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RESEARCH ARTICLE

Dietary protein level influences growth, adult emergence, and susceptibility to bacterial infection in *Hermetia illucens* L. (Diptera: Stratiomyidae) larvae

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Abstract

The larvae of the black soldier fly (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), are grown on diverse residual organic matter that differ in their protein content and contain a plethora of microorganisms. The effect of dietary protein level on the interaction of BSFL with entomopathogenic bacteria remains unexplored. In this study, we investigated the role of dietary protein level on BSFL growth, uric acid accumulation, and adult emergence and the outcome of the host-pathogen interaction between BSFL and the Gram-negative bacterium *Pseudomonas protegens* Pf-5. We formulated three experimental diets in which digestible carbohydrate content was maintained at 50% of dry matter (DM), and crude protein was included at either 10% (low protein), 22.5% (medium protein), or 35% (high protein) DM, respectively. The influence of these diets on larval biomass, accumulation of uric acid in the larval hindgut, and subsequent adult emergence were recorded. In addition, the survival of BSFL fed on these diets was monitored for 64 hours after injection with an LD_{50} dose (1 µl of 2.5×10^3 CFU/ml) of *P. protegens* Pf-5. The biomass of 5-day-old larvae grown on a low-protein diet was higher than that of larvae grown on a high-protein diet. However, no such difference in biomass was observed in 8-day-old larvae. The amount of uric acid in the hindgut of larvae fed on high protein was three-fold and two-fold higher than larvae fed on low- and medium-protein diets, respectively. Adult emergence on a high-protein diet was significantly reduced and delayed compared to low- and medium-protein diets. BSFL fed a high-protein diet displayed significantly lower survival after infection with *P. protegens* Pf-5 than those fed a low-protein diet. Overall, feeding a high-protein diet reduced adult emergence and combined with infection with a Gram-negative bacterium survival of BSF larvae was strongly reduced.

Keywords

adult emergence – black soldier fly – infection – survival – uric-acid

1 Introduction

Nutritional composition is essential in maintaining normal biological processes, including insect growth, development, and immunity (Candian *et al.*, 2023; Vogel *et al.*, 2018). The black soldier fly (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae), is a saprophagous insect that is increasingly grown in mass-rearing facilities for use as livestock feed due to its high nutritional value and ability to feed on diverse organic streams of varying nutritional composition (Barragán-Fonseca *et al.*, 2018b; Lalander *et al.*, 2019; Liu *et al.*, 2018). Although BSF

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larvae (BSFL) are capable of feeding on diets having diverse dietary protein: carbohydrate ratios, their performance varies (Barragán-Fonseca *et al.*, 2018a; Barragán-Fonseca *et al.*, 2021; Beniers and Graham, 2019; Cammack and Tomberlin, 2017; Eggink *et al.*, 2023; Sandrock *et al.*, 2022; Sripontan *et al.*, 2020). Hence, a balanced availability of dietary proteins and carbohydrates is essential for achieving optimal growth in the larval stage and optimal performance in the adult stage of BSF (Barragán-Fonseca *et al.*, 2019; Bellezza Oddon *et al.*, 2022; Beniers and Graham, 2019; Cammack and Tomberlin, 2017; Cheon *et al.*, 2022; Eggink *et al.*, 2023).

The dietary composition of macronutrients procured during the larval phase influences adult development in BSFL (Barragán-Fonseca *et al.*, 2021; Cammack and Tomberlin, 2017). Dietary protein level has a stronger effect on BSF performance than dietary carbohydrate level (Barragán-Fonseca *et al.*, 2021). Provision of dietary protein above 37% of dry matter reduced the survival of BSF larvae (Barragán-Fonseca *et al.*, 2019; Barragán-Fonseca *et al.*, 2021; Chia *et al.*, 2018). Dietary protein level influences the concentration of uric acid in insect excreta (Wang *et al.*, 2022). The accumulation of uric acid resulted in deleterious effects on pupation of *Drosophila melanogaster* (Morimoto, 2022). Therefore, we hypothesized that providing a high amount of dietary protein to BSF larvae could result in uric acid accumulation in the larval hindgut with associated negative side effects.

Besides growth and development, dietary protein level influences the immunological response and survival of insects to bacterial infections (Ponton *et al.*, 2020; Povey *et al.*, 2009). Protein-poor diets are known to improve host survival after bacterial infection (Ponton *et al.*, 2020); the opposite has also been recorded where a high dietary protein level improved host survival after bacterial challenge (Povey *et al.*, 2009; Savola *et al.*, 2021). Protein-rich diets increase the expression of immunity-related genes in BSFL (Vogel *et al.*, 2018), which could prove to be detrimental for growth and survival in the presence of entomopathogens. BSFL possesses a robust immune system (reviewed by Vogel *et al.*, 2022) and until recently no entomopathogens had been reported for this fly species (Joosten *et al.*, 2020). However, last year a report appeared on BSFL mortality after infection by the bacterial entomopathogen *Paenibacillus thiaminolyticus* (She *et al.*, 2023) that is indicative of emerging risks of bacterial infections. The role of dietary protein in modulating the outcome of bacterial infections in BSFL has not yet been investigated.

Based on the outcome of previous experiments (Shah *et al.*, 2023), we selected the Gram-negative bacterium *Pseudomonas protegens* Pf-5 that displays insecticidal activity against dipteran and lepidopteran insects such as *D. melanogaster*, *Musca domestica*, and *Pieris brassicae* (Garrido-Sanz *et al.*, 2023; Loper *et al.*, 2016; Ruiu and Mura, 2021). Due to the use of *P. protegens* Pf-5 as a crop-protection agent, the bacterium could end up in the diet of BSF larvae when grown on agricultural residues or by-products. In this study, BSFL was raised from the egg stage on three levels of dietary protein (i.e. 10%, 22.5%, and 35% protein on a dry matter basis [DM]). The biomass of BSFL was measured for each experimental diet after five and eight days post-hatching. The uric acid content in the hindgut of 12-day-old BSFL grown on these diets was determined colorimetrically using an enzyme-based assay. In addition, the emergence of BSF adults grown on the three protein levels was measured. To assess the effect of dietary protein level on the interaction of BSFL with *P. protegens* Pf-5, we injected 5-day-old larvae with an LD_{50} dose of *P. protegens* Pf-5, and the survival of control and infected groups grown on each of the three diets was monitored at five time points for up to 64 hours post-infection.

2 Materials and methods

Insect rearing and diet composition

BSF eggs laid within a six-hour window, were obtained from a colony maintained at the Laboratory of Entomology, Wageningen University (the Netherlands) in a climate room (27 ± 1 °C, 70 ± 10% RH, L12:D12). The insects used in the experiment were raised from the egg stage onwards on three experimental diets with different protein levels (Table 1). The ingredients used to formulate the experimental diets included chicken feed (Kuikenopfokmeel 1; Kasper Faunafood, Woerden, the Netherlands), potato starch (Duchefa Biosciences, Haarlem, the Netherlands), cellulose (Alphacel non-nutritive bulk, VWR International, Amsterdam, the Netherlands) and casein (Acros Organics, Thermo Fisher Scientific, Landsmeer, the Netherlands); their quantities are presented in Table 1. The nutrient composition of the chicken feed diet is detailed in Supplementary Table S1. For each diet, two clutches of randomly selected BSF eggs (~1,500 eggs) were placed on top of an inverted Petri dish lid (60/15 mm, Greiner Bio-One, Alphen aan den Rijn, the Netherlands). Fifty grams of homogenous diet (Table 1), premixed with 100 ml tap water, was supplied in 480 ml rearing cups (Ø12.5 × 8.5 cm height; Bug-

TABLE 1 Composition of experimental diets with three protein levels used in this study

Dietary ingredient*	Low protein	Medium protein	High protein
Chickenfeed**	50	50	50
Potato Starch	25	25	25
Casein	–	12.5	25
Cellulose	25	12.5	–
Nutrient content (% DM)			
Crude protein	10	22.5	35
Digestible carbohydrates&	45	45	45
P: C ratio	1: 4.5	1: 2	1: 1.3
P + C ratio	55	67.5	80

*Ingredients in g/100 g of diet on dry matter (DM) basis; **50 g of chicken feed contained 10 g of protein and 20 g of digestible carbohydrates; the detailed composition of chicken feed is described in Supplementary Table S1; &Sum of digestible carbohydrates in chicken feed and potato starch.

Dorm insect pots with snap-on lids, MegaView Science Co., Taichung, Taiwan). The snap-on lid of the rearing cup was equipped with nylon mesh (104 × 94 mm and 300 μm aperture) to facilitate ventilation. Rearing cups containing experimental diets and BSF eggs were placed inside a climate cabinet maintained at 27 °C and 65% RH.

Larval biomass

Larval biomass was measured by performing the experiment in three serial experimental blocks, each comprising three rearing containers for each experimental diet. Larval biomass was measured on day five and day eight post-hatching. Twenty BSF conspecifics were randomly selected per container, surface-sterilized with 70% ethanol for 5 s, dried on tissue paper, and weighed using NewClassic MF ML54 (Mettler Toledo, Tiel, the Netherlands) balance. After weight measurement, the 5-day-old larvae were reintroduced to their respective diet. Similarly, twenty 8-day-old BSF larvae were selected randomly from each rearing container, washed, dried, and weighed. The results indicate the average weight of twenty larvae (N = 9 replicates of twenty larvae each).

Uric acid analysis

Ten 12-day-old larvae were randomly selected from each experimental diet, washed with 70% ethanol for 5 s, then two consecutive washes in autoclaved distilled water for 5 s each. The larvae were subjected to a short CO_2 exposure to immobilize them before dissection. The immobilized larvae were placed in the middle of a dissection dish, submerged in autoclaved and pre-chilled (4 °C) phosphate-buffered saline (PBS) solution (Oxoid Chemicals, Thermo Fisher Scientific, Landsmeer, the Netherlands), pinned using Sphinx dissection needles (size 00, Vermandel) and dissected under a stereomicroscope at 20× magnification (Olympus SZX12 Stereomicroscope) using sterile tweezers (Vermandel, Hulst, the Netherlands) and Scalpel blade no. 24 (Swann-Morton, Sheffield, UK). Tweezers and scalpel were cleaned with 70% ethanol between samples to avoid cross-contamination. Intact larval guts were transferred to a clean dissection dish filled with autoclaved and chilled (4 °C) PBS buffer, clipped near the site of attachment of the Malpighian tubules, to obtain the larval hindguts. The hindguts from individual BSFL were placed in a 200 μl Eppendorf tube containing 50 μl pre-chilled (4 °C) PBS buffer and two 2-cm glass beads (pre-cleaned with 100% ethanol). The fresh weight of the individual hindgut was measured using a Mettler Toledo balance, after which the gut tissue was sheared in a TissueLyser II (Qiagen, Venlo, the Netherlands) at 30 oscillations for 2 minutes. The sheared hindgut samples were placed on ice until further processing. The uric acid content of the samples was assessed colorimetrically at wavelength 570 nm using a Uric Acid Assay kit (Cayman Chemicals, MI, USA), following the manufacturer's instructions in a MultiSkan Sky plate reader (Thermo Fisher Scientific, Landsmeer, the Netherlands). The built-in software of the plate reader device calculated the uric acid concentration in the samples against a standard curve over the concentration range 0-1000 μM, using a blank treatment as the baseline. The measured concentration of uric acid (μM) was multiplied with the sample dilution factor and molar mass of uric acid (168.011 g/mol) to determine the

amount of uric acid in the sample volume, expressed as μg uric acid per mg of hindgut tissue.

Adult emergence

Twenty larvae were fed with 50 g of their respective experimental diet (Table 1) and mixed with 100 ml of tap water. The larvae were placed in snap-lid boxes (480 ml volume, Ø 125 mm × height 83 mm; Bugdorm, MegaView Science Co., Taichung, Taiwan) inside a climate cabinet maintained at 27 °C and 65 ± 5% RH. The experiment was performed twice, each with three containers (N = 6), with twenty larvae per container, thus evaluating 120 larvae for each dietary protein treatment. Adult emergence was recorded once every day for each diet for 60 days.

Bacterial culture conditions

A stock of *P. protegens* Pf-5 (kindly provided by Dr. Viriginia Stockwell, Oregon State University, USA) was stored in 50% glycerol at –80 °C. Inoculum was prepared by culturing *P. protegens* Pf-5 on King's Medium B (KMB) agar plates (Merck Millipore, Darmstadt, Germany) and incubating the plate for 48 h at 27 °C. A single bacterial colony was transferred to 10 ml Luria-Bertani (LB) broth (Miller; Merck Millipore) and incubated overnight while shaking at 24 °C and 180 rpm in a rotary shaker (Innova™ 40 Incubator, New Brunswick Scientific, Edison, NJ, USA). One ml was drawn from the culture the following day, centrifuged at 3,500 rpm for 3.5 min, washed once in sterile PBS buffer (Oxoid™, Phosphate Buffered Saline Tablets, Thermo Fisher Scientific, Landsmeer, the Netherlands™), and re-suspended in sterile PBS buffer. OD_{600} of the bacteria suspension was measured using a DS-11 FX + Spectrophotometer Fluorometer (DeNovix, Wilmington, DL, USA), and the culture was then serially diluted to the desired concentration ($OD_{600} = 0.25 \sim 2.5 \times 10^3$ CFU/mL) of *P. protegens* Pf-5.

Infection and monitoring survival

Five-day-old BSF larvae were selected at random from each experimental diet (Table 1) using sterile tweezers (Vermandel, Hulst, the Netherlands), surface-sterilized in 70% ethanol for 5 s, washed two times separately in autoclaved distilled water for 5 s each. Sterilized larvae were placed on a clean paper towel, air-dried for 5 min, and transferred to a sterile Petri dish. Larvae fed on a respective diet were randomly selected and injected with either control (1 μl PBS buffer) or bacteria (1 μl of *P. protegens* Pf-5 suspension) using a needle (ga33/30 mm/pst4-30°; on a microsyringe (Gastight #1705, Hamilton, Bonaduz, Switzerland). Larvae were held between thumb and index finger, and injected with either treatment by piercing the intersegmental membrane between the seventh and eight segment. Control and infected larvae for respective diets were transferred to clean containers (containing 10 g chicken feed + 20 ml sterile distilled water) containing the same diet on which they had been reared previously. The container (volume 550 ml, height 11 cm, top width 10 cm, bottom width 8.5 cm) and the lid (9.5 cm × 5 mm) had a circular vent of 5 cm diameter with nylon mesh (of 1 mm grid size) to facilitate ventilation. The containers were placed in a climate cabinet maintained at 27 °C and 65% RH, and larval survival was monitored across all diet and treatment combinations at five time points (16, 24, 40, 48, and 64) post-injection. The experiment was repeated twice, in which the survival of twenty larvae in each of three containers was monitored (N = 6). For each experimental diet, 120 larvae were subjected to each treatment (i.e. control or bacterial infection). Each experimental block was performed on a different day with different sets of larvae, and the bacterial culture was freshly prepared on the day of the experiment.

Statistical analysis

Statistical analyses were performed using R version 4.3.0 (R Core Team, 2023) in RStudio version 3.5-5 (Posit team, 2024).

Larval biomass: Larval biomass was analyzed using a two-way ANOVA with dietary protein levels and time as explanatory variables. Variance homogeneity and normality of residuals were determined graphically. Contrasts between different dietary protein levels and time were analyzed using the 'emmeans' package (Lenth, 2023). Pairwise comparisons between larvae grown on different experimental diets for five and eight days were made using Sidak adjustment for multiple comparisons.

Uric acid analysis: The effect of different experimental diets on the amount of uric acid per unit mass of larval hindgut was tested using a Kruskal-Wallis test, followed by a Conover's multiple-comparison post-hoc test.

Adult emergence and survival analysis: Adult emergence and larval survival were evaluated with a 'time-to-event' model (Cox, 1972). The model describes the probability of an event per unit time. For adult emergence, the successful emergence of adults from pupae was considered an 'event,' and for survival analysis, larval mortality was considered an 'event'.

The effect of diet on (a) adult emergence and (b) larval survival was evaluated using Cox's proportional

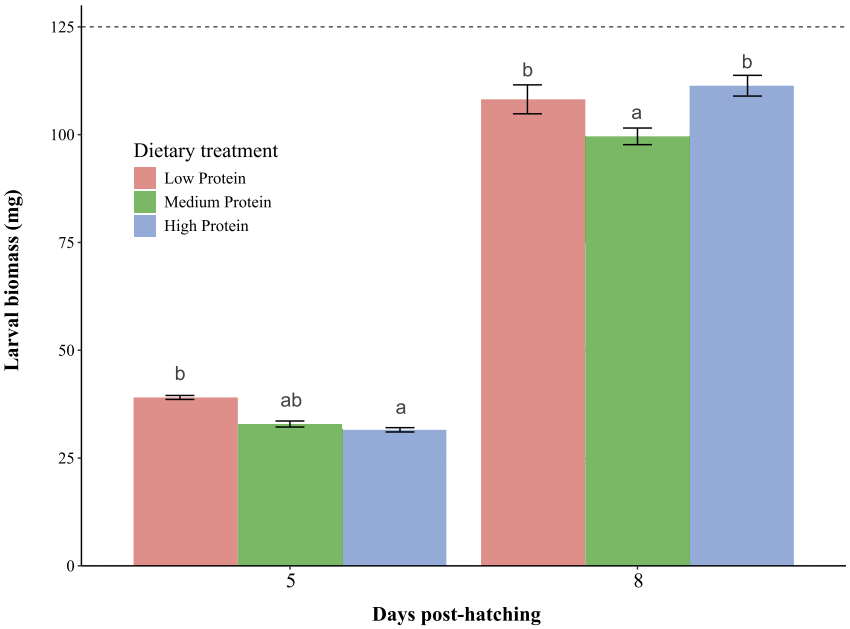

FIGURE 1 Biomass of BSF larvae on day 5 and day 8 post-hatching, grown on diets with different protein levels: low = 10%, medium = 22.5%, high = 35% of total dry matter content. Data represent mean ± standard error; N = 9. Bar values having no letters in common within the two ages differ significantly (Sidak multiple comparisons post-hoc, $P < 0.05$).

hazards model (Cox, 1972) with the 'survminer' package (Kassambara *et al.*, 2023) in R. No difference was observed between experimental blocks when tested with the Cox proportional hazard model. Therefore, the results of survival of control and infected larvae from different experimental blocks were combined and presented as one group in the Kaplan-Meier survival curves. Cox-proportional hazards model was used to quantify the effect size of dietary protein level on both adult emergence and death of larvae. Differences between dietary protein levels on adult emergence and survival were evaluated by pairwise comparison using log-rank comparison (with Bonferroni adjustment). The results of pairwise comparison between diets were extracted using the 'emmeans' package (Lenth, 2023). All graphical plots were generated using the 'ggplot2' package (Wickham, 2016).

3 Results

Dietary protein levels affect larval biomass

Larval biomass was significantly affected by different levels of dietary protein (two-way ANOVA: F-value = 7.934, df = 2, $P < 0.01$) and age (two-way ANOVA: F-value = 2142.434, df = 1, $P < 0.001$). A significant interaction between the effect of diet and age was observed (two-way ANOVA: F-value = 6.7, df = 2, $P < 0.01$).

In 5-day-old larvae, larval biomass was significantly higher when fed on low dietary protein level than when fed on high level of dietary protein (Sidak post-hoc, $P < 0.05$, Figure 1). However, no significant differences were observed in larval biomass between medium and high-protein diets ($P > 0.05$) and between low- and medium-protein diets ($P > 0.05$) in 5-day-old BSF larvae.

The 8-day-old BSF larvae grown on a medium dietary protein level weighed significantly less than larvae grown on low- and high-protein diets (Sidak post hoc, $P < 0.01$). Larval biomass of 8-day-old larvae was not significantly different when grown on low- or high-protein diets (Sidak post-hoc, $P > 0.05$).

Higher content of uric acid in the hindgut of high-protein fed larvae

Dietary protein levels affected the uric acid content found in the larval hindgut (Kruskal-Wallis test: $\chi^2 = 15.316$, df = 2, $P < 0.001$). The concentration of uric acid in the hindgut samples of larvae grown on different dietary protein levels was estimated against a standard curve of known concentrations (Supplementary Figure S1). The concentration of uric acid was normalized to the biomass (mg) of hindgut tissue (Supplementary Table S2). Pairwise comparisons between the diets revealed that uric acid in larvae grown on a high-protein diet was significantly higher than in larvae grown on low- and medium-protein diets (Conover's post-hoc: $P < 0.05$). Larvae fed on a high-protein diet

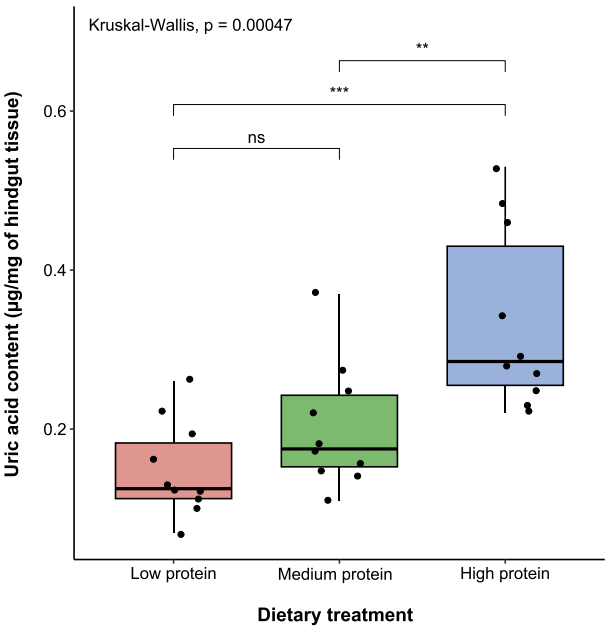

FIGURE 2 Uric acid content (µg per mg of hindgut tissue) of 12-day-old BSF larvae grown on diets with different protein levels: low = 10%, medium = 22.5%, high = 35% of total dry matter content). Differences between dietary treatments are shown for comparisons indicated by brackets: ** $P < 0.01$; *** $P < 0.001$; ns = non-significant (Kruskal-Wallis test followed by Conover's all-pairs rank comparison). Boxplots indicate the median (bold horizontal line), and vertical lines indicate the upper and lower interquartile range.

had the highest uric acid content in their hindgut, three-fold and two-fold higher than larvae grown on low- and medium-protein diets, respectively (Figure 2).

High dietary protein negatively influences adult emergence

Adult emergence, evaluated as the proportion of larvae successfully emerging as adult flies, was significantly affected by dietary protein level (CoxPH: $\chi^2 = 339.2$, df = 2, $P < 0.001$). Pairwise comparisons revealed significant differences in the proportion of adult emergence between the three diets (log-rank comparison with Bonferroni adjustment, $P < 0.05$). Low- and medium-protein diets resulted in 100% adult emergence, while only 30% of the pupae grown on high-protein diets emerged as adults. The median time to adult emergence for low- and medium-protein diet treatment was 32 and 36 days, respectively (Figure 3).

High dietary protein negatively affects larval survival upon pathogen infection

The survival of 5-day-old BSF larvae was significantly affected by treatment (i.e. control and bacteria infection; CoxPH: $\chi^2 = 680.6$, df = 1, p < 0.001) and level of dietary protein (CoxPH: $\chi^2 = 10.58$, df = 2, $P < 0.01$). There was no significant interaction between diet and treatment (Coxph, $P > 0.05$). Low mortality (<2%) was observed in control larvae (Figure 4A). Pairwise comparison to evaluate the effect of different protein levels on the outcome of infection revealed significant differences amongst dietary protein levels. The highest survival of infected BSF larvae was observed when fed on a low-protein diet, and the lowest survival in infected larvae was recorded on a high-protein diet (Figure 4B). Infected larvae fed on a low-protein diet showed significantly higher survival compared to infected larvae grown on a high-protein diet (log-rank pairwise comparison: $P < 0.05$). Survival did not differ between larvae fed on medium-protein and either high- or low-protein diets (log-rank comparison, $P > 0.05$).

4 Discussion

This study aimed to determine the role of different dietary protein levels on BSF growth, uric acid content of larval hindguts, adult emergence, and its effect on larval survival after infection with *P. protegens* Pf-5. We hypothesized that the dietary protein levels would affect the outcome of all the evaluated variables, which was confirmed by the results. The biomass of 5-day-old larvae was significantly higher on a low-protein diet than on a high-protein diet. However, the biomass of BSF larvae no longer differed on these diets when larvae were eight days old. High dietary protein resulted in three- and two-fold higher uric acid content of the larval hindgut than larvae fed on low and medium dietary levels, respectively. A high protein diet also resulted in a significant reduction and delay of adult emergence. Lastly, we observed that the 5-day-old BSF larvae fed on a high-protein diet died faster after infection with *P. protegens* Pf-5 than BSFL fed on a low-protein diet.

Our findings on the effect of dietary protein influencing larval biomass agree with previously reported findings from Barragán-Fonseca *et al.* (2019), who noted that diets with P:C 1:3 resulted in the highest larval biomass, similar to the findings of Cheon *et al.* (2022). Barragán-Fonseca *et al.* (2018a) tested a range of P:C ratios and observed that dietary protein had a stronger influence on larval and adult body mass than dietary carbohydrate content. Bellezza Oddon *et al.* (2022) reported the highest prepupal weight for larvae grown at 14% dietary protein level, which was close to the dietary protein content provided in our low protein diets (10%). Prepupal weight decreased significantly with increasing dietary

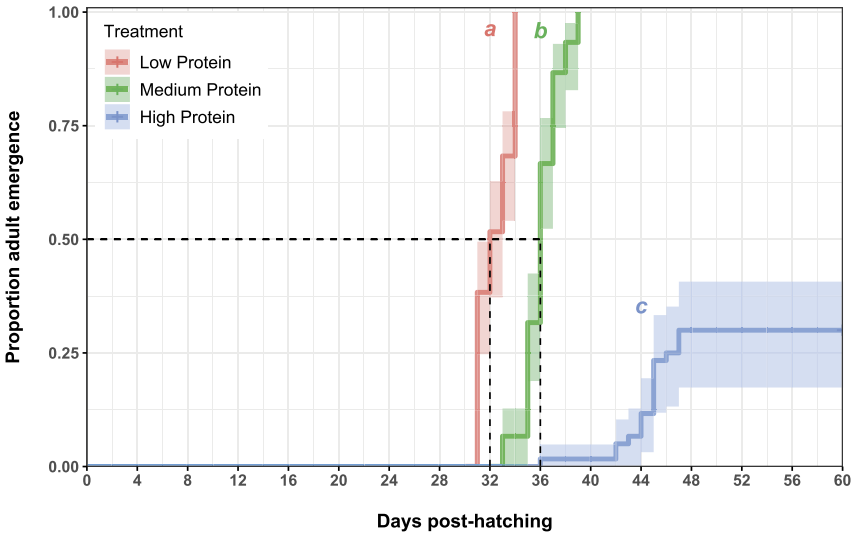

FIGURE 3 Emergence of BSF adults from larvae grown on diets with different protein levels: low = 10%, medium = 22.5%, high = 35% of total dry matter content. Emergence of adults was evaluated over a period of 60 days post-hatching. Colored bands around the respective dietary treatments indicate 95% confidence intervals. Letters indicate the outcome of pairwise comparisons between dietary treatments; different letters indicate significant differences in adult emergence between different dietary protein levels (log-rank comparison, $P < 0.05$).

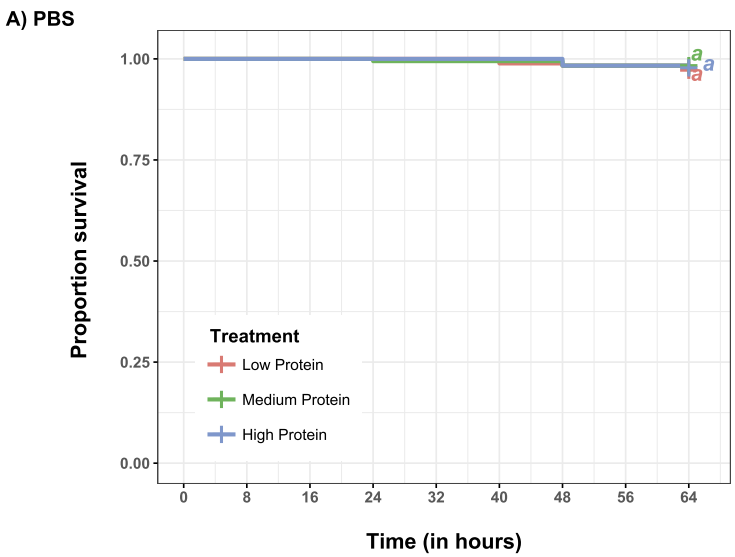

FIGURE 4 Kaplan-Meier survival curves for larvae grown on diets with different protein levels: low = 10%, medium = 22.5%, high = 35% of total dry matter content, and subjected to either of two treatments: (A) PBS buffer (= control), and (B) injection of P. protegens Pf-5 (2-3 CFU per larva). Letters next to the respective curves indicate significant differences between diets (pairwise log-rank comparison, $P < 0.05$). The curves represent survival for 120 larvae per experimental diet, performed in two separate blocks.

protein levels (Bellezza Oddon *et al.*, 2022). Provision of extreme protein-biased diets such as fish remains (P:C 90:1) resulted in lower biomass yield compared to larvae grown on kitchen waste (P:C 1:3) (Nguyen *et al.*, 2015). In contrast to our findings, Beniers and Graham (2019) reported a significant increase in larval biomass on protein-biased diets (P:C ratios of 1:65-1:2). However, these diets resulted in lower survival (55.6% and 70%, respectively). Similar to Barragán-Fonseca *et al.* (2018a), Beniers and Graham (2019) also noted that the provision of additional dietary protein did not result in increased levels of crude protein content in the larval body, which was strictly maintained between 34.13-37.96% DM despite feeding across a wide range of P:C ratios. Maximum biomass yield in BSF larvae is observed within a range of P:C ratio from 1:2 to 1:4 (Barragán-Fonseca *et al.*, 2019; Barragán-Fonseca *et al.*, 2021; Bellezza Oddon *et al.*, 2022; Cheon *et al.*, 2022; Eggink *et al.*, 2023). On the other hand, the provision of excessive dietary carbohydrate (P:C 1:9) increased devel-

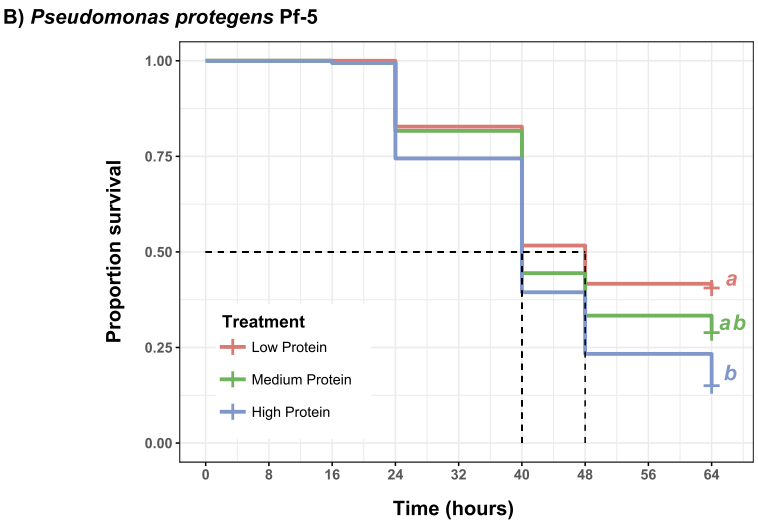

FIGURE 4 (Continued.)

opment time while reducing final weight and crude protein content in the BSF larvae (Eggink *et al.*, 2023). Collectively, these results indicate that while BSF larvae can navigate their developmental processes across a wide range of P:C ratios, the provision of extremely protein- or carbohydrate-biased diets negatively affects larval growth and survival post-bacterial infection.

Besides biomass yield, dietary protein and carbohydrate composition also influence the metabolism and development of multiple insect species (Galenza *et al.*, 2016; Musselman *et al.*, 2018; Obata and Miura, 2015; Zanotto *et al.*, 1993). However, the provision of high dietary protein levels leads to the formation of secondary metabolites, such as uric acid, that are usually excreted and deposited in the hindgut or Malpighian tubules of insects, resulting in renal pathologies (Dow and Davies, 2006; Zanotto *et al.*, 1993). The accumulation of uric acid in *D. melanogaster* larvae reduced the proportion of pupal development into adults (Morimoto, 2022). An elevated level of uric acid was observed in the excreta of *Helicoverpa armigera* (Hübner) larvae when fed on a protein-biased (P:C 2.5:1) diet in comparison to larvae fed on a carbohydrate-biased (P:C 1:2.5) diet (Wang *et al.*, 2022). Uric acid concentration in *H. armigera* excreta correlated with dietary protein levels, with uric acid concentration decreasing at lower protein level (Wang *et al.*, 2022). Elevated levels of dietary purines are linked to increased uric acid levels, whose accumulation in the excretory system resulted in shortened lifespan of *D. melanogaster* (Lang *et al.*, 2019).

Multiple studies report the effect of dietary protein levels on adult emergence in BSFL (Barragán-Fonseca *et al.*, 2019; Barragán-Fonseca *et al.*, 2021; Bellezza Oddon *et al.*, 2022; Cammack and Tomberlin, 2017; Cheon *et al.*, 2022; Holmes *et al.*, 2013). Feeding BSFL on protein-biased diets composed of fish-rendering (P:C 9:1) and pig-liver (P:C 16.2: 1), resulted in extremely high pre-adult mortality of 99.7% and 84.6%, respectively (Nguyen *et al.*, 2013). In agreement with our findings, Cheon *et al.* (2022) and Barragán-Fonseca *et al.* (2019) also reported an extended pre-adult development time and shortest development time on high and low-protein diets, respectively. Deleterious effects of elevated dietary proteins were observed on the emergence of adult BSF (Barragán-Fonseca *et al.*, 2019; Barragán-Fonseca *et al.*, 2021; Cammack and Tomberlin, 2017; Nguyen *et al.*, 2013). Extreme protein-biased diets, such as fish rendering (90:1), resulted in 100% BSFL mortality during the pupal stage (Nguyen *et al.*, 2013). Nguyen *et al.* (2013) reported that adult development time in BSF fed on fish increased to 55-65 days, strikingly similar to the development time observed in our study for BSF larvae grown on high-protein diets. Along similar lines (Nguyen *et al.*, 2013), we observed a distinct developmental delay and reduced emergence in BSFL fed on a high-protein diet, while low- and medium-protein diets led to 100% adult emergence.

Macronutrient concentration influences the outcome of host-pathogen interactions in multiple insect species (Galenza *et al.*, 2016; Hudson *et al.*, 2019; Lee *et al.*, 2006; Manjula *et al.*, 2020; Musselman *et al.*, 2018; Pan *et al.*, 2018; Ponton *et al.*, 2020). Adult *D. melanogaster* actively switched their dietary protein intake (from P:C 1:4 to 1:10), leading to higher survival during infection with *Micrococcus luteus* (Ponton *et al.*, 2020). A shift to a low protein and high carbohydrate diet was linked to the upregulation of key antimicrobial peptide (AMP) encoding genes (Ponton *et*

al., 2020), positively influencing the survival of infected *Drosophila*. Likewise, a high dietary carbohydrate (glucose) level was also linked to increased longevity and survival in *Drosophila* adults with a chronic infection with *Vibrio cholerae* C6706 (Galenza *et al*., 2016). Elevated levels of dietary protein were detrimental to *Drosophila* survival during infection with *V. cholerae* (Galenza *et al*., 2016), in line with our findings about the negative effect of a protein-rich diet on BSFL survival. Interestingly, elevated carbohydrate levels (glucose) not only improved immunity but also increased the median lifespan of adult w^{1118} *D. melanogaster* by 25% compared to adults fed on control diets (Galenza *et al*., 2016), while protein-rich (casein) diets reduced *Drosophila* longevity significantly. A carbohydrate-rich diet improved the survival of *Spodoptera litura* larvae infected by *Enterobacter hormaechei* (Manjula *et al*., 2020). Diets that are extremely low in protein (3%) resulted in poor survival of *Drosophila* after injection with *Pseudomonas entomophila* (Savola *et al*., 2021).

Feeding on protein-rich diets induced elevated expression of immunity-related antimicrobial peptides in BSFL (Vogel *et al*., 2018). The upregulation of AMP-encoding genes could be linked to additional metabolic and physiological expenses in the insect larva, increasing mortality in infected individuals or incurring developmental costs at later life stages. This could explain the poor survival of infected BSFL when fed on a protein-rich diet in our study and merits further exploration. In addition, feeding on high levels of dietary protein reduces the bacterial and fungal diversity in the gut of BSFL (Chen *et al*., 2023), possibly increasing their susceptibility to infections.

The opposite has also been found, where the provision of high levels of dietary protein improved the survival of infected insects (Lee *et al*., 2006; Povey *et al*., 2009). The survival of *Spodoptera exempta* larvae upon infection with *Bacillus subtilis* was significantly improved on protein-biased diets (P:C 5:1) compared to carbohydrate-biased diets (P:C 1:5) (Povey *et al*., 2009). A similar improvement in the survival of *Spodoptera littoralis* larvae was observed post-infection with nucleopolyhedrovirus on protein-rich diets (P:C 5:1) compared to carbohydrate-rich diets (P:C 1:5) (Lee *et al*., 2006). However, it must be noted that the insects in these studies were grown on a basic diet until the start of the experiment and were only transferred to protein-biased diets after infection, which may have allowed too short a time span for the new diet to actually affect pathogen growth or insect survival. Infected insects make choices in their terminal investment by increasing egg laying post-infection (Hudson *et al*., 2019). Hudson *et al*. (2019) provided adult *D. melanogaster* with either 14% or 31% DM dietary protein and then infected them orally with *Pseudomonas aeruginosa*. The difference in dietary protein levels did not affect post-infection survival in adult *D. melanogaster*. However, infected *D. melanogaster* adults increased their egg production when fed with a high-protein (31% DM) diet compared to increased egg-to-adult viability when grown on a low-protein (14% DM) diet, pointing to a trade-off between offspring production and viability.

It is not straightforward to make comparisons between studies that determine the role of dietary proteins in modulating host-pathogen interactions. Macronutrient composition of the diet has only in some cases been reported on a DM basis, precluding straightforward comparisons. A standardized description of diet formulation is required to evaluate performance between studies (Bosch *et al*., 2020). Additionally, the choice of a particular host-pathogen pair may affect the outcome in terms of insect survival, as diet alters various components of the host response and pathogen performance, and these relationships vary between insect-pathogen combinations (Cotter *et al*., 2011; Lee *et al*., 2006; Miller and Cotter, 2018; Povey *et al*., 2009). Other factors such as choice of infection route (oral or injection), differences in caloric content between formulated diets, mode of protein provision (as solid or liquid), choice of substrates for supplementing dietary protein and carbohydrate, and differences in the developmental stage of insects (adult or larvae) used for infection (Savola *et al*., 2021), complicate the comparison of outcomes of different studies. Infection through physical wounds has been reported in other insect species (Maciel-Vergara *et al*., 2021), however, no such incidences have been reported in BSFL. Although the risk of infection through wounding remains low or negligible in BSFL, generating acute infection through injection represents an extreme scenario whose outcome may instruct insect growers for similar scenarios in the future. To understand the relationship between dietary protein and insect survival upon infection, further work across multiple host and pathogen combinations is required to fully comprehend the role of diet in modulating host-pathogen dynamics.

In conclusion, we observed that a high level of dietary protein (i.e. 35% of total dry matter) did not necessarily increase larval biomass within an observation period of 8-days post-hatching. Adult emergence was delayed and reduced in BSFL fed on high protein levels, consistent with previous findings of Barragán-Fonseca *et al*. (2019). Three-fold higher uric acid concentrations were found

in BSFL hindgut when fed on a high-protein diet compared to those fed on a low-protein diet. High-protein diets also resulted in poor survival of BSFL infected with *P. protegens* Pf-5 compared to infected larvae grown on a low-protein diet. Hence, it is best to avoid excess dietary protein as it would not only delay and reduce adult emergence but also increase the susceptibility of BSFL to pathogens.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.25887547

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Conflict of interest

The authors have no conflict of interest to declare.

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