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Biochemical and Immune Modulation in Mice Fed Defatted Black Soldier Fly (*Hermetia illucens* L.) Larvae Meal

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ABSTRACT

Black soldier fly larvae (BSFL; *Hermetia illucens* L.) have gained attention as a sustainable, high-protein natural product with potential applications in animal feed. Defatted BSFL meal, a byproduct of oil extraction, offers enhanced nutritional profiles while minimizing excess dietary fat. Despite its growing use, its effects on blood biochemistry, hematology, and tissue morphology in mammals remain underexplored. Addressing this gap, this study evaluated the impact of defatted BSFL meal supplementation (5%, 10%, 15%, and 20%) on growth, hematological, biochemical, and antioxidant parameters, and histology in mice over 30 days. Mice fed up to 15% BSFL supplementation showed no significant differences in red blood cell counts, hemoglobin, liver enzymes (Alanine Transaminase and Aspartate Transaminase), antioxidant enzymes (Superoxide Dismutase and Catalase), or Malondialdehyde content compared to controls. However, at 20% supplementation, white blood cell counts significantly increased (14.00×10^3/µL vs. 8.95×10^3/µL in control), suggesting mild immunostimulation. Histological evaluation revealed slight hepatic vacuolation and villus erosion at 20% BSFL, while lower BSFL levels maintained normal tissue architecture. Ventricular wall thickness and villus height were preserved at 5–15% BSFL supplementation but declined slightly at 20%. In conclusion, moderate inclusion of defatted BSFL meal supported stable physiological and biochemical parameters without inducing toxicity. These findings highlight the feasibility of utilizing defatted BSFL meal as an environmentally friendly, functional protein source in animal feed, contributing to sustainable nutrition and offering potential applications for both livestock and human health.

Keywords: BSFL, Defatted, Hematology, Antioxidant, Histology

Introduction

Natural products derived from black soldier fly larvae (BSFL; *Hermetia illucens* L.) have become a focal point in sustainable animal nutrition research. BSFL can efficiently transform organic waste into high-quality biomass, offering an environmentally friendly method for producing protein-rich feed ingredients with a balanced essential amino acid profile and protein content.[1,2] The BSFL protein content is 559.9 g/kg and the crude lipid content is 18.6 g/kg.[3] BSFL have demonstrated potential health benefits in animal models, including improved metabolic function and enhanced gut health, largely attributed to its bioactive compounds, such as peptides and antimicrobial proteins.[4] BSFL are effective in transforming diverse types of biological material detritus into insect biomass,[5] and the cultivation of BSFL is an environmentally friendly approach to biogenically converting various forms of waste.[1] BSFL biomass, such as defatted BSFL meal, can be used as animal feed.

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Defatted BSFL, a byproduct of oil extraction from larvae, offers a low-fat, high-protein material that retains the functional benefits of BSFL while addressing issues related to high dietary fat content. This defatted product is especially relevant in research focused on its effects on physiological parameters, such as blood biochemistry and hematology, which are indicators of general health and metabolic state in animals.[6,7] Although BSFL has been extensively studied in various animal models, the effects of defatted BSFL on specific health markers, such as blood biochemistry and hematology, are still not fully understood. Translational research using mouse models provides an opportunity to explore the effects of BFSL under controlled conditions, offering insights into potential applications for both animal and human health. Research on BSFL has emphasized its capacity to boost feed efficiency, foster intestinal health, and augment immunological responses in poultry and aquaculture species. In addition, dietary supplementation with BSFL modulates lipid metabolism and oxidative stress biomarkers, suggesting broader systemic benefits.[8] However, defatting alters the chemical composition of BSFL, potentially influencing its bioactivity and effects on health markers. Previous studies have revealed that the application of live BSFL did not negatively affect the growth or blood characteristics of medium-growing chickens.[9] Previous research has also reported several beneficial effects of a diet incorporating BSFL meal, including reduced plasma blood cholesterol levels, increased diversity of microbial species, and improved intestinal uptake, up to 50% BSFL meal.

Though it is possible to include BSFL meal in the diets of certain carnivorous fish species without any negative consequences, numerous studies have revealed that replacing more than 50% of fish meal with BSFL meal can lead to detrimental effects. These effects are mostly attributed to the high quantities of chitin and crude fat present in the BSFL diets.[10] Beagles that consumed 5% and 15% BSFL showed decreased activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and malondialdehyde (MDA). Substituting 25% of fish meal with BSFL meal in the feed of juvenile golden pompano (*Trachinotus ovatus*) enhanced the consumption of food, increased fat accumulation in the liver, and enhanced the healthy balance of gut flora. This substitution did not negatively impact growth or gastrointestinal health; however, BSFL meal negatively impacts both development and intestinal health when the replacement level reaches 50%.[11] Antioxidants neutralize free radicals, increasing the response of the immune system and reducing the risk of disease in livestock. Huang[12] stated that fed mice with BSFL meal, which led to significant results on SOD and CAT, including its ability to stabilize free radicals by reducing MDA levels.

In poultry models, defatted BSFL has been linked to improved nutrient digestibility and favorable changes in serum biochemical parameters, including reductions in cholesterol and improvements in liver function markers.[13] However, such studies remain limited, and evidence from mammalian models, particularly in terms of hematological and biochemical parameters, is sparse.

Despite promising results from animal feed studies, little is known about how defatted BSFL affects blood biochemistry and hematology in mammals. Current research has primarily focused on whole BSFL or its crude extracts, leaving the effects of defatted preparations underexplored. Furthermore, the potential of defatted BSFL to serve as a functional food ingredient or therapeutic agent in mammalian systems has yet to be fully characterized. This study addresses this gap by evaluating the effects of defatted BSFL on blood biochemistry, hematology, and the digestive system in mice. The results add to the growing body of research that supports the use of defatted BSFL meal as a sustainable and useful ingredient. Specifically, it provides insights into its potential applications in improving animal and human health through dietary interventions.

Materials and Methods

Chemicals and BSFL preparation

Chemicals of analytical grade were acquired from Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich (USA). Eggs of the black soldier fly were procured from a local farmer at BSFL, Ahasa, Samarinda, Ltd., situated in East Kalimantan, Indonesia. After 4 days, the eggs metamorphosed and matured into juvenile larvae. The juvenile larvae were cultivated for 7 days in a plastic enclosure at approximately 28°C and relative humidity of 60–70%. During this stage, chicken pellets served as the feeding substrate.

BSFL substrate and rearing

Palm kernel meal (PKM; Manunggal, Ltd., Kalimantan Timur, Indonesia) was subjected to fermentation with effective microorganism 4 (EM4). The fermentation process included a mixture of 180 g molasses, 279 mL EM4, 2.5 L water, and 2.5 kg PKM. The mixture was enclosed in a plastic bag, which was sealed and left undisturbed at ambient temperature to facilitate fermentation. A fructose concentration of 5% is suitable for achieving optimal development and nutrient composition of BSFL.[1]

Five plastic chambers (L 24 x W 15 x H 6 cm) were used to rear 3000 7-day-old BSFL with 200 larvae in each chamber (with three replications). To attain a humidity level of 60–70%, water was introduced to each substrate and the temperature was maintained at approximately 28°C. BSFL in each chamber were supplied with a specific quantity of substrate in accordance with the methodology outlined in a previous study by Hoc[14] with some adjustments. Rearing proceeded until 90% of the larvae entered the prepupal phase, which occurred around day 22. Prepupal BSFL were harvested for BSFL meal preparation and analyzed for nutritional content.

BSFL meal and test diet preparation

Harvested BSFL were cleaned by washing and soaking in hot water for 30 s. BSFL were placed in an oven and dried at a maximum temperature of 60°C for 24 h. The dried BSFL were pressed using an oil-press machine to separate the meal from the oil. The defatted BSFL meal was mixed with factory feed at levels of 5%, 10%, 15%, and 20%. A total of 700 mL of water was added to the feed compound (1 kg) and stirred until it was completely mixed. The mixture was then placed in a pelletizing machine to form pellets. The pellets were placed in a food dehydrator for ± 22 h until dry pellets were obtained. The dry pellets were placed in a plastic container at room temperature until use. Proximate analysis of the test diet was performed to determine the nutritional content.

Proximate analysis of BSFL

Proximate analysis of BSFL, a standardized method,[15] was used to determine the basic nutritional composition, including moisture, ash, crude protein, crude fat, and crude fiber. The procedure begins with sample preparation in which the BSFL are first dried (usually oven-dried at 60–105°C) to a constant weight to determine the moisture content by weighing a known quantity of the sample before and after drying and calculating the percentage of weight loss attributable to water content.

For ash content, the dried BSFL samples were incinerated in a muffle furnace at around 550–600°C for 4–6 hours. This process oxidizes all organic matter, leaving behind inorganic minerals, and the ash percentage was calculated by comparing the weight of the residue to the initial dry weight of the sample. The crude protein content was measured using the Kjeldahl method, which involves digesting the sample in concentrated sulfuric acid to convert nitrogen into ammonium sulfate. The solution was distilled and titrated to determine the total nitrogen content, which was multiplied by a nitrogen-to-protein conversion factor (6.25) to estimate the crude protein levels.

Crude fat was extracted using Soxhlet apparatus with a non-polar solvent (petroleum ether). The fat content was determined gravimetrically by evaporating the solvent and weighing the remaining lipid fraction. For crude fiber analysis, the defatted sample was subjected to sequential digestion with dilute acid and alkali to simulate the human digestive process, leaving behind the indigestible fibrous material. The residue was filtered, dried, weighed, and ashed, and the difference in weight before and after ashing represents the fiber content.

Mice treatment

Twenty-five male mice (BALB/c, 2 months old, average body weight: 30 g) were acclimatized in the laboratory for 7 days. The mice were randomized freely to five groups (n = 5 per group): the control group (C) and the BSFL meal supplementation groups (P1: 5%; P2: 10%; P3: 15%; and P4: 20%). All mice were weighed on the first day of the study to measure their initial body weight prior to treatment. The control group was provided with 100% factory feed. All mice were fed a total of 16 g daily. Feeding was performed twice a day at 07:00 and 16:00, local time, for 30 days. All mice were given free access to drinking water.

Growth measurement

Mice were weighed three times: once at the beginning of the study and on days 15 and 30. The mice were weighed using a digital scale.

Hematological and biochemical blood analysis

A blood profile determination of the mice was performed at the end of the trial to evaluate the health of the test animals. Blood was withdrawn through the orbital sinus using a microhematocrit. The blood sample was placed in a tube containing EDTA as an anticoagulant. The blood profiles—namely, white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), hemoglobin (Hb), and hematocrit (HCT)—were determined using a hematology analyzer. To separate the serum from the blood, the blood samples were withdrawn and centrifuged at 3500 rpm for 15 min. The serum was separated and placed in an Eppendorf tube for ALT and AST determination.

For ALT measurements, the substrate solution consisted of 200 mM L-alanine and 2 mM α-ketoglutarate in phosphate buffer (pH 7.4), while

for AST measurements, L-aspartate replaced L-alanine in an otherwise similar solution. In a clean test tube, 0.5 mL of the respective substrate solution was mixed with 0.1 mL of the serum sample. The mixture was incubated at 37°C for 30 min. Following incubation, 0.5 mL of 1 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 1 N hydrochloric acid was added to each tube to react with the keto acids (pyruvate for ALT, oxaloacetate for AST) produced during transamination. The mixture was left to stand at room temperature for 20 min to ensure complete development of the hydrazone complex. The reaction was terminated by the addition of 5.0 mL of 0.4 N sodium hydroxide (NaOH), and the tubes were allowed to stand for another 10 min to stabilize the color. The ALT and AST levels were measured using the Biolis 24i Premium (Tokyo Boeki, Japan).[16,17]

Enzyme antioxidants evaluation

Antioxidant enzymes activities (SOD and CAT) and MDA contents of the mice were measured on the last day of the study. Blood (n = 3 mice per group) was withdrawn using a similar procedure to the blood profile analysis to obtain serum from the blood. Serum was analyzed for SOD (Superoxide Dismutase Activity Assay Kit, catalog number BC0170, Solarbio, China), CAT (Catalase Assay Kit, catalog number BC0200, Solarbio, China), and MDA (Malondialdehyde Content Assay Kit, catalog number BC0020, Solarbio, China) levels were measured using the Microplate Reader (HBS-1101, Guangdong Kangyu Medical Instrument Co., Ltd, China).

Prior to the SOD assay,[18] the kit reagents were prepared and equilibrated as follows. Reagents I and III and the working Reagent V (prepared by dissolving Reagent IV powder into Reagent V buffer per kit instructions) were warmed to 37°C for ≥5 min, while Reagent II (xanthine oxidase solution) and plasma samples were kept on ice. The reaction mixture for each test was assembled by combining 90 µL of mouse plasma with 240 µL of Reagent I, 6 µL of Reagent II, 180 µL of Reagent III, 30 µL of Reagent V, and 480 µL distilled water (final volume ≈1.026 mL). A control reaction was prepared identically but without Reagent II (xanthine oxidase), replacing it with 6 µL of water, to account for any absorbance not due to the enzymatic reaction. Two blank reactions were also included: Blank 1 contained all reagents (including Reagent II) but no plasma (plasma replaced with 90 µL water), and Blank 2 contained all reagents except Reagent II. All tubes were mixed thoroughly and incubated at room temperature (~25°C) for 30 min. After incubation, each mixture was transferred to a 1 mL cuvette, and absorbance at 560 nm was measured against a distilled-water blank using a spectrophotometer. The absorbance differences were then calculated as follows: for the test reaction, ΔA_sample = A_test – A_control, and for the blanks, ΔA_blank = A_blank1 – A_blank2. The percentage inhibition of Nitro Blue Tetrazolium (NBT) reduction by SOD was computed by the formula:

$$P\ (\%) = \frac{[(\Delta A\_blank - \Delta A\_sample)]}{\Delta A\_blank}\ x100$$

The SOD activity in the original plasma sample (U/mL) was calculated using the kit equation for serum/plasma:

$$SOD\ (U/mL) = 11.4 \times \left[\frac{P}{(1-P)}\right] x\ F,$$

where P is the inhibition fraction and F is the dilution factor of the sample.

For each reaction in the CAT assay, 20 µL of serum sample was mixed with 1 mL of working H_2O_2 solution (prepared by diluting the 250 mM H_2O_2 reagent provided in the kit with CAT Assay Buffer) and incubated at 37°C for 1 min to allow catalase to act on the substrate. The reaction was terminated by adding 1 mL of ammonium molybdate solution (Reagent III). For the control, the molybdate solution was added to the H_2O_2 solution first, followed by the serum sample, to quench the reaction immediately to account for background absorbance. A blank tube containing all reagents except serum was used to calibrate the spectrophotometer (zero absorbance). After mixing thoroughly, 200 µL of the final reaction mixture was transferred to a 96-well microplate or a quartz cuvette, and the absorbance was read at 405 nm using a spectrophotometer.

The catalase activity (U/mL) was calculated using the following formula provided by the manufacturer:

$$CAT\ activity\ (U/mL) = \frac{(Acontrol\text{-}Asample)}{Acontrol}\ x\ VT \div (VS \times t)$$

where Acontrol and Asample are the absorbance values of the control and test reactions respectively, VT is the total reaction volume (2.0 mL), VS is the serum sample volume (0.02 mL), and t is the reaction time (1 min). The resulting activity was normalized to the protein concentration of each serum sample (determined separately by the Bicinchoninic Acid-BCA method) and is expressed as units per milligram of protein (U/mg protein).[19]

Meanwhile, in MDA analysis, 200 µL of serum from each sample was pipetted into a clean tube and 600 µL of the kit's MDA working reagent (containing thiobarbituric acid-TBA) was added. For the reagent blank, 200 µL of distilled water replaced the sample. Then, 200 µL of Reagent III (provided in the kit) was added to both the sample and blank tubes. All tubes were tightly capped and incubated at 100°C for 60 min (to drive the TBA–MDA condensation reaction). After heating, tubes were cooled on ice and centrifuged at 10,000×g for 10 min at room temperature to pellet any precipitate. One milliliter of the clear supernatant was transferred to a quartz cuvette for spectrophotometric measurement.

The absorbance of each sample was measured at 450 nm, 532 nm, and 600 nm using a UV-Vis spectrophotometer (the instrument was zeroed with distilled water prior to measurements). The 532 nm reading corresponds to the red MDA–TBA adduct, while the 600 nm reading accounts for non-specific turbidity. A reading at 450 nm was taken to correct for interfering color from reducing sugars, as recommended by the kit.[20] Absorbance differences were calculated as:

$\Delta A_{450} = A_{450}(sample) - A_{450}(blank)$,
$\Delta A_{532} = A_{532}(sample) - A_{532}(blank)$, and
$\Delta A_{600} = A_{600}(sample) - A_{600}(blank)$.

The MDA concentration in nmol/mL was then calculated according to the kit formula:

$$MDA\ (nmol/mL) = 5\ x\ [6.45\ (\Delta A_{532} - \Delta A_{600}) - 1.29\Delta A_{450}]$$

The results were normalized to the total protein content and MDA is expressed as nmol per mg protein. Thus, MDA (nmol/mg protein) was obtained by dividing the above nmol/mL value by the protein concentration (mg/mL) of the sample.

Histology

At the end of the experiment, three mice from each group were sacrificed by cervical dislocated and dissected. The ventriculus, intestine, and liver were collected. Tissue samples were fixed in 10% neutral buffered formalin for 24–48 hours to preserve the cellular architecture. Following fixation, samples were processed through a graded series of ethanol (70–100%) for dehydration, cleared in xylene, and embedded in paraffin wax to facilitate sectioning. Paraffin blocks were sectioned at 4–5 µm thickness using a rotary microtome. The sections were mounted on glass slides, deparaffinized in xylene, and rehydrated through a descending alcohol series. Hematoxylin and eosin (H&E) staining[21] was performed. Slides were stained with hematoxylin to visualize the cell nuclei (blue-purple), followed by eosin to stain the cytoplasmic and extracellular components (pink). Stained sections were dehydrated, cleared, and coverslipped using a mounting medium.

Histological preparations were examined using a light microscope to observe the histological structures in each organ. In the intestine, observations were made to determine the damage and structure of the villi, which included the height of the villi (µm) and the depth of the crypt (µm), while in the ventriculus, observations were made to observe the damage and thickness of the ventriculus wall using magnifications of 100x, respectively. In the liver, hepatic damage was assessed by the occurrence of degeneration (cell swelling) and necrosis (cell death). Observations for all tissues were made in five different fields of view of the histological preparations using a microscope at 100x magnification.

Ethical Statement
The Ethic Commission for Health Research, Faculty of Medicine, Universitas Mulawarman, Samarinda, Kalimantan Timur, Indonesia, granted approval (No: 263/KEPK-FK/X/2024) for the in vivo study.

Data analysis
The growth measurement data, hematological and biochemical blood analysis, and measurement of ventricular wall thickness, villus height, and intestine crypt depth were analyzed using SPSS version 22 software (SPSS, Inc., USA). ANOVA was used to identify significant variations among the treatment groups. Following the identification of significant differences through ANOVA analysis, a Duncan multiple range test (DMRT) was performed. A *p*-value of < 0.05 was taken to be significant. Histological images were descriptively evaluated.

Results and Discussion

Nutritional profile of BSFL
Proximate analysis of the BSFL reared using fermented PKM revealed high protein and fat contents. BSFL obtained in this research are suitable as animal feed in which the protein (35.33%) and fat (33.75%) content can potentially replace fishmeal (Table 1). The protein levels in the diet increased with higher BSFL inclusion from 19.00% in the control group to 27.24% in the P4 group, suggesting better dietary suitability for growth and development. These results are similar to a previous study that reported that fishmeal can be substituted with several alternative sources, such as rapeseed (20.9% protein).[22]

Table 1: Nutrition content of the BSFL reared using fermented palm kernel meal

Nutrition content	Value (%)
Moisture	4.76
Ash	9.07
Protein	35.33
Fat	33.75
Fiber	34.79

The supplementation of defatted BSFL meal in the diet substantially enhanced protein levels. BSFL are a rich source of high-quality protein, and similar studies have shown its efficacy in promoting protein enrichment in livestock diets. Kondo[23] reported that the ideal feed content for young mice comprised 25–35% protein, which is required for maintaining metabolic health. Finke[24] also reported that BSFL meal supplementation significantly increased protein content in poultry and aquaculture feed formulations.
The fiber content slightly increased in the BSFL groups, from 6.00% in the control group to 7.20–7.86% in the BSFL groups (Table 2). A higher protein and slightly increased fiber content in the diet can promote better growth and digestion in mice. In addition, fiber supports gut health, reducing the risk of gastrointestinal disorders. A consistent fat content across the groups (5.12–5.58%) ensured sufficient energy without risking obesity.

Table 2: Proximate analysis of the mice diet with various substitutions level of the defatted BSFL meal in the factory feed

Groups	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Fiber (%)
C	14.00	8.00	19.00	5.12	6.00
P1	6.15	94.89	20.48	5.43	7.86
P2	5.35	94.97	23.00	5.58	7.38
P3	5.41	95.04	23.84	5.58	7.20
P4	4.91	94.98	27.24	5.42	7.30

Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal)

Proximate analysis of the test diet showed that the moisture content significantly decreased with increased BSFL, ranging from 14.00% in the control group to 4.91% in P4 (20% defatted BSFL meal). The reduced moisture content in the BSFL diets, particularly in P4 compared with the control moisture content, indicates improved shelf stability. This aligns with past findings that highlight the lower moisture content in insect-based meals as a key factor in extending the storage life of feed.[25] The high ash content in BSFL diets (94.89–95.04%) compared to the control (8.00%) also reflects the mineral richness of BSFL, particularly calcium and phosphorus. A high mineral content promotes bone health and vitality in animals. Further, the ash content increased dramatically with BSFL supplementation, from 8.00% in the control to 94.89–95.04% in the BSFL groups (P1–P4, 5–20% defatted BSFL meal).
Studies have consistently demonstrated the beneficial effects of BSFL as an alternative protein source in animal diets. Van Huis[26] demonstrated improved growth rates in animals fed diets supplemented with BSFL, owing to superior protein digestibility. Schiavone[27] found that replacing 50% soybean meal with BSFL meal in poultry diets resulted in comparable or superior growth performance. Thus, substituting factory feed with defatted BSFL meal in mouse diets significantly improves nutritional quality, particularly protein content.

Growth parameters
The impact of incorporating defatted BSFL meal on the body weight of mice was evaluated over a 30-day period. The statistical evaluation showed no significant differences ($p > 0.05$) on body weight among the groups, although the trends suggest some potential impacts of BSFL inclusion levels (Table 3).

Table 3: Body weight of mice treated with various concentration of the defatted BSFL meal substitutions for 30 days

Groups	Weight (g)		
	Initial	Day 15th	Day 30th
C	28.80± 0.58^a	30.40± 1.26^a	31.20± 1.42^a
P1	28.80±0.58^a	33.60± 0.92^a	33.60± 0.92^a
P2	29.20± 0.48^a	32.80± 1.06^a	34.60± 0.87^a
P3	29.40± 0.40^a	34.20± 1.06^a	34.60± 0.87^a
P4	29.40± 0.40^a	33.40± 0.50^a	34.00± 0.83^a

Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). Data are presented as the mean ± standard error of the mean. No significant differences ($p>0.05$) are indicated by means in the same column that are followed by the same superscript letter (a).

Initially, all groups had comparable weights, ranging from 28.80 ± 0.58 g (C, P1) to 29.40 ± 0.40 g (P3, P4). On day 15, the highest body weight was seen in the P3 group (34.20 ± 1.06 g), while the control group had the lowest weight (30.40 ± 1.26 g); the P1, P2, and P4 groups displayed intermediate weight gains (33.40–33.60 g). On day 30, either mice in the P2 (10% defatted BSFL meal) or P3 (15% defatted BSFL meal) groups achieved the highest final weights (34.60 ± 0.87 g), followed closely by P4 (34.00 ± 0.83 g). The control group showed the lowest weight gain (31.20 ± 1.42 g).
Although not significant, the trends in body weight suggest that dietary inclusion of BSFL meal positively influenced growth in mice. Groups supplemented with moderate levels of BSFL (10–15% defatted BSFL meal) exhibited the greatest weight gain, consistent with prior research emphasizing the growth-promoting properties of BSFL. For example, Schiavone[27] reported significant weight gain in broilers fed diets containing 10–15% defatted BSFL meal, which they attributed to its high protein content and digestibility. Similarly, BSFL is rich in essential amino acids and contributes to muscle development and energy metabolism in livestock and laboratory animals.[28]
The highest weight gains in this study were observed in the P2 and P3

groups (10–15% defatted BSFL meal), suggesting that these levels may optimize nutritional benefits without negatively affecting feed intake or digestibility. Excessive inclusion (P4, 20%, defatted BSFL meal) did not result in additional growth, which aligns with previous findings that high levels of insect meal might reduce feed efficiency because of altered palatability or nutrient assimilation.[6]

BSFL meal is a rich source of bioavailable protein, essential fatty acids, and micronutrients such as calcium and phosphorus. These components likely contributed to the observed trends in weight gain, as proteins are critical for muscle growth and repair, whereas fats provide sustained energy. The slight reduction in weight gain at the highest inclusion level (20% defatted BSFL meal) may reflect a ceiling effect, in which nutrient absorption or utilization becomes less efficient at higher dietary concentrations.

Blood analysis

The effects on blood profiles of mice treated with different supplementation of defatted BSFL meal for 30 days are shown in Table 4. The WBC count in group C was 8.95×10³/µL, while those in mice fed the defatted BSFL-supplemented diet ranged from 8.08×10³/µL in group P3 (15% defatted BSFL meal) to 14.00×10³/µL in group P4 (20% defatted BSFL meal), which was significantly higher than the control. However, there were no significant differences in RBC, HB, and HCT values between groups. The platelet values were highest in the control group (848.5×10³/µL) but there were no significant differences between groups (Table 4).

Table 4: Blood profile of mice treated with various concentration of the defatted BSFL meal substitutions for 30 days

Groups	WBC (10^3/µL)	RBC (10^6/µL)	PLT (10^3/µL)	HB (g/dL)	HCT (%)
C	8.95 ± 0.51^a	9.97±0.14^a	848.50±183.50^b	15.30 ±0.40^a	0.46±0.02^a
P1	9.07 ± 2.98^a	7.67±0.89^a	540.50±11.50^a	11.80 ±1.10^a	0.36±0.36^a
P2	9.58 ± 1.23^a	7.21±0.20^a	677.00±169.00^a	11.20 ±0,60^a	0.33±0.20^a
P3	8.08 ± 3.38^a	7,82±0.16^a	406.00±123.00^a	12.10 ±0.30^a	0.37±0.14^a
P4	14.00±3.45^b	8.46±0.38^a	624.50±77.50^a	12.95 ±1.30^a	0.40±0.24^a

Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). Results are shown as the mean ± standard error. Means followed by the same superscript letter (a) in the same column indicate no significant differences ($p>0.05$). WBC: White Blood Cell, RBC: Red Blood Cell, PLT: Platelet, HB: Hemoglobin, HCT: Hematocrit.

The inclusion of defatted BSFL meal in mouse diets showed a variable impact on blood profile parameters. An increase in the WBC count was observed in group P4 (20% defatted BSFL meal). The elevated WBC value suggests a mild immunostimulatory effect, possibly due to bioactive compounds, such as chitin, antimicrobial peptides, and other immune-enhancing components present in BSFL. This is in line with the study of Rumpold and Schlüter[29], who indicated that insect-based meals could enhance innate immunity by promoting the activity of immune cells. However, the lack of significant differences in the other groups suggests that the effect is dose-dependent and unlikely to induce systemic inflammation.

The RBC, hemoglobin, and hematocrit values did not show any significant differences between the groups. The RBC values were slightly lower in P2 (10% defatted BSFL meal) and P3 (15% defatted BSFL meal), but these variations remained within the normal range for mice, suggesting that the defatted BSFL meal did not impair erythropoiesis or oxygen transport capacity. Sawicka[30] reported similar findings that insect-based protein sources maintain erythrocyte function without causing anemia or other hematological disorders in livestock and rodent models.

The PLT value demonstrated a decreasing trend with higher defatted BSFL meal inclusion, but the differences were not statistically significant. The control group recorded the highest PLT value (848.5×10³/µL), whereas the lowest (406.0×10³/µL) was recorded in P3 (15% defatted BSFL meal). Such variations might indicate minor dietary effects on platelet production, but these changes were not severe enough to impair hemostatic function. Similar findings by Siddiqui[2] emphasize that insect-based diets are hemostatically neutral, making them safe for consumption at different inclusion levels.

Biochemical blood status

We next examined the impact of including defatted BSFL meal at different levels on ALT and AST in mice over a 30-day period. There were no significant changes ($p > 0.05$) in ALT and AST levels between groups (Table 5), suggesting that defatted BSFL meal does not impair liver function, supporting its safety as a feed ingredient. This finding is consistent with Schiavone[27], who reported no significant alterations in ALT and AST levels in broilers fed defatted BSFL meal at varying levels. Similarly, partially defatted BSFL meal did not affect liver enzyme activity in test animals, indicating good hepatic tolerance across species.[31] However, Fikri[32] observed decreased ALT and AST levels in chickens fed BSFL-based diets, attributing these effects to bioactive compounds in BSFL, such as antimicrobial peptides and chitin. The absence of significant changes in the present study may reflect species-specific metabolic responses or differences in the experimental conditions, including diet composition and feeding duration.

Table 5: Biochemical status of mice treated with various levels of defatted BSFL meal substitution for 30 days.

Groups	ALT (IU/L)	AST (IU/L)
C	363.00 ± 18.00^a	133.00 ± 7.00^a
P1	340.31 ± 11.31^a	98.37 ± 6.37^a
P2	326.81 ± 15.18^a	106.87 ± 2.12^a
P3	379.0 ± 11.00^a	108.50 ± 6.50^a
P4	356.0 ± 17.00^a	113.50 ± 4.50^a

Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). Results are shown as the mean ± standard error. Means followed by the same superscript letter (a) in the same column indicate no significant differences ($p>0.05$). ALT: *Alanine Amino Transaminase* and AST: *Aspartate Amino Transaminase*

Antioxidant status

The SOD and CAT are critical enzymes that mitigate oxidative stress. The lack of significant changes in their activity suggests that the inclusion of BSFL did not enhance or impair antioxidant defenses in the mice (Figure 1). These findings suggest that supplementing factory feed with defatted BSFL meal does not significantly impact antioxidant enzyme activities in mice. This is consistent with previous research on other species. For instance, Li[33] investigated the effects of replacing up to 50% fish meal with defatted BSFL meal in the diets of juvenile Jian carp (*Cyprinus carpio* var. Jian) and found no significant changes in antioxidant enzyme activities, including SOD and CAT. Similarly, defatted BSFL meal as an alternative protein source for juvenile Japanese seabass (*Lateolabrax japonicus*) led to no significant differences in antioxidant responses compared to control diets.[34]

In contrast, some studies have reported enhanced antioxidant enzyme activities with insect meal supplementation. Dietary inclusion of BSF or dynastid beetle meals significantly improved non-specific immune responses and antioxidant enzyme activities in Pacific white shrimp (*Litopenaeus vannamei*).[35] The discrepancy between these findings and the current study may be attributed to species-specific metabolic differences, variations in diet composition, or differences in the levels of insect meal inclusion. Similarly, Chen[36] reported enhanced antioxidant activity in chickens fed BSFL, which they attributed to the bioactive compounds present in the larvae. The absence of such effects in the current study could be due to the short duration (30 days), relatively moderate inclusion levels (5–20% defatted BSFL meal), or a balanced basal diet providing sufficient antioxidant capacity. In addition, Makkar[37] emphasized that, while insects are safe and

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nutritionally rich, their effects on physiological and biochemical parameters are not always statistically significant, particularly in short-term studies.

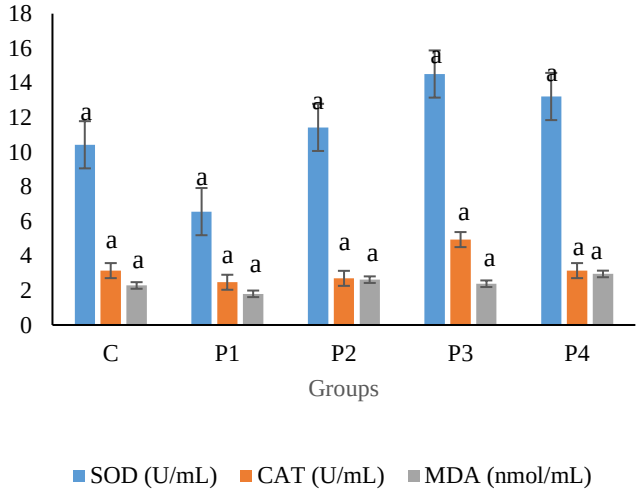

Figure 1: Antioxidant enzyme activity of mice treated with various levels of BSFL inclusion for 30 days. Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). The same superscript letter (a) above the bar for the same enzyme indicates no significant differences ($p>0.05$). SOD: *Superoxide Dismutase*, CAT: *Catalase*, and MDA: *Malondialdehyde*.

Furthermore, the absence of significant changes in MDA levels in this study indicates that the substitution of defatted BSFL meal did not induce lipid peroxidation in mice. This aligns with past findings that there were stable MDA levels in Jian carp fed diets containing defatted BSFL meal.[33] The presence of bioactive compounds, such as chitin, in insect meals has been suggested to confer antioxidant properties. However, in the current study, this did not translate into measurable changes in antioxidant enzyme activities, possibly because of the specific physiological responses of mice or the duration and level of dietary exposure.

Histology

We also investigated the effects of a defatted BSFL meal on ventricular wall thickness, villus height, and intestinal crypt depth in mice after 30 days of feeding with varying levels of defatted BSFL meal (Table 6). The control group, which received no defatted BSFL meal, displayed a ventricular wall thickness of 1256.72 ± 159.83 µm. Mice in groups P1 (5% defatted BSFL meal) and P2 (10% defatted BSFL meal) showed no significant difference in ventricular wall thickness (1244.23 ± 68.23 and 1279.25 ± 57.58 µm, respectively) compared to the control. However, mice in groups P3 (15% defatted BSFL meal) and P4 (20% defatted BSFL meal) exhibited significantly reduced ventricular wall thickness (845.54 ± 77.93 and 820.80 ± 110.81 µm, respectively), indicating a threshold for the effect of higher BSFL inclusion levels. For villus height, the control group (378.77 ± 6.90 µm) and P2 (378.86 ± 5.90 µm) showed similar results, both of which were significantly ($P < 0.05$) greater than those in P1 (287.73 ± 6.24 µm). Mice in group P3 (377.74 ± 6.58 µm) also maintained a villus height comparable to the control, while P4 (322.22 ± 9.01 µm) showed a moderate ($P < 0.05$) reduction. Crypt depth analysis revealed that mice in groups P2 (149.14 ± 3.33 µm) and P3 (147.63 ± 4.40 µm) were similar to the control mice (141.22 ± 1.82 µm), while mice in P1 (129.91 ± 3.12 µm) and P4 (129.07 ± 5.01 µm) exhibited a significant reduction.

Table 6: Mean Ventricular Wall Thickness, Villus Height, and Intestine Crypt Depth of Mice (*Mus musculus* L.) fed various inclusion of defatted BSFL meal for 30 Days

Groups	Parameters		
	Ventricular Wall Thickness (µm)	Villus Height (µm)	Intestine Crypt Depth (µm)
C	1256.72±159.83^{b}	378.77±6.90^{c}	141.22±1.82^{b}
P1	1244.23±68,23^{b}	287.73±6.24^{a}	129.91±3.12^{a}
P2	1279.25±57.58^{b}	378.86±5.90^{c}	149.14±3.33^{b}
P3	845.54±77.93^{a}	377.74±6.58^{c}	147.63±4.40^{b}
P4	820.80±110.81^{a}	322.22±9.01^{b}	129.07±5.01^{a}

Note: Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). Results are shown as the mean ± standard error. Means followed by the same superscript letter (a) in the same column indicate no significant differences ($p>0.05$).

Histological analysis of the ventriculus, intestines, and liver of mice fed varying concentrations of defatted BSFL meal showed distinct changes in tissue morphology across the experimental groups (Figure 2). In the control group, the villi were well-formed with intact mucosal layers and abundant Paneth cells. In group P1 (5% defatted BSFL meal), there were minimal changes in villus height and crypt depth compared to the control. Groups P2 (10% defatted BSFL meal) and P3 (15% defatted BSFL meal) demonstrated a slight reduction in villus height, along with mild erosion in some sections. Paneth cells were still observable but appeared slightly reduced. In group P4 (20% defatted BSFL meal), there was more pronounced villus erosion, reduced villus height, and deeper crypts compared to the other groups. In addition, the number of Paneth cells was notably lower.

The reduction in villus height and increase in crypt depth in mice fed defatted BSFL meal, particularly mice in the P4 (20% defatted BSFL meal) group, might indicate mild mucosal irritation or adaptive remodeling of the intestinal lining. Basili[38] observed that insect-based diets could lead to subtle structural changes in the gut mucosa, possibly owing to the presence of chitin and other bioactive components in insect meals.

The decrease in Paneth cell numbers in the P3 (15% defatted BSFL meal) and P4 (20% defatted BSFL meal) groups is noteworthy. Paneth cells play a pivotal role in intestinal immunity and maintain the gut microbiota balance by secreting antimicrobial peptides. This reduction suggests altered gut homeostasis at higher BSFL meal concentrations. This is consistent with the findings of Finke[24], who highlighted that dietary chitin, while beneficial in moderation, may overstimulate gut-associated lymphoid tissues at high doses, potentially depleting epithelial protective cells. The mild erosion observed in group P4 (20% defatted BSFL meal) may result from increased dietary fiber and chitin levels, as these compounds can pose a challenge to digestive enzymes. Despite these changes, the overall intestinal architecture remained intact, indicating that the defatted BSFL meal was not inherently toxic at the tested concentrations.

The disruption of sinusoidal structures in group P4 (20% defatted BSFL meal) suggests that high BSFL inclusion may exceed the metabolic capacity of the liver, potentially causing oxidative stress or inflammation. Wang[39] noted that although medium-chain fatty acids in BSFL have beneficial antimicrobial and metabolic effects at moderate levels, excessive intake could overwhelm hepatic processes. This could explain the progression from mild to severe histological changes as the BSFL inclusion increased.

The histological observation of mice also revealed clear differences in liver histology between the control group and experimental groups P1–P4 (5-20% defatted BSFL meal). In group C (100% factory feed), the liver tissue exhibited normal hepatic architecture, characterized by distinct central veins, sinusoids, and hepatocytes with clear nuclei, confirming the healthy and functional state of the liver under a standard diet.

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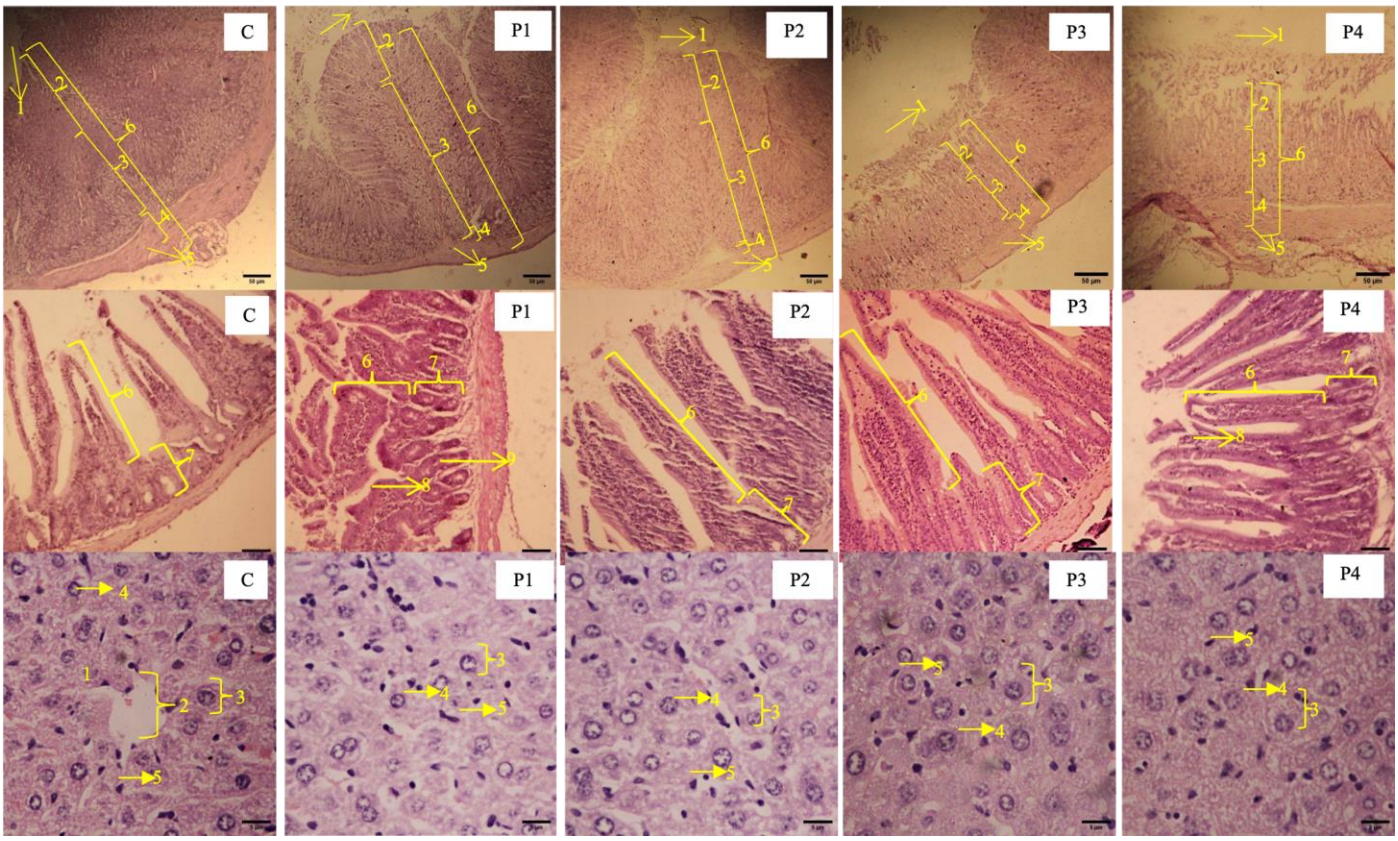

Figure 2: Ventriculus (Upper row), Intestine (Middle row), and Liver (Lower row) Histology of Mice (*Mus musculus*) after fed various level of defatted BSFL inclusion in the diet for 30 days. Control (no defatted BSFL meal), P1 (95% factory feed + 5% defatted BSFL meal), P2 (90% factory feed + 10% defatted BSFL meal), P3 (85% factory feed + 15% defatted BSFL meal), and P4 (80% factory feed + 20% defatted BSFL meal). Paraffin method and Harris Haematoxylin & Eosin staining. Image Description (Ventriculus): 1. Lumen; 2. Tunica Mucosa; 3. Tunica Submucosa; 4. Tunica Muscularis; 5. Tunica Serosa; 6. Ventriculus Wall Thickness; Image description (Intestine): 6. Villi Height; 7. Crypt Depth. 8. Erosion of villi; 9. Paneth cells are still visible in large numbers. Image description (Hepar): 1. Sinusoids; 2. Central Veins; 3. Hepatocytes; 4. Hepatocyte Nuclei; 5. Endothelial Cells

Dose-dependent changes were observed in the experimental groups. In group P1 (5% defatted BSFL meal), the liver histology remained close to normal, with only minimal changes, such as a slight enlargement of the central veins and hepatocyte nuclei. Group P2 (15% defatted BSFL meal) displayed moderate vacuolation within the hepatocytes, which may indicate an early adaptive metabolic response. Group P3 (15% defatted BSFL) showed more prominent vacuolation and irregular sinusoidal structures, whereas group P4 (20% defatted BSFL meal) exhibited the most significant changes, including hepatocyte degeneration and sinusoidal disorganization. These changes in response to the dietary inclusion of defatted BSFL suggest a dose-dependent impact on hepatic function and structure. The mild changes in mice group P1 (5% defatted BSFL meal) indicated that low levels (5%) of defatted BSFL meal were well tolerated and did not compromise health. However, the more significant alterations in mice groups P2 (10% defatted BSFL meal) to P4 (20% defatted BSFL meal) reflect the liver's response to metabolic challenges posed by higher BSFL inclusion.

Vacuolation in mice in groups P2 (10% defatted BSFL) and P3 (15% defatted BSFL meal) may indicate lipid accumulation, which has been linked to dietary changes involving high-fat or high-protein ingredients. The defatted BSFL meal contains a high concentration of medium-chain fatty acids (e.g., lauric acid) and proteins, which can influence lipid metabolism. Similar findings reported mild hepatic steatosis in fish fed diets containing insect-derived proteins. While these changes can be adaptive, excessive lipid accumulation, as observed in mice fed 20% defatted BSFL meal, may lead to hepatocyte damage and impaired liver function over time.[40]

The nutritional benefits of BSFL meal as a protein source are due to its high amino acid content and digestibility. However, over-supplementation may introduce an excess of chitin, a structural polysaccharide found in insect exoskeletons, which could interfere with nutrient absorption and gut health.[39] The observed reduction in villus height at higher inclusion levels corroborates the study of Kim[41], who showed that excessive insect-based proteins could impair gut morphology by altering the microbiota composition and increasing intestinal stress markers.

The crypt depth trends aligned with the villus height results, as both parameters are indicators of intestinal health and turnover rate. The mice in P2 and P3 maintained optimal crypt depths comparable to the control, consistent with studies highlighting the role of moderate insect meal supplementation in supporting gut integrity.[42] In contrast, a reduction in the crypt depths of mice in the P4 group may reflect the adverse effects of prolonged exposure to high chitin content or other indigestible components, leading to compromised nutrient absorption and intestinal adaptation.

Conclusion

These findings have important implications for animal nutrition and feed formulation. Defatted BSFL meal, when included in moderate amounts, has potential benefits owing to its protein content and bioactive compounds. However, higher concentrations might require careful management to avoid adverse effects on gut morphology and

function. This could involve optimizing BSFL processing techniques to reduce chitin content or incorporating prebiotics and probiotics to support gut health. This study also demonstrated that dietary supplementation of factory feed with defatted BSFL meal up to 15% is safe and beneficial in mice, maintaining stable hematological, biochemical, antioxidant, and histological parameters. At 20% supplementation, minor immunostimulatory effects and mild hepatic and intestinal tissue alterations were observed, suggesting a threshold for optimal inclusion. The defatted BSFL meal offers a diet with a protein-rich alternative, supporting growth without compromising physiological health. These findings emphasize the potential of defatted BSFL as a viable protein source in mammalian nutrition. Future research should explore long-term feeding trials, functional health benefits, and optimized processing methods.

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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