% dietary BSFL when fed to turkeys. Similarly, Popova et al. [41] found positive growth performance of chickens fed 5% BSFL, although some changes in fatty acid composition in meat of broilers are possible [42]. However, in these studies the diet with BSFL was formulated with different amounts of maize, wheat, amino acids and BSFL in order to meet the SBM nutrients. A meta-analysis of published data [43] showed that the daily WG starts decreasing above 10% insect meal inclusion, which can partially explain the results from our study whereby at 16% BSFL inclusion, WG was reduced compared to the SBM control.

The reduced production performance of BSFL fed birds in the present study may be associated with the observed reduced DMD, FD, ADFD, AMEn and the reduced ability of birds to utilise dietary energy. Despite the many functional benefits associated with chitin (e.g., as a potential prebiotic, as well as antimicrobial, antiviral and antifungal agent), it has often been characterised as an anti-nutritional factor [31]. Although chickens have been shown to produce chitinase in the proventriculus and hepatocytes [44], the digestibility of chitin seems to be limited [45], particularly in young birds [46]. Additionally, chitin comprises 3.0% to 6.8% non-protein nitrogen (NPN), meaning that the standard 6.26 N-to-CP conversion factor overestimates protein [47]. This is likely the case in the present study whereby no adjustment was made during feed formulation and therefore true CP in the BSFL diet was likely lower than in the SBM diet, possibly explaining the lower WG. Estimated levels of chitin in the present study were 55.3 g/kg in the BSFL sample, which is similar to values obtained by chemical chitin determinations of similar products [48].

Saturated fats are poorly digested by young birds in comparison to unsaturated [49], however, whilst insect fat contains a greater content of saturated fatty acids compared to soya oil and therefore could reduce AMEn and FD, this is not consistent with the findings of others [50]. The importance of modifying dietary formulations specifically for insect meal with regards to NPN and fatty acid profile, must therefore be an important requirement of future feed formulation algorithms to ensure accuracy.

Similar to the present study, Lokman et al. [51] reported that feeding chitin and chitosan did not affect FI but reduced overall WG. However, Longvah et al. [52], found that high concentrations of chitin reduced FI and inhibited the use of protein in rats. Gariglio et al. [53] found a decrease in overall ND during the starter period, but an increase in FD during the growing and finishing periods when feeding partially defatted BSFL meal to Muscovy ducks. Bovera et al. [19] and Cutrignelli et al. [20] similarly reported reduced DMD, ND and organic matter digestibility of diets containing BSFL when fed to laying hens. Research on the villus morphometry of Ross 308 broilers [54] and laying hens [55] fed insect meal reports decreased small intestinal villus height, which usually reduces nutrient absorption and growth of birds [56]. Indeed, Biasato et al. [57] found that high inclusion levels of insect meal (about 15% or over) result in a partial reduction of microbial complexity, reduction of potentially beneficial bacteria, selection of bacteria with mucolytic activity and a decrease in villi mucins. These further suggest that the observed reduction in energy, nutrient availability and bird growth in our study may be associated with the chitin content of BSFL in the diet.

The method of processing the insect meal also impacts its feeding value [12]. The BSFL meal used in our study was exposed to 120 °C for approximately 20 min. The melting temperature varies for different proteins, but temperatures above 41 °C will compromise the structure of many proteins and denature them [58], although the impact on individual AAs is less clear. Further damage to sensitive nutrients, e.g., vitamins and lysine, may also result from prolonged steam pelleting during subsequent diet manufacture [59], often performed commercially. For these reasons, care must be taken when choosing insect products for inclusion in poultry diets based on knowledge of their rendering technique, to ensure nutritional value is preserved as formulated. Further research into standardising insect rendering and production methods should be prioritised by the industry.

Research by Vieira and Lima [60] showed that birds fed all-vegetarian diets had an increase in water intake compared to birds fed animal-protein-containing diets. Water intake may be affected by several factors, including changes in the levels of some nutrients. The basal nutrient profile of the two diets in the present study was very similar, with the exception of potassium, which was double in the SBM (vegetarian) diet. Soybean meal contains twice the amount of potassium of BSFL meal [10,61]. Potassium is well recognised to positively affect water intake and increase excreta moisture [60–62]. High moisture in excreta is a major factor related to instances of foot pad dermatitis (FPD) [63], thus hypothesising that dietary inclusion of insect meal may improve litter quality and reduce the incidences of FPD. Foot health of poultry has not only welfare implications but also a significant economic impact on broiler production. Chicken feet are a major export product for the poultry industry. For example, the value of USA-only chicken feet exports to China is over USD 500 million per year [64]. Further research is therefore required to confirm the use of insect meal in preventing FPD and the subsequent financial benefits associated with this.

The use and development of various feed additives, including enzymes, is a topic of ongoing research in poultry nutrition [65,66] to improve protein and carbohydrate digestibility, resulting in improved growth performance [67] and potentially improved litter quality as a consequence of enhanced nutrient utilisation. Research on the use of feed additives, alongside processing techniques and refining insect meal inclusion levels to reduce the negative impact of chitin and improve/preserve the nutrient availability of insect meal for poultry, is essential in the future.

5. Conclusions

The present study shows that dietary inclusion of 16% BSFL meal as a complete replacement of SBM reduced dietary available energy and nutrient digestibility, leading to reduced growth performance of broiler chickens. These effects may have been associated with high dietary chitin content and inaccuracies in dietary formulation assumptions regarding NPN and fat digestibility values. However, feeding BSFL reduced excreta moisture content, suggesting that BSFL meal may be beneficial in improving litter quality. Apparently, our hypothesis was not confirmed, and it seems that a partial replacement of SBM with BSFL may be more appropriate than a complete replacement unless all other factors (e.g., insect processing technology, feed additive supplementation, mineral balance and AA supplementation) have been considered. Further research into standardising insect rendering and production methods should be prioritised by the industry.

Author Contributions: Conceptualisation and experimental design, V.R.P., S.C., N.K., S.C.M. and S.P.R.; resources, S.C. and N.K.; data curation, S.C., N.K. and V.R.P.; laboratory analysis, A.S., I.M.W., S.C. and N.K.; statistical analysis, V.R.P., S.C.M. and A.S.; writing—original draft preparation, V.R.P., S.C.M. and I.M.W.; writing—review and editing, V.R.P., S.C.M., I.M.W. and S.P.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted at the poultry research facility of Trakia University (Stara Zagora 6000, Bulgaria) and approved by the University Research Ethics Committee. The study was designed in compliance with the guidelines of the European and Bulgarian legislation regarding the protection of animals used for experimental and other scientific purposes put into law in Bulgaria with Regulation 20/2012. The manuscript has been prepared in compliance with the ARRIVE 2.0 guidelines [68].

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available on reasonable request from the corresponding author.

Acknowledgments: The authors acknowledge the technical assistance of the staff from the Elizabeth Creak laboratories, Harper Adams University, for supporting the majority of the laboratory analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. FAOSTAT Statistical Database. Available online: https://www.fao.org/statistics/en/ (accessed on 13 May 2022).
2. National Research Council (NRC). *Nutrient Requirements of Poultry*, 10th ed.; National Academy Press: Washington, DC, USA, 1994.
3. European Soy Monitor. Researched by B. Kuepper and M. Riemersma of Profundo. IDH, The Sustainable Trade Initiative and IUCN. National Committee of the Netherlands. Available online: https://www.idhsustainabletrade.com/uploaded/2019/04/European-Soy-Monitor.pdf (accessed on 20 May 2022).
4. Grossi, S.; Massa, V.; Giorgino, A.; Rossi, L.; Dell'Anno, M.; Pinotti, L.; Avidano, F.; Compiani, R.; Rossi, C.A.S. Feeding Bakery Former Foodstuffs and Wheat Distiller's as Partial Replacement for Corn and Soybean Enhances the Environmental Sustainability and Circularity of Beef Cattle Farming. *Sustainability* **2022**, *14*, 4908. [CrossRef]
5. Abdulla, J.M.; Rose, S.P.; Mackenzie, A.M.; Ivanova, S.G.; Staykova, G.P.; Pirgozliev, V.R. Nutritional value of raw and micronised field beans (*Vicia faba* L. var. *minor*) with and without enzyme supplementation containing tannase for growing chickens. *Arch. Anim. Nutr.* **2016**, *70*, 350–363. [CrossRef]
6. Whiting, I.; Pirgozliev, V.; Rose, S.P.; Karadas, F.; Mirza, M.W.; Sharpe, A. The temperature of storage of a batch of wheat distillers dried grains with solubles samples on their nutritive value for broilers. *Br. Poult. Sci.* **2018**, *59*, 76–80. [CrossRef]
7. Karkelanov, N.; Chobanova, S.; Dimitrova, K.; Whiting, I.M.; Rose, S.P.; Pirgozliev, V. Feeding value of de-hulled sunflower seed meal for broilers. *Acta Agrophys.* **2020**, *27*, 31–38. [CrossRef]
8. Watts, E.S.; Rose, S.P.; Mackenzie, A.M.; Pirgozliev, V.R. Investigations into the chemical composition and nutritional value of single-cultivar rapeseed meals for broiler chickens. *Arch. Anim. Nutr.* **2021**, *75*, 209–221. [CrossRef] [PubMed]
9. Józefiak, A.; Benzertiha, A.; Kieronczyk, B.; Lukomska, A.; Wesollowska, I.; Rawski, M. Improvement of cecal commensal microbiome following the insect additive into chicken diet. *Animals* **2020**, *10*, 577. [CrossRef]
10. Kierończyk, B.; Rawski, M.; Mikołajczak, Z.; Homska, N.; Jankowski, J.; Ognik, K.; Józefiak, A.; Mazurkiewicz, J.; Józefiak, D. Available for millions of years but discovered through the last decade: Insects as a source of nutrients and energy in animal diets. *Anim. Nutr.* **2022**, *11*, 60–79. [CrossRef] [PubMed]
11. Petkov, E.; Ignatova, M.; Popova, T. Layers' performance and egg hatchability as affected by the dietary inclusion of two meals of black soldier fly (*Hermetia illucens*). *J. Insects Food Feed.* **2022**, *8*, 1077–1084. [CrossRef]
12. Liceaga, A.M. Processing insects for use in the food and feed industry. *Curr. Opin. Insect. Sci.* **2021**, *48*, 32–36. [CrossRef] [PubMed]
13. Józefiak, A.; Engberg, R.M. Insect proteins as a potential source of antimicrobial peptides in livestock production. A review. *J. Anim. Feed Sci.* **2017**, *26*, 87–99. [CrossRef]
14. Benzertiha, A.; Kieronczyk, B.; Rawski, M.; Jozefiak, A.; Kozłowski, K.; Jankowski, J.; Józefiak, D. Tenebrio molitor and Zophobas morio full-fat meals in broiler chicken diets: Effects on nutrients digestibility, digestive enzyme activities, and cecal microbiome. *Animals* **2019**, *9*, 1128. [CrossRef]
15. Makkar, H.P.; Tran, G.; Heuzé, V.; Ankers, P. State-of-the-art on use of insects as animal feed. *Anim. Feed Sci. Technol.* **2014**, *197*, 1–33. [CrossRef]
16. Maurer, V.; Holinger, M.; Amsler, Z.; Früh, B.; Wohlfahrt, J.; Stamer, A.; Leiber, F. Replacement of soybean cake by *Hermetia illucens* meal in diets for layers. *J. Insects Food Feed.* **2016**, *2*, 83–90. [CrossRef]
17. Onsongo, V.O.; Osuga, I.M.; Gachuiri, C.K.; Wachira, A.M.; Miano, D.M.; Tanga, C.M.; Ekesi, S.; Nakimbugwe, D.; Fiaboe, K.K.M. Insects for income generation through animal feed: Effect of dietary replacement of soybean and fish meal with black soldier fly meal on broiler growth and economic performance. *J. Econ. Entomol.* **2018**, *111*, 1966–1973. [CrossRef] [PubMed]

18. Marono, S.; Loponte, R.; Lombardi, P.; Vassalotti, G.; Pero, M.E.; Russo, F.; Gasco, L.; Parisi, G.; Piccolo, G.; Nizza, S.; et al. Productive performance and blood profiles of laying hens fed *Hermetia illucens* larvae meal as total replacement of soybean meal from 24 to 45 weeks of age. *Poult. Sci.* **2017**, *96*, 1783–1790. [CrossRef] [PubMed]
19. Bovera, F.; Loponte, R.; Pero, M.E.; Cutrignelli, M.I.; Calabrò, S.; Musco, N.; Vassalotti, G.; Panettieri, V.; Lombardi, P.; Piccolo, G.; et al. Laying performance, blood profiles, nutrient digestibility and inner organs traits of hens fed an insect meal from *Hermetia illucens* larvae. *Res. Vet. Sci.* **2018**, *120*, 86–93. [CrossRef]
20. Cutrignelli, M.I.; Messina, M.; Tulli, F.; Randazzo, B.; Olivotto, I.; Gasco, L.; Loponte, R.; Bovera, F. Evaluation of an insect meal of the Black Soldier Fly (*Hermetia illucens*) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. *Res. Vet. Sci.* **2018**, *117*, 209–215. [CrossRef]
21. European Commission (EC). *No 1069/2009 of the European Parliament and the Council of 21 October 2009 Laying down Health Rules as Regards Animal by-Products and Derived Products Not Intended for Human Consumption and Repealing Regulation (EC) No 1774/2002 (Animal by-Products Regulation)*; European Union: Brussels, Belgium, 2009.
22. European Commission (EC). *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes*; European Union: Brussels, Belgium, 2010.
23. AOAC (Association of Analytical Communities). *Official Method of Analysis, 934.01, Vacuum Oven*, 18th ed.; AOAC International: Gaithersburg, MD, USA, 2006.
24. AOAC (Association of Analytical Communities). *Official Method of Analysis, 990.03, Protein (Crude) in Animal Feed, Combustion Method*, 18th ed.; AOAC International: Gaithersburg, MD, USA, 2006.
25. AOAC (Association of Analytical Communities). *Official Method of Analysis, 945.16, Oil in Cereal Adjuncts*, 18th ed.; AOAC International: Gaithersburg, MD, USA, 2005.
26. Pirgozliev, V.; Mansbridge, S.C.; Rose, S.P.; Mackenzie, A.M.; Beccaccia, A.; Karadas, F.; Ivanova, S.G.; Staykova, G.P.; Oluwatosin, O.O.; Bravo, D. Dietary essential oils improve feed efficiency and hepatic antioxidant content of broiler chickens. *Animal* **2019**, *13*, 502–508. [CrossRef]
27. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef] [PubMed]
28. Priyankarage, N.; Rose, S.P.; Silva, S.S.P.; Pirgozliev, V.R. The efficiency of energy retention of broiler chickens and turkeys fed on diets with different lysine concentrations. *Br. Poult. Sci.* **2008**, *49*, 721–730. [CrossRef]
29. Abdulla, J.M.; Rose, S.P.; Mackenzie, A.M.; Pirgozliev, V. Variability of amino acid digestibility in different field bean cultivars for broilers. *Br. Poult. Sci.* **2021**, *62*, 596–600. [CrossRef]
30. Leone, J.L. Collaborative study of the quantitative determination of titanium dioxide in cheese. *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 535–537. [CrossRef] [PubMed]
31. Hahn, T.; Roth, A.; Febel, E.; Fijalkowska, M.; Schmitt, E.; Arsiwalla, T.; Zibek, S. New methods for high-accuracy insect chitin measurement. *J. Sci. Food Agric.* **2018**, *98*, 5069–5073. [CrossRef]
32. Hill, F.W.; Anderson, D.L. Comparison of metabolizable energy and productive energy determinations with growing chicks. *J. Nutr.* **1958**, *64*, 587–603. [CrossRef]
33. Cullere, M.; Tasoniero, G.; Giaccone, V.; Miotti-Scapin, R.; Claeys, E.; De Smet, S.; DalleZotte, A. Black soldier fly as dietary protein source for broiler quails: Apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. *Animal* **2016**, *10*, 1923–1930. [CrossRef]
34. Mahmoud, A.E.; Morel, P.C.H.; Potter, M.A.; Ravindran, V. The apparent metabolisable energy and ileal amino digestibility of black soldier fly (*Hermetia illucens*) larvae meal for broiler chickens. *Br. Poult. Sci.* **2023**, *64*, 377–383. [CrossRef]
35. Matin, N.; Utterback, P.; Parsons, C. True Metabolisable Energy and Amino Acid Digestibility of Black Soldier Fly Larvae Meals, Cricket Meal, and Mealworms Using a Precision-Fed Rooster Assay. *Poult. Sci.* **2021**, *100*, 101146. [CrossRef] [PubMed]
36. Finke, M.D. Estimate of chitin in raw whole insects. *Zoo Biol.* **2007**, *26*, 105–115. [CrossRef] [PubMed]
37. Pirgozliev, V.; Mirza, M.W.; Rose, S.P. Does the effect of pelleting depend on the wheat sample when fed to chickens? *Animal* **2016**, *10*, 571–577. [CrossRef]
38. Yang, Z.; Pirgozliev, V.R.; Rose, S.P.; Woods, S.; Yang, H.M.; Wang, Z.Y.; Bedford, M.R. Effect of age on the relationship between metabolizable energy and digestible energy for broiler chickens. *Poult. Sci.* **2020**, *99*, 320–330. [CrossRef]
39. Lalev, M.; Hristakieva, P.; Mincheva, N.; Oblakova, M.; Ivanova, I. Insect meal as alternative protein ingredient in broiler feed. *Bulg. J. Agric. Sci.* **2022**, *28*, 743–751.
40. Lalev, M.; Mincheva, N.; Oblakova, M.; Hristakieva, P.; Ivanova, I.; Atanassov, A.; Petrova, A. Effects of insect and probiotic-based diets on turkeys' production, health, and immune parameters. *Bulg. J. Agric. Sci.* **2020**, *26*, 1254–1265.
41. Popova, T.; Petkov, E.; Ignatova, M. Effect of black soldier fly (*Hermetia illucens*) meals in the diet on the growth performance and carcass composition in broilers. *J. Insects Food Feed.* **2021**, *7*, 369–376. [CrossRef]
42. Popova, T.L.; Petkov, E.; Ignatova, M. Effect of black soldier fly (*Hermetia illucens*) meals on the meat quality in broilers. *Agric. Food Sci.* **2020**, *29*, 177–188. [CrossRef]
43. Moula, N.; Detilleux, J. A meta-analysis of the effects of insects in feed on poultry growth performances. *Animals* **2019**, *9*, 201. [CrossRef]

44. Suzuki, M.; Fujimoto, W.; Goto, M.; Morimatsu, M.; Syuto, B.; Toshihiko, I. Cellular expression of gut chitinase mRNA in the gastrointestinal tract of mice and chickens. *J. Histochem. Cytochem.* **2002**, *50*, 1081–1089. [CrossRef]
45. Hossain, S.; Blair, R. Chitin utilisation by broilers and its effects on body composition and blood metabolites. *Br. Poult. Sci.* **2007**, *48*, 33–38. [CrossRef]
46. Ijaiya, A.T.; Eko, E.O. Effect of replacing dietary fish meal with silkworm (Anaphe infracta) caterpillar meal on performance, carcass characteristics and haematological parameters of finishing broiler chicken. *Pak. J. Nutr.* **2009**, *8*, 850–855. [CrossRef]
47. Janssen, R.H.; Vincken, J.P.; van den Broek, L.A.; Fogliano, V.; Lakemond, C.M. Nitrogen-to-protein conversion factors for three edible insects: Tenebrio molitor, Alphitobius diaperinus, and *Hermetia illucens*. *J. Agric. Food Chem.* **2017**, *65*, 2275–2278. [CrossRef]
48. Schiavone, A.; De Marco, M.; Martínez, S.; Dabbou, S.; Renna, M.; Madrid, J.; Hernandez, F.; Rotolo, L.; Costa, P.; Gai, F.; et al. Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (*Hermetia illucens* L.) meal for broiler chickens: Apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 51. [CrossRef] [PubMed]
49. Hakansson, J. Factors affecting the digestibility of fats and fatty acids in chicks and hens. *Swed. J. Agric. Res.* **1974**, *4*, 33–47.
50. Kierończyk, B.; Rawski, M.; Józefiak, A.; Mazurkiewicz, J.; Świątkiewicz, S.; Siwek, M.; Bednarczyk, M.; Szumacher-Strabel, M.; Cieślak, A.; Benzertiha, A.; et al. Effects of replacing soybean oil with selected insect fats on broilers. *Anim. Feed Sci. Tech.* **2018**, *240*, 170–183. [CrossRef]
51. Lokman, I.H.; Ibitoye, E.B.; Hezmee, M.N.M.; Goh, Y.M.; Zuki, A.B.Z.; Jimoh, A.A. Effects of chitin and chitosan from cricket and shrimp on growth and carcass performance of broiler chickens. *Trop. Anim. Health Prod.* **2019**, *51*, 2219–2225. [CrossRef] [PubMed]
52. Longvah, T.; Mangthya, K.; Ramulu, P. Nutrient composition and protein quality evaluation of eri silkworm (Samia ricinii) prepupae and pupae. *Food Chem.* **2011**, *128*, 400–403. [CrossRef]
53. Gariglio, M.; Dabbou, S.; Biasato, I.; Capucchio, M.T.; Colombino, E.; Hernandez, F.; Madrid Sanchez, J.; Martinez, S.; Gai, F.; Caimi, C.; et al. Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy duck. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 37. [CrossRef]
54. Biasato, I.; Gasco, L.; De Marco, M.; Renna, M.; Rotolo, L.; Dabbou, S.; Capucchio, M.T.; Biasibetti, E.; Tarantola, M.; Sterpone, L.; et al. Yellow mealworm larvae (Tenebrio molitor) inclusion in diets for male broiler chickens: Effects on growth performance, gut morphology, and histological findings. *Poult. Sci.* **2018**, *97*, 540–548. [CrossRef] [PubMed]
55. Moniello, G.; Ariano, A.; Panettieri, V.; Tulli, F.; Olivotto, I.; Messina, M.; Randazzo, B.; Severino, L.; Piccolo, G.; Musco, N.; et al. Intestinal morphometry, enzymatic and microbial activity in laying hens fed different levels of a *Hermetia illucens* larvae meal and toxic elements content of the insect meal and diets. *Animals* **2019**, *9*, 86. [CrossRef]
56. Laudadio, V.; Passantino, L.; Perillo, A.; Loprestu, G.; Passantino, A.; Khan, R.U.; Tufarelli, V. Productive performance and histological features of intestinal mucosa of broiler chickens fed different dietary protein levels. *Poult. Sci.* **2012**, *91*, 265–270. [CrossRef]
57. Biasato, I.; Ferrocino, I.; Dabbou, S.; Evangelista, R.; Gai, F.; Gasco, L.; Cocolin, L.; Capucchio, M.T.; Schiavone, A. Black soldier fly and gut health in broiler chickens: Insights into the relationship between cecal microbiota and intestinal mucin composition. *J. Anim. Sci. Biotechnol.* **2020**, *11*, 11. [CrossRef]
58. Creighton, T.E. *Proteins, 2nd ed*; Freeman: New York, NY, USA, 1993.
59. Bergeron, A.N.; Boney, J.W.; Moritz, J.S. The effects of diet formulation and thermal processes associated with pelleting on 18-day broiler performance and digestible amino acid concentration. *J. Appl. Poult. Res.* **2018**, *27*, 540–549. [CrossRef]
60. Vieira, S.L.; Lima, I.L. Live performance, water intake and excreta characteristics of broilers fed all vegetable diets based on corn and soybean meal. *Int. J. Poult. Sci.* **2005**, *4*, 365–368.
61. Youssef, I.M.; Beineke, A.; Rohn, K.; Kamphues, J. Effects of high dietary levels of soybean meal and its constituents (potassium, oligosaccharides) on foot pad dermatitis in growing turkeys housed on dry and wet litter. *Arch. Anim. Nutr.* **2011**, *65*, 148–162. [CrossRef]
62. Smith, A.; Rose, S.P.; Wells, R.G.; Pirgozliev, V. Effect of excess dietary sodium, potassium, calcium and phosphorus on excreta moisture of laying hens. *Br. Poult. Sci.* **2000**, *41*, 598–607. [CrossRef]
63. Mirza, M.W.; Pirgozliev, V.; Rose, S.P.; Sparks, N.H.C. Dietary modelling of nutrient densities: Effect and response in different growing phases on growth performance, nutrient digestibility, litter quality and leg health in turkey production. *Worlds Poult. Sci. J.* **2016**, *6*, 161–190.
64. The Poultry Site. Chinese Appetite for Chicken Paws Proves a Boon for US Poultry Industry. 2021. Available online: https://www.thepoultrysite.com/news/2021/07 (accessed on 10 May 2023).
65. Pirgozliev, V.; Rose, S.P.; Ivanova, S. Feed additives in poultry nutrition. *Bulg. J. Agric. Sci.* **2019**, *25*, 8–11.
66. Oso, A.O.; Williams, G.A.; Oluwatosin, O.O.; Bamgbose, A.M.; Adebayo, A.O.; Olowofeso, O.; Pirgozliev, V.; Adegbenjo, A.A.; Osho, S.O.; Alabi, J.O.; et al. Effect of dietary supplementation with arginine on haematological indices, serum chemistry, carcass yield, gut microflora, and lymphoid organs of growing turkeys. *Livest. Sci.* **2017**, *198*, 58–64. [CrossRef]

67. Pirgozliev, V.R.; Mansbridge, S.C.; Whiting, I.M.; Abdulla, J.M.; Rose, S.P.; Kljak, K.; Johnson, A.; Drijfhout, F.; Atanasov, A.G. The Benefits of Exogenous Xylanase in Wheat–Soy Based Broiler Chicken Diets, Consisting of Different Soluble Non-Starch Polysaccharides Content. *Poultry* **2023**, *2*, 123–133. [CrossRef]
68. Percie du Sert, N.; Hurst, V.; Ahluwalia, A.; Alam, S.; Avey, M.T.; Baker, M.; Browne, W.J.; Clark, A.; Cuthill, I.C.; Dirnagl, U.; et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research*. *J. Cereb. Blood Flow Metab.* **2020**, *40*, 1769–1777. [CrossRef] [PubMed]