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Impact of dietary protease on energy, nutrient availability, and performance of broilers fed diets containing black soldier fly larvae meal

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ABSTRACT - The experiment aimed to assess the impact of exogenous protease in diets formulated with soybean meal (SBM) or black soldier fly larvae meal (BSFLM) on nutrient retention and broiler performance. Forty-eight female Ross 308 chicks were assigned to a randomized block design study using a 2 × 2 factorial arrangement, with four treatments in total. The main factors considered were the protein source and the presence or absence of protease. Each experimental diet was fed to six pens of two birds per pen, following randomization. Dietary apparent metabolizable energy (AME), N-corrected AME (AMEn), dry matter (DMR), nitrogen (NR) and fat (FR) retention coefficients were measured. Daily feed intake (FI), weight gain (WG) and feed conversion ratio (FCR) were assessed from 28 to 38 d of age. Data were analyzed by two-way ANOVA following a factorial arrangement. Birds fed protease consumed more feed ($P = 0.036$) and tended to have greater ($P = 0.058$) AMEn intake. There was a diet × protease interaction; birds fed the protease supplemented SBM diet grew faster than other groups ($P = 0.021$). Compared with the required 195 g/kg dietary crude protein (CP), the analyzed CP was 183 g/kg in the SBM diet and 203 g/kg in the BSFLM diet. Feeding diets based on BSFLM tended ($P = 0.061$) to lead to lower feed efficiency, e.g. higher FCR. The diets based on BSFLM also had lower ME ($P<0.05$) and lower FR ($P = 0.001$). The disparity in dietary CP likely confounded the results; therefore, conclusions regarding protease effects are limited. Further research on the impact of protease in low protein BSFLM-based diet may further elucidate the nutritive value of these enzymes for broilers.

Keywords: enzyme, growth performance, insect meal, metabolizable energy, poultry

1. Introduction

Crude protein (CP) is a vital nutrient in poultry nutrition, essential for maintaining bird welfare, supporting optimal growth, and facilitating key physiological functions (Dilelis et al., 2021; Lalev et al., 2023). Modern broilers require diets containing more than 20% high-quality crude protein (CP), well-balanced in amino acids (AA), during the starter and grower phases (Aviagen, 2022). Soybean meal (SBM) is usually incorporated into poultry diets, but is expensive for those who import it, which impacts the cost of broiler production (Pirgozliev et al., 2024). While numerous research efforts are focused on replacing SBM with more accessible and cost-effective locally produced alternative protein sources, their use is often limited by suboptimal amino acid profiles, reduced nutrient availability, and lower nutrient yield (Whiting et al., 2019; Watts et al., 2021). However, recent research has demonstrated that insects are a promising high-quality sustainable source of dietary CP and fat for poultry and other nonruminant animals (Al-Qazzaz et al. 2016; Bovera et al., 2018; Lalev et al., 2023; Yordanova et al., 2024). The meal produced from insects is rich in CP and AA, but contains chitin, which is considered as an antinutrient for poultry, reducing dietary energy and CP availability (Lubitz et al., 1943; Kobayashi and Itoh, 1991; Lokman et al., 2019).

The use of dietary enzymes such as phytase, protease, and carbohydrase is a well-established strategy to enhance nutrient and energy utilization, thereby improving the overall quality and efficiency of poultry diets (Šimić et al., 2023). Protease is a less extensively studied enzyme, but experiments with broilers fed SBM-based diets have shown that protease supplementation can improve growth performance (Angel et al., 2011; Cowieson et al., 2016; Xu et al., 2017), as well as dietary metabolizable energy, apparent ileal AA and CP digestibility (Adebiyi and Olukosi, 2015; Stefanello et al., 2016; Watts et al., 2020). However, the results of supplementing protease in insect meal-containing diets for fish (Bolton et al., 2021), pigs (Go et al., 2022) and laying hens (Facey et al., 2024) have not demonstrated efficacy expected for commercial animal production.

Updated information on the effects of supplementary protease in insect meal–containing diets for broilers is required to inform modern diets that include insect-derived ingredients. Accordingly, the objective of this study was to investigate the effects of exogenous protease in isocaloric and isonitrogenic diets formulated with and without insect meal on the dietary apparent metabolizable energy (AME), N-corrected AME (AMEn), dry matter (DMR), nitrogen (NR) and fat (FR) retention coefficients. Daily feed intake (FI), weight gain (WG) and feed conversion ratio (FCR) were also determined.

2. Material and methods

All animal procedures were carried out in accordance with the ethical guidelines for animal experimentation outlined in European Directive 2010/63/EU and its implementation in Bulgaria under Regulation 20/2012. The study was conducted at the poultry research facility of Trakia University (Stara Zagora 6000, Bulgaria; 42°23'54" N, 25°34'17" E) and received approval from the University Research Ethics Committee.

2.1. Insect meal sample and experimental diets

The insect meal used in this study was derived from larvae of the Black Soldier Fly (*Hermetia illucens* L.; Diptera: Stratiomyidae; BSFLM) and was sourced from Hexafly™ (Navan, Co. Meath, Ireland) (Table 1). To ensure good hygiene and biosecurity, the production process followed EC regulations (Regulation E.C. No 1069/2009) as previously described (Chobanova et al., 2023; Chobanova et al., 2024). Two isocaloric and isonitrogenic diets, with approximately 50 kcal metabolizable energy (ME) and 24 g CP less than the breeder recommendation (Aviagen Ltd, Edinburgh, UK), were formulated to have 13.17 MJ/kg ME and 171 g/kg CP (Table 2). One of the diets was formulated with the main component being 480 g/kg wheat, 200 g/kg maize, 150 g/kg SBM and 80 g/kg sunflower meal (control diet; CD). The other diet was formulated with 520 g/kg wheat, 200 g/kg maize, 45 g/kg SBM, 75 g/kg sunflower meal and 100 g/kg BSFLM (experimental diet; ED). Each of the two diets was divided into two portions: one served without modification, and the other supplemented with 1 g/kg of an exogenous protease enzyme (RONOZYME® ProAct, DSM). RONOZYME® ProAct is a serine protease enzyme produced by *Bacillus licheniformis* with a minimum protease activity of 75 000 PROT/g. Four diets in total were fed *ad libitum* in mash form. The insect meal was provided in a finely milled form by the supplier, while the cereal grains were ground using a hammer mill fitted with a 6-mm screen.

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)	
Dry matter	972.7	Arginine	21.36
Gross energy (MJ/kg)	21.15	Histidine	17.21
Crude protein (N × 5.60)	454	Isoleucine	20.43
Crude fat	171	Leucine	33.31
Ash	122	Lysine	32.61
Acid detergent fiber	79.5	Methionine	9.08
Acid detergent lignin	20.4	Phenylalanine	20.75
Chitin	59.1	Threonine	18.92
Minerals		Valine	29.21
Calcium (g/kg)	39.7	Tryptophane	7.68
Magnesium (g/kg)	4.0	Dispensable amino acids (g/kg)	
Phosphorus (g/kg)	11.9	Alanine	30.38
Potassium (g/kg)	13.5	Aspartic acid	47.41
Sulphur (g/kg)	4.3	Cystine	3.32
Copper (mg/kg)	23.9	Glycine	25.09
Manganese (mg/kg)	132.3	Glutamic acid	46.89
Sodium (mg/kg)	77.0	Proline	24.93
Zinc (mg/kg)	120.7	Serine	19.54
		Tyrosine	30.36

2.2. Birds and experimental design

Sixty day-old Ross 308 female broiler chicks were obtained from a commercial hatchery (Martivo - Rumen Kirchev Ltd., Sliven, Bulgaria) and housed in a single-floor pen, where they were fed a proprietary commercial broiler diet until 28 d of age. At 28 d, 48 birds, excluding those that were unusually light or heavy, i.e. 10% below or above of the 1493 g flock mean weight at this age, were randomly assigned to 24 pens (60 × 60 × 60 cm), with two birds per pen. Each pen featured a wire-mesh floor and was equipped with individual feeders and drinkers. The birds were maintained under standard broiler rearing conditions (Aviagen Ltd., Edinburgh, UK). Each dietary treatment was provided to six pens, with the pen serving as the experimental unit. Body weight and feed intake were recorded at placement, 28 d, and at the conclusion of the trial at 38 d. From these data, FI, WG, and FCR were calculated. Excreta were collected over four consecutive days (35 to 38 d of age), oven-dried at 60 °C, milled, and analyzed to determine dietary AMEn, total tract dry matter, and nutrient retention coefficients. Feed intake was also monitored throughout this collection period.

2.3. Laboratory analysis

Dry matter (DM), nitrogen (N), fat as ether extract, ash, and minerals in diets were determined as explained elsewhere (Pirgozliev and Bedford, 2013; Whiting et al., 2022). Crude protein in BSFLM was calculated as 5.60 × N (Janssen et al., 2017). The gross energy (GE) values in feed and excreta samples were determined with an isoperibol bomb calorimeter (Pirgozliev et al., 2006). The amino acid content of the BSFLM was analyzed by SSNIFF Spezialdiäten GmbH in accordance with EC Directive 2000/45/EC for tryptophan (European Union, 2000) and Directive EC/98/64 (L 257/16) for the remaining AAs (European Union, 1998). Acid detergent fiber (ADF) and acid detergent lignin (ADL) in BSFLM, as well as neutral detergent fiber (NDF) in the diets, were analyzed following the methodology outlined by Van Soest et al. (1991). The chitin in BSFLM was calculated as previously described (Hanh et al., 2018). Dietary AME and AMEn values were determined using standard procedures (Hill and Anderson, 1958). Daily AMEn intake and nutrient retention coefficients were measured as described elsewhere (Cullere et al., 2016; Cutrignelli et al., 2018).

Table 2 - Basal dietary composition of diets fed to broiler chickens either as the control (CD) or containing Black Soldier Fly larvae meal (ED)

Ingredient (g/kg)	Control (CD)	Insect meal (ED)
Maize (8.2% CP)	200.00	200.00
Wheat (10.9% CP)	480.00	520.00
Sunflower meal (36% CP)	80.00	75.00
Soybean meal (46% CP)	150.00	45.00
Black Soldier Fly larvae meal (45.4% CP)	-	100.00
L-Lysine (78%)	2.75	2.35
DL-Methionine (99%)	1.50	1.30
L-Threonine (98%)	1.25	1.40
Sodium chloride (NaCl)	2.50	2.50
Sodium bicarbonate	2.50	2.50
Vitamin and mineral premix[1]	2.00	2.00
Choline chloride (60%)	2.00	2.00
Monocalcium phosphate	8.50	7.50
Calcium carbonate	15.00	14.50
Sunflower oil	52.00	23.95
Total	1000	1000
Calculated analysis (as fed)		
Metabolizable energy (MJ/kg)	13.17	13.18
Crude protein (g/kg)	171	171
Crude fat (g/kg)	70.7	59.0
Ca (g/kg)	8.5	8.5
P (g/kg)	6.1	6.1
Lysine (g/kg)	9.5	9.5
Methionine + Cysteine (g/kg)	8.9	8.7
Determined analysis (as fed)		
Dry matter (g/kg)	896	902
Gross energy (MJ/kg)	17.09	16.77
Nitrogen (g/kg)	29.2	32.8
Crude protein (g/kg)[2]	183	203
Ether extract (g/kg)	73.9	63.0
Neutral detergent fiber	141	180

[1] The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by the breeder. The premix provided (units/kg diet) the following: vit. A (retinyl acetate) 10,000 IU; vit. D3 (cholecalciferol) 2,000 IU; vit. E (dl-α-tocopherol) 25 mg; vit. K3 (menadione) 1.5 mg; vit. B1 (thiamin) 1.0 mg; vit. B2 (riboflavin) 3.5 mg; vit. B6 (pyridoxine-HCl) 1.0 mg; vit. B12 (cyanocobalamin) 15 µg; niacin 30 mg; D-pantothenic acid 12 mg; choline chloride 350 mg; folic acid 0.8 mg; biotin 0.1 mg; iron 50 mg; copper 10 mg; manganese 60 mg; zinc 54 mg; iodine 0.7 mg; selenium 0.1 mg.
[2] The crude protein for the vegetable protein sources was obtained as N × 6.25 and as N × 5.6 for the Black Soldier Fly larvae meal.

2.4. Statistical analysis

Variables were analyzed by twoway ANOVA using a 2 × 2 factorial arrangement (dietary IM × supplementary protease) employing GenStat 21st edition software package. Data are expressed as means and their pooled standard errors (SEM). Residuals were checked for normality and homogeneity prior to ANOVA. Results were considered significant at P<0.05 by F-test.

The proposed mathematical model was as follows:

$$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + \mathcal{E}_{ijk},$$

in which Y_{ijk} = value observed in the insect meal use i (i = 1, 2), supplementary protease j (j = 1, 2,), and repetition k (k = 1, 2, 3, ..., 6); μ = overall mean of the experiment; a_i = fixed effect of the insect meal i (i = 1,2); b_j = fixed effect of supplementary protease j (j = 1, 2); $(ab)_{ij}$ = fixed effect of the interaction between insect meal i (i = 1, 2) and supplementary protease j (j = 1, 2); and $\mathcal{E}_{ijk}$ = random error in the insect meal i (i = 1, 2), supplementary protease j (j = 1, 2), and repetition k (k = 1, 2, 3, ...,6).

3. Results

Crude protein was the main constituent of the BSFLM meal, followed by crude fat (Table 1). Among the essential AAs, leucine, lysine, and valine were present in the highest concentrations, while tryptophan was found at the lowest level (Table 1). Aspartic and glutamic acids were the main dispensable AAs, with cystine having the lowest content (Table 1).

Compared to the calculated values, determined dietary CP was 7.0 and 18.7% higher in CD and ED, respectively than formulated (Table 2). Conversely, the determined values of oil in CD and ED were respectively 4.3 and 6.3% lower compared with calculated values (Table 2). It can be assumed that the observed differences were due to the composition of the actual ingredients used in the present study and the values given by the diet formulation software for the same ingredients. The diets contained similar GE, but the ED had approximately 28% more NDF than the CD (Table 2).

The mean body weight of the birds was 1493 g (SD ± 153.2) at start (at 28 d old) and 2096 g (SD ± 207.4) at the end of the study (at 38 d old) (data not shown). Overall, birds fed ED containing BSFLM, tended to have lower feed efficiency (higher FCR; P = 0.061) compared to birds fed CD (Table 3). Protease fed birds consumed 8.4% more feed than birds fed unsupplemented diets (P = 0.036; Table 3). There was a BSFLM by protease interaction regarding daily WG, as birds fed protease supplemented SBM-based diet reached 28.2% greater WG (P = 0.021) than birds fed the unsupplemented SBM-based diet (Table 3). However, in the BSFLM diet there was no response to protease for WG (P>0.05; Table 3). The AME and AMEn of the CD were more than 1.3 MJ greater than those of the BSFLM based ED (P<0.05), and there was no response to protease (P>0.05) and no response to protease was observed (P>0.05; Table 3). Birds fed protease supplemented diets consumed 9.6% more AMEn than those fed the unsupplemented feed (P = 0.036). There was no response (P>0.05) of DMR and NR to dietary type or protease, although the CD had a greater FR coefficient (P = 0.001; Table 3).

Table 3 - The effect of diets on apparent metabolizable energy (AME), AME corrected for N retention (AMEn), AMEn intake, total tract dry matter (DMR), nitrogen (NR) and fat (FR) retention coefficients determined with female Ross 308 broilers

Treatment	FI (g/b/d DM)	WG (g/b/d)	FCR (g:g)	AME (MJ/kg DM)	AMEn (MJ/kg DM)	AMEn intake (MJ/d)	DMR	NR	FR
BSFLM									
no	109.2	61.2	1.812	13.66	13.03	1.42	0.684	0.571	0.839
yes	113.6	58.3	1.960	12.19	11.51	1.30	0.646	0.550	0.730
SEM	2.78	2.08	0.0521	0.379	0.355	0.046	0.0204	0.0220	0.0192
Protease									
no	106.9	55.9	1.934	12.82	12.18	1.30	0.661	0.551	0.795
yes	115.9	63.6	1.838	13.03	12.36	1.43	0.670	0.570	0.774
SEM	2.78	2.08	0.0521	0.379	0.355	0.046	0.0204	0.0220	0.0192
BSLFM × Protease									
no no	101.8	53.6a	1.921	13.73	13.10	1.33	0.689	0.571	0.869
no yes	116.6	68.8b	1.702	13.59	12.96	1.51	0.680	0.571	0.809
yes no	112.1	58.2a	1.947	11.92	11.26	1.26	0.632	0.530	0.720
yes yes	115.2	58.4a	1.974	12.46	11.75	1.35	0.660	0.569	0.739
SEM	3.93	2.94	0.0737	0.536	0.502	0.065	0.0289	0.0311	0.0272
P-value									
BSFLM	0.278	0.384	0.061	0.014	0.008	0.085	0.206	0.505	0.001
Protease	0.036	0.019	0.211	0.709	0.724	0.058	0.757	0.549	0.464
BSFLM × Protease	0.153	0.021	0.114	0.537	0.539	0.483	0.530	0.542	0.166

BSFLM - Black Soldier Fly larvae meal; SEM - pooled standard errors of mean.
Growth performance variables were obtained between 28 and 38 d of age; metabolizable energy and retention coefficients were obtained for the last three days of the study.
a,b - Means followed by different lowercase letters in a column differ significantly from each other by the F test (P<0.05).

4. Discussion

The analyzed composition of BSFLM fell within the expected ranges reported in previous studies (Cullere et al., 2016; Bovera et al., 2018; Mahmoud et al., 2023; Chobanova et al., 2023). However, variations in fat, protein, mineral, and chitin contents are well recognized and primarily influenced by factors such as the larvae's age and the rearing substrate used (Makkar et al., 2014; Kieronczyk et al., 2022). The Ca content, which forms part of the total ash, corresponds with the elevated ash levels observed in the BSFLM sample of this study. The ADF content of BSFLM is commonly linked to the chitin present in insects. To improve the accuracy of chitin quantification, ADF values can be adjusted to account for amino acids (Finke, 2007) or acid detergent lignin (ADL) content (Hahn et al., 2018). The chitin levels observed here were consistent with previous findings (Schiavone et al., 2017; Chobanova et al., 2023), although variability is expected depending on the specific chitin determination method employed.

The discrepancies observed between the analyzed and calculated CP and fat contents of the diets are likely due to variations in the nutrient composition of the actual ingredients used compared to the values employed in the formulation software. The determined AME in CD was 13.24 MJ/kg on an as-fed basis, in agreement with the calculated values, but the AME of ED was 12.15 MJ/kg, or 1.0 MJ lower than the expected. Although BSLFM has a relatively high ME value (Bovera et al., 2018; Cutrignelli et al., 2018; Chobanova et al., 2024), dietary fiber influences AME in poultry diets, as high fiber content is generally associated with for lower AME (Pirgozliev et al., 2016). Dietary fiber fractions can correlate with chitin content in insect meals, a nitrogen-containing carbohydrate that is nutritionally unavailable to birds (Lubitz et al., 1943). In this study, the ED contained 28% more NDF compared with the CD, which may explain the lower AME and AMEn values in ED. Kobayashi and Itoh (1991) and Lokman et al. (2019) suggested that chitin itself may contribute to the low production and metabolizable energy. Previous research also found a reduced dietary nutrient and energy availability in diets containing over 10% insect meal (Bovera et al., 2018; Cutrignelli et al., 2018; Chobanova et al., 2023). Yang et al. (2025) verified that chitinase is effective to improve the nutrient digestibility in growing pigs. Therefore, the use of chitinase in diets with BSFLM can be an alternative to mitigate the negative effects of chitin on digestibility.

The birds remained healthy throughout the study, with no mortalities recorded. However, their body weights at both the start and end of the trial were lower than the Ross 308 female broiler target weights, which may be attributed to the use of mash feed instead of pelleted feed and housing in small groups (Pirgozliev et al., 2016; Yang et al., 2020). Despite this, these factors were not considered to compromise the study's objectives. Although mash diets can potentially lead to selective feeding of certain ingredients, this effect is expected to be minimal in practice, and no data on selective feeding were collected in the current study.

Dietary CP content is an important factor in elucidating the effectiveness of the protease supplementation. Proteases typically improve performance in birds fed low CP diets by releasing additional available amino acids and fulfilling the needs of the animals. In this study, despite the intention to produce low CP diets, the ED contained 203 g/kg vs 195 g/kg required CP. Thus, the birds had enough dietary CP for growth and maintenance, such that additional release of amino acids from the protease was not essential. In the current study, it was not possible to measure ileal AA digestibility, however this may be explored in future work. In the absence of ileal AA digestibility data, the positive response of the CD to protease may be due to the greater suitability of the enzyme to the plant-based diet and to the 6% reduction of CP in CD compared with the ED.

Most of the published literature on the feeding value of protease when supplemented to diets is based on vegetarian protein sources. Although in some reports supplementary protease did not improve performance or nutrient digestibility in broilers and turkeys (Freitas et al., 2011; Walk et al., 2018), in other reports the enzyme improved growth performance (Angel et al., 2011; Cowieson et al., 2016; Xu et al., 2017), dietary AMEn, and apparent ileal AA and CP digestibility (Adebiyi and Olukosi, 2015; Stefanello et al., 2016; Watts et al., 2020). However, the lack of response of the ED to the enzyme regarding WG requires further explanation. Proteases are categorized into six main classes: aspartate, cysteine, glutamate, metallo, serine, and threonine proteases. Among these, serine proteases account for over one-third and are characterized by endoproteolytic activity that typically relies on a catalytic triad composed of aspartate, histidine, and serine residues (Walk et al., 2018). The protease used in this study is from the serine group and was developed and very successfully used in vegetable/SBM-based diets but may not be so efficient when the diet contains a high amount of animal CP, such as insect meal. Lu et al. (2025) concluded that the cecal microbiome diversity is enhanced in laying hens fed high levels of BSFLM, linking it to suboptimal protein digestion and an undesired increase in protein fermentation, which can be partially addressed by protease supplementation. Bolton et al. (2021) did not find changes in the performance of fish fed 150 to 300 g/kg insect meal with and without exogenous protease (produced by thermophile bacteria) in an eight-week feeding study. Additionally, Go et al. (2022) did not find differences in growth performance of pigs when fed diets containing insect meal with and without supplementary alkaline serine endopeptidase for four weeks. Also, after feeding 130 g/kg insect meal to laying hens for 20 weeks, Facey et al. (2024) concluded that the supplementary protease, possessing acid, neutral, and alkaline activity and produced by *Bacillus licheniformis*, did not improve growth or performance in hens. Walk et al. (2018) reported that novel protease supplementation improved AA digestibility in vegetable diets, but no changes in growth performance of birds were observed. Since the high CP dietary content in our study may compromise the efficiency of protease, it is not possible to draw firm conclusions regarding protease effects in insect meal–based diets.

This study investigated a commercially available protease and insect meal for broiler production using the widely available Ross 308 broiler model. The research indicates that there is a need for further research in this area to make insect meal a consistent and viable alternative protein to soybean meal.

5. Conclusions

Feeding 10% BSFLM reduced dietary metabolizable energy for broiler chickens but did not affect other variables, except fat retention. An interaction was observed between dietary type and protease supplementation whereby protease was effective in SBM diets but not in BSFLM diets. Research on the impact of protease low-protein BSFLM diets, rather than protein sufficient diets, is recommended.

Data availability

The data that support the results of this study are available from the corresponding author upon reasonable request.

Author contributions

Conceptualization: Chobanova, S.; Karkelanov, N.; Mansbridge, S. C.; Rose, S. P. and Pirgozliev, V. R. **Data curation:** Chobanova, S. and Whiting, I. M. **Formal analysis:** Karkelanov, N. and Whiting, I. M. **Investigation:** Chobanova, S.; Karkelanov, N. and Pirgozliev, V. R. **Methodology:** Rose, S. P. and Pirgozliev, V. R. **Project administration:** Chobanova, S. and Pirgozliev, V. R. **Validation:** Mansbridge, S. C.; Rose, S. P. and Pirgozliev, V. R. **Writing – original draft:** Mansbridge, S. C. and Pirgozliev, V. R. **Writing – review & editing:** Stringhini, J. H. and Mello, H. H. C.

Conflict of interest

The authors declare no conflict of interest.

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