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RESEARCH ARTICLE

# Effect of rosemary (*Rosmarinus officinalis*) supplement on the growth characteristics and larval metabolism of black soldier fly (*Hermetia illucens* L.)

*M. Kannan*[1,2], *T. Vitenberg*[1], *R. Schweitzer*[2,3], *S. Khatib*[2,3] *and I. Opatovsky*[1,2*]

[1]Laboratory of Insect Nutrition and Metabolism, The Department of Nutrition and Natural Products, MIGAL-Galilee Research Institute, Kiryat Shmona, Israel; [2]Department of Animal Science, Faculty of Sciences and Technology, Tel-Hai Academic College, Upper Galilee, Israel; [3]Laboratory of Natural Compounds and Analytical Chemistry, MIGAL-Galilee Research Institute, Kiryat Shmona, Israel; *itaio@migal.org.il

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## Abstract

The black soldier fly (BSF), *Hermetia illucens*. L (Diptera: Stratiomyidae), is often harnessed to transform organic waste into nutrient-rich larval biomass, providing an alternative animal feed in the aquaculture industry. In Israel, rosemary (*Rosmarinus officinalis*) is a common ornamental plant in gardens that also demonstrates insecticidal and repellent properties and medicinal value. This study focuses on utilizing rosemary waste to produce nutrient-enriched BSF larvae for animal feed and potential medical applications. We evaluated BSF larval survivability, adult emergence and metabolomics following supplementation with different percentages of rosemary leaves (4%, 15%, and 20%, relative to 25 g of the total Gainesville diet). Surprisingly, we observed 95-99.6% larval survivability in the rosemary treatments, comparable to the control. Larval body weight slightly decreased by 4%, 12%, and 20%, respectively, measured as 219 ± 3 mg, 220 ± 3 mg, and 219 ± 4 mg, respectively, compared to the control (249 ± 5 mg) ($\bar{x}$ ± SE; $P = 1.83 \times 10^{-7}$). There was a significant difference in adult emergence at 10 days from larval addition ($P = 0.002$), but no difference after 16 days ($P = 0.15$). Metabolomics analysis unveiled the over-accumulation of metabolites in BSF larvae after rosemary supplementation linked to aminoacyl-tRNA biosynthesis, glutathione metabolism, phenylalanine metabolism, arginine biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthesis, butanoate metabolism, and arginine and proline metabolism. Conversely, purine metabolism, tyrosine metabolism, and pantothenate and CoA biosynthesis were down-accumulated. Rosemary supplementation also led to the over-accumulation of antioxidant metabolites (rosmarinic acid, carnosic acid, and caffeic acid), suggesting that including rosemary powder in organic compost slightly reduced larval body weight but shortened adult developmental time and enhancing the nutritional value of BSF larvae. However, further molecular studies are necessary to understand BSF resistance and other biochemical changes resulting from rosemary inclusion in their diet.

## Keywords

animal feed – growth parameters – metabolomics – rosemary (*Rosmarinus officinalis*) – secondary metabolites

## 1 Introduction

The black soldier fly (BSF; *Hermetia illucens*) is a detritivorous insect that flourishes in diverse organic matter environments (Vitenberg and Opatovsky, 2022) and has gained prominence as a bio-converter due to its larval capacity to consume various types of organic waste. These larvae are reared as protein-rich feed for fish, pigs, and poultry (Barragan-Fonseca *et al.*, 2017; Mani *et al.*, 2023a) and contribute to biofuel production through their fat content (Tumpa *et al.*, 2021). Additionally, BSF larvae produce a variety of antimicrobial peptides with potential biomedical applications (Elhag *et al.*, 2017; Moretta *et al.*, 2020; Van Moll *et al.*, 2022; Vogel *et al.*, 2018; Xia *et al.*, 2021). Despite their already substantial nutritional value, the antioxidant and antimicrobial compounds in BSF larvae may be potentially enhanced by incorporating natural medicinal plant materials into their diets. Thus, BSF larvae make a significant contribution to the animal feed industry.

In Israel, rosemary (*Rosmarinus officinalis* L.), a member of the Lamiaceae family, is widely cultivated for its ornamental and medicinal properties. Despite its abundance, it is often discarded as waste during gardening maintenance. Rosemary is rich in phytochemicals, including phenols, flavonoids, antioxidants, and bioactive compounds such as rosmarinic acid, carnosol, carnosic acid, and caffeic acid (Almela *et al.*, 2006; Fontana *et al.*, 2013; Ngo *et al.*, 2011). These bioactive compounds prevent the oxidative stress caused by reactive oxygen species, thus benefitting many animals (Brewer *et al.*, 2014; Kumar, 2019). Furthermore, rosemary exhibits antimicrobial properties effective against *Staphylococcus aureus* and *Streptococcus* spp (Bajalan *et al.*, 2017; Nakagawa *et al.*, 2020). Beyond its impact on microorganisms, rosemary has been linked to a diverse array of health benefits in animals and humans. These include antidepressant, antihypertensive, antiproliferative, antibacterial, antiatherogenic, hypocholesterolemic, hepatoprotective, and anti-obesity properties (Afonso *et al.*, 2013; Harach *et al.*, 2010; Kwon *et al.*, 2006; Sasaki *et al.*, 2013; Tai *et al.*, 2012; Ullevig *et al.*, 2011; Wang *et al.*, 2012). A previous study indicated that supplementing post-distillation residue from Mediterranean aromatic medicinal plants (lavender, oregano, rosemary, and olive oil) with agri-food by-products, such as rice bran, wheat bran, potato peel, solid biogas, and olive oil processing residue, did not adversely affect the survival of *Tenebrio molitor* larvae. Furthermore, this supplementation was demonstrated to increase larval dry weight (Andreadis *et al.*, 2022).

Therefore, incorporating rosemary garden waste as a supplement in the diet of BSF larvae presents significant advantages. This method has the potential to reduce the risk of BSF larval infection by various pathogens while efficiently utilizing diverse types of organic waste (Jensen and Lecocq, 2023; Joosten *et al.*, 2020; Lecocq *et al.*, 2021; Mani *et al.*, 2023c). The inclusion of rosemary ensures the production of high-quality animal feed as well as introducing antimicrobial and antioxidant properties to the larvae (Andreadis *et al.*, 2022). Beyond enhancing the nutritional value of the BSF, the incorporation of natural phenolics derived from plants such as rosemary emerges as a potential catalyst for robust growth. Moreover, this approach aligns with the dual benefit of managing solid waste effectively, contributing to a cleaner environment. In this study, we examined BSF growth parameters such as larval weight, survival, and adult emergence, and conducted a metabolomics analysis after feeding BSF larvae on the Gainesville diet supplemented with rosemary under laboratory conditions.

## 2 Materials and methods

### *Rearing the larvae and rosemary treatment*

Newly hatched larvae were obtained from FreezeM, Nachshonim, Israel, and were rinsed with sterile deionised water using a strainer. The third instar larvae were raised for the initial five days on 200 g of the Gainesville diet, comprising 50% wheat bran, 30% alfalfa meal, and 20% corn, with 400 mL sterilised deionised water (Hogsette, 1992). Simultaneously, rosemary leaves were collected from a street garden, lyophilised, and powdered using a blender. After five days, 50 young larvae were transferred to a Drosophila rearing flask, with 25 g of the same diet and 50 mL water supplemented with 4%, 12%, or 20% rosemary powder, labelled as treatments R1, R3, and R5, respectively. Measured larval growth parameters included body weight in mg (ten individuals per replicate), and percent larval survival and adult emergence.

### *Metabolite extraction and metabolomics analysis*

On day ten after releasing the larvae into the rosemary treatment, we collected 30 larvae, washed them with running water, and removed excess moisture using tissue paper. Ten clean larvae were lyophilised and powdered using a pestle and mortar remaining larvae were stored at −80 °C for the further use. For metabolomics analysis, we focused on the control and 20% (R5) rosemary treatments (Five replicates per treatment). We resuspended approximately 100 mg larval powder in 1 mL 80% methanol with 0.1% formic acid, following a previously described method (Mani *et al.*, 2023b). The mixture was vortexed at room temperature for 1 h, then centrifuged at 12,000 rpm for 15 min at 4 °C. The clear supernatant (80% methanol) was transferred to a new Eppendorf tube, and the samples were diluted to 53% methanol using liquid chromatography–mass spectroscopy (LC-MS) grade water. The extracts were filtered using a 0.20-μm membrane filter (JET BIOFIL Guangzhou Jet Bio-Filtration Co., Ltd, Guangzhou, China) and stored at −80 °C until further use. Untargeted metabolomics analysis employed quality control (QC) procedures to ensure data quality. A pooled matrix was prepared by mixing 100 μL of each experimental sample to generate QC samples (four replicates). An experimental blank, prepared using acidified aqueous methanol extraction buffer instead of samples was also included.

Ultra-high-performance liquid chromatography (UHPLC) analysis: The samples were analysed by injecting 5 μL of the extracted solutions into a UHPLC connected to a photodiode array detector (Dionex Ultimate 3000), with a reverse-phase column (ZORBAX Eclipse plus C18, 3.0*100 mm, 1.8 μm). The mobile phase comprised (A) DDW with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient was initiated with 2% B, increased to 30% B over 4 min, increased to 40% B over 1 min, maintained isocratic at 40% B for 3 min, increased to 50% over 6 min, increased to 55% over 4 min, increased to 95% B over 5 min and maintained isocratic at 98% B for 6 min. Phase B was then returned to 5% over 2 minutes and the column was allowed to equilibrate at 5% B for 5 min before the next injection. The flow rate was 0.4 mL/min, and the column temperature was set to 30 °C.

Mass spectroscopy/Mass spectroscopy (MS/MS) analysis: MS analysis was performed with a heated electrospray ionisation (HESI-II) source connected to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The ESI capillary voltage was set to 3,900 V, capillary temperature to 350 °C, gas temperature to 350 °C and gas flow to 35 mL/min. The mass spectra (*m/z* 67-1000) were acquired in negative and positive-ion mode with high resolution (FWHM = 70,000). The $MS^1$ parameters were: resolution 70,000, AGC target $3E^6$, maximum IT 100 ms, scan range 67-1000 *m/z*; the $MS^2$ parameters were: resolution 17,500, AGC target $1E^5$, maximum IT 50 ms, loop count 5, MSX count 1, isolated window 1, collision energy (CE) 20, 50 and 80 EV; the data dependent (dd) settings were: minimum AGC $8E^3$, apex trigger 6 to 16 s, exclude isotope on, dynamic exclude 8.0 s.

The LC-MS spectrum was used for peak determination and peak area integration. Each sample peak area was normalised to the quality controls using Compound Discoverer 3.3 (Thermo Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA). Peak areas with relative standard deviation (RSD) > 50% in the pooled QC samples were excluded, and peak areas with RSD < 50% were normalised using the QC samples. Compound structure annotation was done via high resolution mass spectra (HRMS) based on the MzCloud, ChemSpider and Mass list databases using $MS^2$ data. Compounds with no $MS^2$ data were annotated using $MS^1$ data with the same databases mentioned above. Principal Components Analysis (PCA) was performed using Compound Discoverer 3.3 to assess the homogeneity of replicates and variation between treatment groups. Furthermore, we conducted a literature review (Afonso *et al.*, 2013; Almela *et al.*, 2006; Harach *et al.*, 2010; Isman *et al.*, 2008; Manville *et al.*, 2023; Mena *et al.*, 2016; Salman *et al.*, 2018; Tai *et al.*, 2012; Vaquero *et al.*, 2012; Yesilbag *et al.*, 2012) to identify the key metabolites present in the rosemary plant. This review aimed to deepen our understanding of the potential transfer of these metabolites into the BSF larvae following rosemary treatments.

### *Pathway enrichment analysis*

We analysed the metabolic pathways of annotated metabolites (both over- and down-accumulated) in rosemary-fed BSF larvae using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml, McGill University, Montreal, Canada; Pang *et al.*, 2021), which integrates with the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway database. For pathway analysis, we utilised the KEGG library specific to the model organism *Drosophila melanogaster*. The hypergeometric test was employed for overrepresentation analysis, and pathway topology analysis was based on out-degree centrality. We selected significantly affected pathways based on either *P*-values from pathway enrichment analysis or impact values from pathway topology analysis as described previously (Zhang *et al.*, 2022).

### *Feature-Based Molecular Networking (FBMN) using the Global Natural Products Social Molecular Networking (GNPS) platform*

Molecular networking based on $MS^2$ spectra was initiated using the FBMN workflow (Nothias *et al.*, 2020) on GNPS (https://gnps.ucsd.edu) (Wang *et al.*, 2016). The $MS^2$ data-dependent spectral files were converted from .Raw to .mzML using ProteoWizard (https://proteowizard.sourceforge.io; ProteoWizard Software Foundation, CA, USA). The mass spectrometry data were pre-processed using MZmine3 for feature detection and analysis. The data pre-processing involved mass detection, chromatogram reconstruction and deconvolution, isotope grouping, alignment, and gap filling. The following parameters were used: $MS^1$ and $MS^2$ noise levels were set to $5.0 \times 10^4$ and $2.5 \times 10^3$, respectively; minimum absolute height was set to $5.0 \times 10^4$, minimum scans were set to 4, *m/z* tolerance was set to 5 ppm, and RT tolerance was set to 0.4 min. Aligned feature lists were exported as MS/MS files (.mgf format) and quantification tables (.csv format) of aligned features and related chromatographic peak areas, following GNPS documentation on FBMN (https://ccms-ucsd.github.io/GNPSDocumentation/). The following network parameters were used: precursor ion mass tolerance was set to 0.02 Da, MS/MS fragment ion tolerance was set to 0.02 Da, minimum pairs was set to cosine 0.6, minimum matched fragments was set to 4, network topK was set to 10, maximum connected component size was set to 100, maximum shifts between precursors' 100 Library search parameters and minimum matched peaks was set to 4, and score threshold was set to 0.6.

The resulting molecular network was established with edges filtered to have a cosine score above 0.6 and more than four matched peaks. Edges between two nodes were retained if each of the nodes appeared in the other's respective top 10 most similar nodes. The maximum size of a molecular family was capped at 100, and the lowest scoring edges were removed from molecular families until the family size was below this threshold. To validate the network, spectra were searched against GNPS spectral libraries (Horai *et al.*, 2010; Wang *et al.*, 2016). Library spectra were filtered similarly to the input data. Matches between network spectra and library spectra were retained if they had a cosine score above 0.6 and at least four matched peaks. The MS/MS spectra were annotated using DEREPLICATOR (Mohimani *et al.*, 2018). Visualization of the molecular networks was conducted using Cytoscape (Shannon *et al.*, 2003).

### *Statistical analysis*

The differences in larval body weight, percent larval survival and percent adult emergence were analysed using One-Way ANOVA followed by post-hoc Tukey tests (Hammer *et al.*, 2001). The differential accumulation of metabolites derived from the rosemary was analysed statistically using MetaboAnalyst 5.0 which includes inbuilt statistical tools such as one-way ANOVA followed by post-hoc Tukey tests ($P \leq 0.05$).

## 3 Results

### *Growth characteristics of BSF following consumption of rosemary powder*

Our primary aim was to utilise rosemary waste to produce nutrient-enriched BSF larvae, suitable for animal feed production. Surprisingly, we observed significant differences in larval survival among the treatments ($F_{df=3} = 3.837$, $P = 0.03$). However, all larval survival values (95.2 ± 0.7% for R1, 97.2 ± 0.96% for R3, 99.6 ± 0.3 for R5 and 99.2 ± 0.6% for control, $\bar{x}$ ± SE) were similar to the control, with the only significant difference being between the R1 and R5 treatments (Figure 1A). Larval body weight showed a slight reduction at R1, R3 and R5, measuring 219 ± 3, 220 ± 3, and 219 ± 4 mg, respectively, compared to the control (249 ± 5 mg, $\bar{x}$ ± SE) ($F_{df=3} = 15.91$; $P < 0.001$) (Figure 1B). There was a notable difference in adult emergence at 10 days from the day of larval addition ($F_{df=3} = 7.5$, $P = 0.002$), but no significant difference after 16 days ($F_{df=3} = 2$; $P = 0.15$) (Figure 1C) among the treatment groups.

### *Distribution of the detected metabolites*

Using Compound Discoverer 3.3 to analyse the LC-MS data a total of 5,170 features were detected after normalisation with an RSD < 50%. Approximately 2,880 features were annotated, while the remainder were uncharacterised. Among the 2,880 annotated features, 600 were annotated through $MS^2$ data (393 with fragment similarity > 80%, 207 with fragment similarity between 50 and 80%) while the others were annotated by the ChemSpider database using isotope abundance and mass accuracy. The PCA clearly revealed the variation among the treatment groups with PCA1 representing 52.9%, PCA2 representing 15.2%. and the replicates of each treatment tending to group together, indicating that the generated metabolic data were reliable (Figure 2). Interestingly, the rosemary treatment group contained 1,846 over-accumulated metabolites and 691 down-accumulated metabolites compared to the control group (Figure 3). The list of differentially accumulated compounds with name, formula, molecular weight, retention time, *m/z* score, KEGG identity, etc, is provided in Supplementary Table S1.

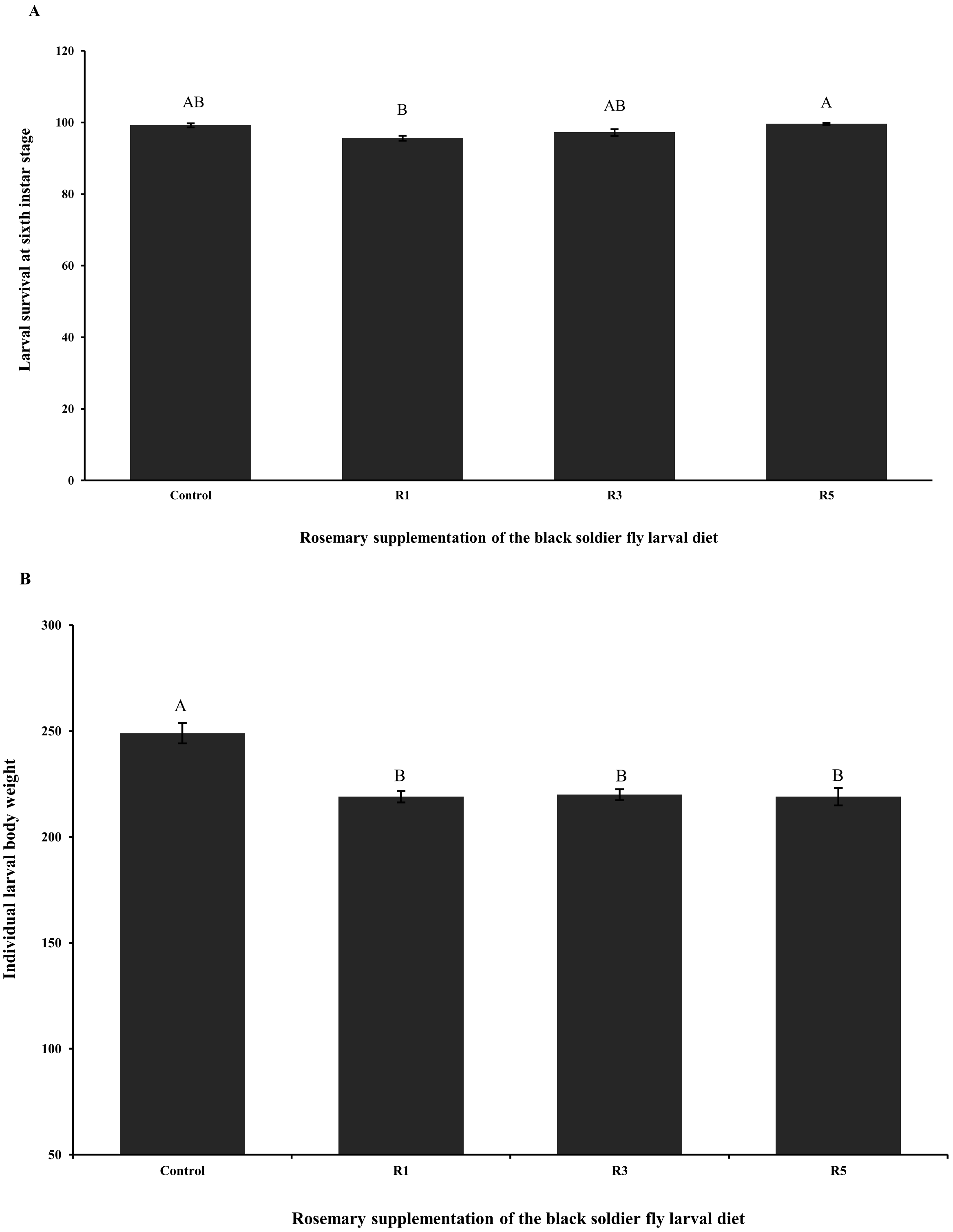

FIGURE 1 (A) Impact of rosemary supplementation on percent BSF larval survival, (B) Larval body weight in mg and (C) percent adult emergence. All values are $\bar{x}$ ± SE. Different letters above the bars represent significant differences between treatments ($P < 0.05$). The labels R1, R3 and R5 correspond to the quantity of rosemary powder (4%, 12% and 20%, respectively) supplemented to the BSF diet. Five replicates were used for each treatment.

### *Rosemary derived metabolites accumulation in the BSF larva*

Metabolites underwent both over- and down-accumulation following rosemary treatment compared to the control. To decipher the impact, Compound Discoverer 3.3 was employed for KEGG metabolic pathway analysis. The findings unveiled 19 enriched pathways, each containing less than 50 metabolites/pathways (Figure

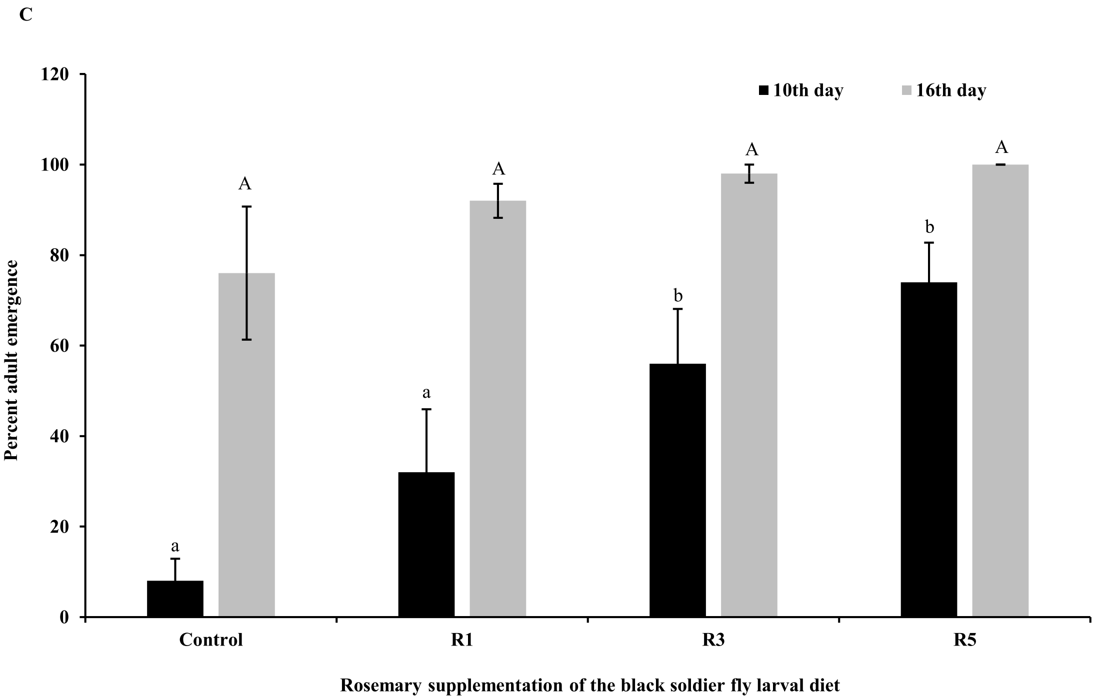

FIGURE 1 (Continued.)

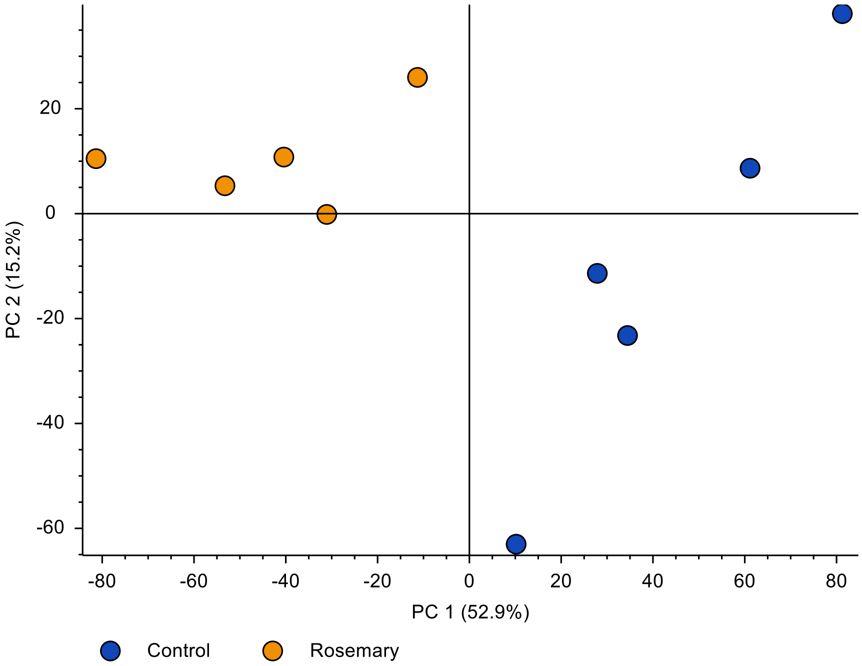

FIGURE 2 Principal component analysis (PCA) of detected metabolites in black soldier fly larvae fed with a rosemary-supplemented diet, with five biological replicates (dots). The *x*-axis (PC1) and *y*-axis (PC2) represent variance among treatments. Blue dots indicate control samples; orange dots indicate rosemary treatment samples. Five replicates were used for each treatment.

4). Notably, the "other category" pathway included over 150 metabolites, including various non-significantly enriched metabolic pathways (Figure 4). Delving deeper into the analysis, a conspicuous shift in the super pathway of rosmarinic acid biosynthesis became evident in the rosemary-treated group compared to the control. This lends strong support to the notion that these altered metabolites could be attributed to the supplementation of rosemary. Further examination of the BSF larval metabolome through MetaboAnalyst 5.0 revealed notable significant overaccumulation of rosemary-specific metabolites, such as rosmarinic acid

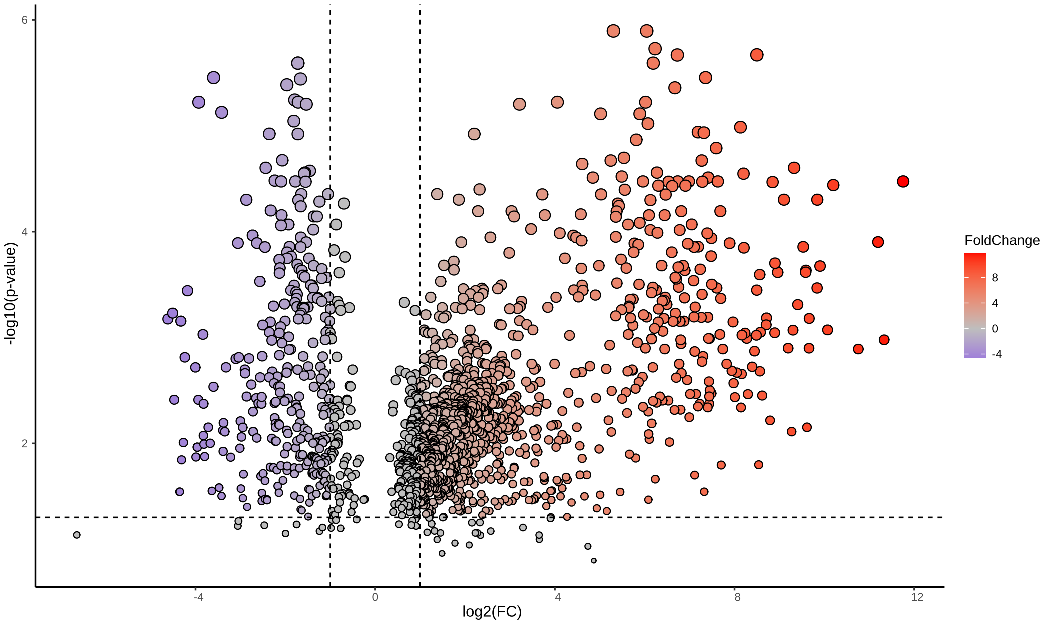

FIGURE 3 Volcano graph presenting significantly over- and down-accumulation of metabolites ($P \leq 0.05$) in the rosemary group compared to the control group with peak area difference of log2 > 0.1. Dots in red (n = 1,846) and green (n = 691) are over- and down-accumulated metabolites, respectively.

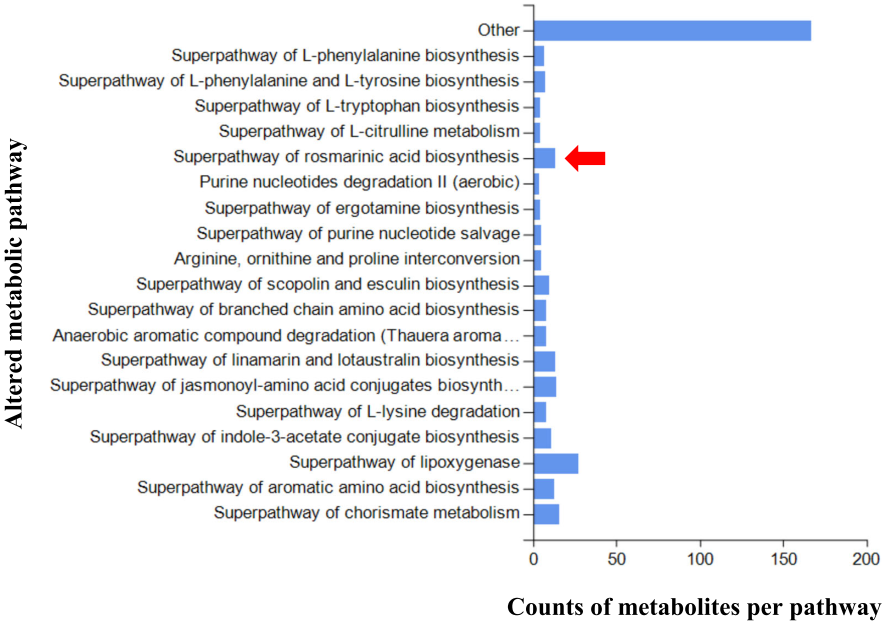

FIGURE 4 Significantly over- and down-accumulated metabolites belong to different metabolic pathways in rosemary-fed black soldier fly larvae compared to the control. The *x*-axis and *y*-axis represent the count of metabolites per pathway and metabolic pathway, respectively. The red color box indicates enrichment in rosmarinic acid biosynthesis after rosemary supplementation in the BSF larvae, as revealed through Compound Discoverer 3.3 software. The super pathway of rosmarinic acid biosynthesis is not present in the insect system, thus metabolite accumulation in the larvae is due to inclusion of rosemary in the diet.

($P = 0.003$), carnosic acid ($P = 0.001$), carnosol ($P = 8.33\text{e}{-}05$), caffeic acid ($P = 0.003$), apigenin ($P = 0.0007$), ursolic acid ($P = 0.03$), luteolin ($P = 0.002$), and humulone ($P = 0.006$), following rosemary ingestion. Conversely, volatile compounds such as caryophyllene oxide ($P = 0.04$) and eucalyptol ($P = 0.006$) were significantly down-accumulated (Figure 5).

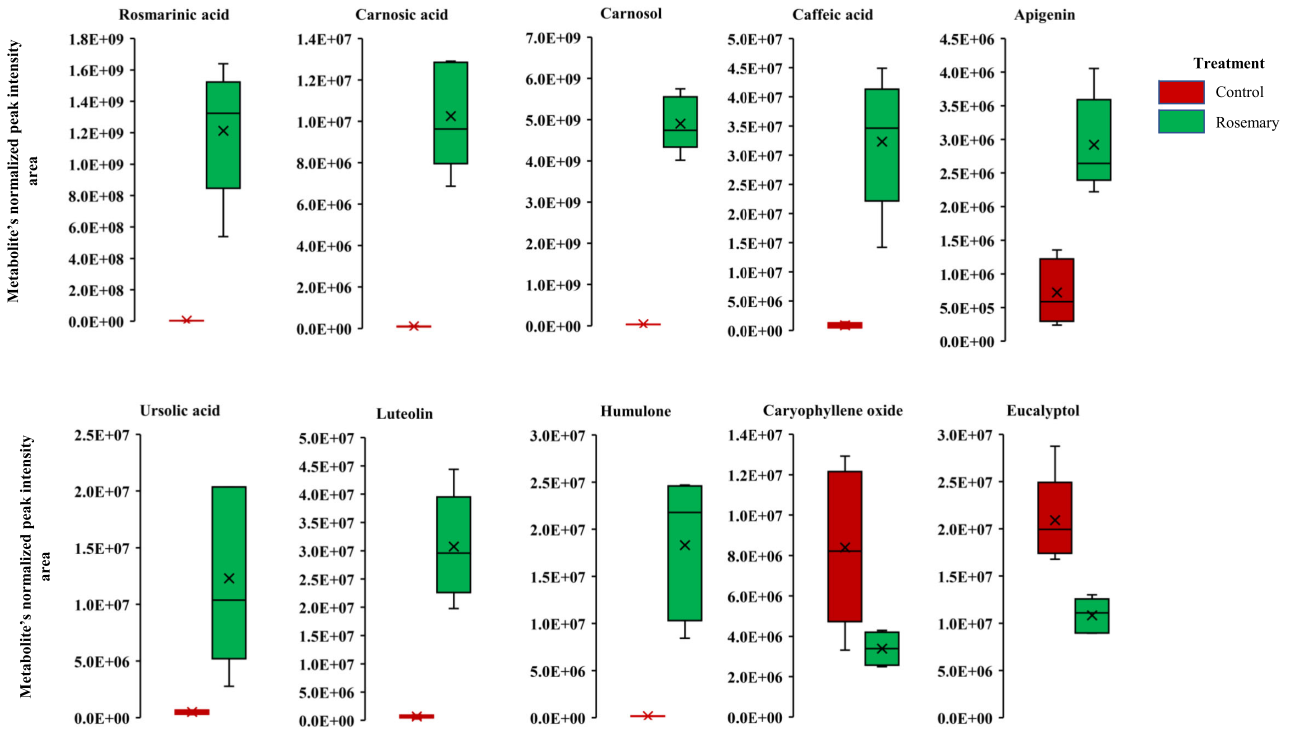

FIGURE 5 Differential accumulation of rosemary-related compounds in rosemary-treated and control groups.

### *Effect of rosemary treatment on BSF larval metabolic pathways using MetaboAnalyst 5.0*

Metabolites such as amino acids, vitamin or enzyme cofactors and nitrogenous bases were significantly over- and down-accumulated by rosemary supplementation (Table 1). Metabolites that were over-accumulated in the BSF larvae after supplementation with 20% rosemary powder were highly associated with aminoacyl-tRNA biosynthesis, glutathione metabolism, phenylalanine metabolism, arginine biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, butanoate metabolism and arginine and proline metabolism. Metabolites that were significantly down-accumulated in the larvae after supplementation with 20% rosemary were mostly linked to three metabolic pathways: purine metabolism, tyrosine metabolism, and pantothenate and CoA biosynthesis. These metabolic pathways were selected based on significant enrichment ratios using $P \leq 0.05$.

### *Metabolite clustering between treatments using FBMN and GNPS platform*

To validate the findings from LC-MS and Compound Discoverer 3.3 indicating differences between rosemary-supplementation and the control, we subjected the LC-MS/MS spectral data to FBMN analysis using GNPS. The results unveiled several clusters which are common as well as specific to each treatment (Supplementary Figure S1). Interestingly, we found clustering of the rosemary-derived metabolites carnosol, oleoylglycine analogues, aminoacid dipeptides, oleanolic acid analogues and γ-Aminobutyric acid (GABA) fatty acid analogues (Figure 6), highlighting differences between rosemary-supplemented samples, blanks and control samples.

## 4 Discussion

This study underscores the significance of employing BSF larvae to transform waste from herbal plants, particularly rosemary, into larvae that are not only nutritionally rich but also possess antioxidative properties. Our research explores the impacts of rosemary on the growth characteristics and metabolomics of BSF larvae. Typically, rosemary oil is known for its metabolites, acting as insecticidal, fumigant, and repellent agents against various insects (Hannour *et al.*, 2018; Isman *et al.*, 2008; Li *et al.*, 2021; Ong *et al.*, 2020; Salman *et al.*, 2018; Yang *et al.*, 2020). Surprisingly, the rosemary treatment did not positively impact the survival of BSF larvae. However, it did lead to a minor decrease in larval body weight. This outcome may be linked to the fact that rosemary powder has a weaker effect than the extracted oil, which has demonstrated promising insecticidal activity in various insects (Isman *et al.*, 2008;

TABLE 1 Effect of rosemary supplementation on differential accumulation of metabolites and their metabolic pathways in black soldier fly (BSF) larvae

| Pathway | Metabolites | Total metabolites annotated to each pathway | Total metabolites detected and annotated to each pathway in the present study | *P*-value |
|---|---|---|---|---|
| Over-accumulated metabolites in rosemary-supplemented BSF | | | | |
| Aminoacyl-tRNA biosynthesis | L-Histidine; L-Phenylalanine; Glycine; L-Methionine; L-Valine; L-Isoleucine; L-Tyrosine; L-Glutamate | 48 | 8 | 0.0001 |
| Glutathione metabolism | Glutathione; Glycine; L-Glutamate; 5-Oxoproline; L-Ornithine | 26 | 5 | 0.0001 |
| Phenylalanine metabolism | L-Phenylalanine; Phenethylamine; L-Tyrosine | 7 | 3 | 0.0002 |
| Arginine biosynthesis | L-Glutamate; L-Citrulline; L-Ornithine | 12 | 3 | 0.001 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | L-Phenylalanine; L-Tyrosine | 4 | 2 | 0.003 |
| Butanoate metabolism | 4-Aminobutanoate; L-Glutamate; Succinate semialdehyde | 14 | 3 | 0.003 |
| Arginine and proline metabolism | 4-Aminobutanoate; D-Proline; L-Glutamate; L-Ornithine | 31 | 4 | 0.003 |
| Down-accumulated metabolites in rosemary-supplemented BSF | | | | |
| Purine metabolism | Xanthine; Adenosine; Urate; Adenine | 63 | 4 | 0.01 |
| Tyrosine metabolism | L-Adrenaline (octopamine- and tyramine-like molecule); Fumarate; 4-Hydroxyphenylacetate | 33 | 3 | 0.01 |
| Pantothenate and CoA biosynthesis | Pantetheine; Pantothenate | 18 | 2 | 0.03 |

Jahanian *et al.*, 2022; Yang *et al.*, 2020). While rosemary supplementation has been reported to reduce rat body weight through plasma lipid reduction (Vaquero *et al.*, 2012), a similar mechanism in BSF larvae warrants further exploration in future studies. Notably, the rosemary treatment significantly accelerated the adult emergence of BSF. Similarly, a recent study that introduced propolis (a resinous substance that bees collect from flowers and sap to use as a sealant in their hives) to the bran diet of BSF reported accelerated larval growth and adult emergence, although reproductive behaviours have yet to be studied (Bakaaki *et al.*, 2023).

Interestingly, the rosemary treatment significantly affected metabolites in various metabolic pathways within BSF larval metabolism. The findings highlight that the over- and down-accumulation of metabolites

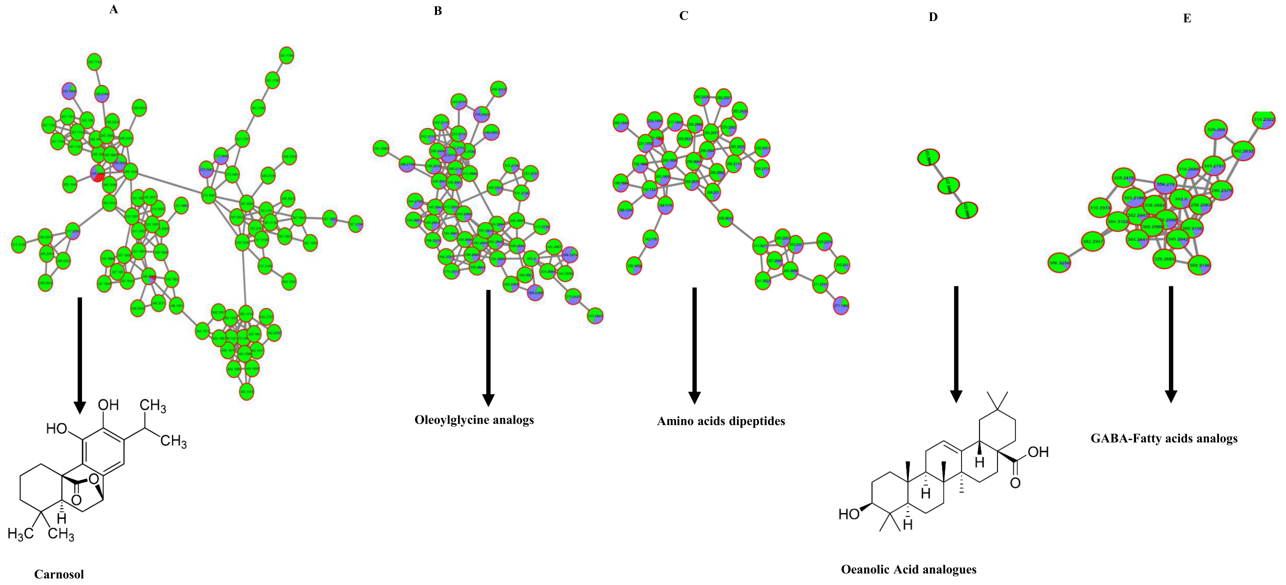

FIGURE 6 Cytoscape spectral similarity networking of the molecular features analysed by FBMN using the GNPS platform. The colours of the nodes represent features detected in the rosemary-supplemented group (green), control group (blue), and blanks (red). The molecular network contains mostly features detected in the rosemary-supplemented group and related to molecular families that are carnosol analogues (A), oleoylglycine analogues (B), amino acid dipeptides (C), oleanolic acid analogues (D) and γ-aminobutyric acid fatty acid analogues (E).

primarily pertains to amino acid metabolism (phenylalanine, arginine, aminoacyl tRNA synthesis), indicating a major shift induced by the rosemary treatment. A recent study indicated that supplementing an agri-food by-products diet with 10% Mediterranean aromatic medicinal plant residue, including rosemary, decreased the protein content in *Tenebrio molitor* larva (Andreadis *et al.*, 2022). Amino acids, fundamental to protein synthesis, play crucial roles in tissue and organ development and act as key regulators of enzymes and hormones (Colinet and Renault, 2014). Aminoacyl-tRNAs, essential precursors for protein synthesis (Ibba and Dieter, 2000), were significantly over-accumulated in response to rosemary treatment, suggesting a substantial improvement in protein synthesis. The above study also reported increased fat and energy content of the substrate while protein content decreased (Andreadis *et al.*, 2022). The over-accumulated metabolites, related to the pathways of phenylalanine, tyrosine, and tryptophan biosynthesis, as well as phenylalanine metabolism, emerged as key contributors to generating metabolites associated with the immune response (Bernays and Woodhead, 1984; Fuchs *et al.*, 2014; Mani *et al.*, 2023b).

The over-accumulation of metabolites from phenylalanine metabolism in BSF larvae fed with rosemary may be linked to enhanced immunity and survival, consistent with previous findings (Bernays and Woodhead, 1984). Notably, metabolites, particularly L-tyrosine, derived from phenylalanine metabolism, play a vital role in the robust formation of cuticles and melanisation of pathogens – a crucial phase in the innate immune response in insects (Brunet, 1980). Intriguingly, the addition of phenylalanine to insect diets has been shown to increase nymphal body weight in the desert locust, *Schistocerca gregaria*, by 30%, and support cuticle production in the immature stage of the grasshopper, *Phoetaliotes nebrascensis* (Bernays and Woodhead, 1984; Behmer and Joern, 1993). We note that BSF larvae lack the shikimate pathway for synthesizing aromatic amino acids (phenylalanine, tyrosine, and tryptophan), necessitating acquisition from rosemary supplementation. This supplementation could potentially enhance the survival of BSF larvae, especially in mass rearing conditions (Herrmann and Weaver, 1999).

Rosemary-fed BSF larvae exhibit over-accumulation of metabolites from the arginine biosynthesis pathway, crucial for converting citrulline/ornithine into arginine, ultimately leading to the biosynthesis of proline (Reddy and Campbell, 1969), which serves as fuel for insect flight (Michalkova *et al.*, 2014; Teulier *et al.*, 2016), contributes to thermotolerance, and influences egg laying in honey bees (Bouchebti *et al.*, 2022). We hypothesise that the observed over-accumulation of arginine biosynthesis and arginine and proline metabolism-related metabolites in rosemary-fed larvae accelerates adult development. Interestingly, recent studies suggest that supplementing honeybees with L-arginine through their diet enhances immune activation in response to artificial wounding and injection of a bacterial toxin (lipopolysaccharide) (Negri *et al.*, 2017). This finding implies that feeding rosemary to BSF larvae may support their immune system. The metabolite glutathione, a critical regulator of mitochondrial metabolism and a free radical scavenger, plays a protective role against oxidative stress induced by insecticides in arthropods (Pavlidi *et al.*, 2018). The observed over-accumulation of glutathione in the rosemary treatment suggests potential side effects in BSF larvae, as evidenced by the slight reduction in larval body weight.

Rosemary supplementation significantly decreased the levels of purine metabolism-related metabolites, including xanthine, hypoxanthine, adenine, and guanine. Purine undergoes hydrolysis to form adenine and guanine, essential for the synthesis of DNA and RNA in the body's cells. The remaining purine is further degraded into xanthine and hypoxanthine (Hou *et al.*, 2021). Adenosine, a purine metabolite, plays a crucial role in stress response signalling, contributing to cytoprotection, immune response, regeneration, and the balance of energy metabolism (Borea *et al.*, 2016). This outcome strongly suggests that the larvae undergo stress during the rosemary treatment. The down-accumulation of metabolites, such as L-adrenaline (epinephrine or an octopamine and tyramine-like molecule) from tyrosine metabolism, which act as neurotransmitters, may play a critical role in memory formation, larval locomotion, and aggression in BSF (Roeder, 2005). However, further studies are needed to comprehensively understand the molecular-level implications of decreased purine and tyrosine metabolism in BSF larvae subjected to rosemary supplementation.

Metabolomics analysis clearly revealed significant over-accumulation of rosemary-derived metabolites, particularly rosmarinic acid, carnosic acid, carnosol, and caffeic acid, in rosemary-supplemented BSF larvae compared to the control. This result suggests that the larvae acquired these metabolites through the ingestion of rosemary, as the BSF larva lacks the biosynthetic mechanism to produce them (Figure 4). These rosemary-derived metabolites, known for their antioxidant properties (Abo Ghanima *et al.*, 2020; Bajalan *et al.*, 2017) are phenolic compounds, derivatives of the phenylpropanoid pathway. The observed over-accumulation of rosmarinic acid in BSF larvae after rosemary supplementation suggests a potential mechanism involving increased proline levels. Proline, serving as a reductant by replacing $NADH_2$, may drive metabolite precursors (such as over-accumulated phenylalanine and down-accumulated tyrosine) towards the shikimate and phenylpropanoid pathway, ultimately leading to rosmarinic acid production (De-Eknamkul and Ellis, 1987; Mizukami *et al.*, 1992; Yang and Shetty, 1998). It appears that rosemary supplementation promotes both BSF adult development and direct accumulation of antioxidants; however, the connection between rosmarinic acid and the proline pathway remains unclear. Further studies are necessary to investigate whether phenylalanine ammonia-lyase gene activity in BSF larvae supports BSF resistance or affects rosmarinic acid metabolism. Overall, the results of this study strongly advocate for the inclusion of rosemary in the BSF diet to augment the nutritional value of BSF larvae for animal feed preparation.

## 5 Conclusions

In conclusion, our study introduces an eco-friendly approach to managing garden waste from rosemary plants by incorporating them into the BSF diet. This addition enhances the production of secondary metabolites with significant biological activity, particularly antioxidants, in addition to protein and fat. These findings underscore the economic potential of BSF larval biomass as a nutrient-enriched substrate, particularly valuable for agri-food companies involved in animal feed production. The integration of rosemary into BSF compost, along with diverse organic waste in appropriate proportions, holds promising prospects for upscaling. This approach, coupled with the inclusion of waste from other medicinal plants, can contribute to a zero-waste model, transforming biomass waste into high-value animal feed. This has potential applications in both aquaculture and human food industries. Furthermore, future research should explore the impact of rosemary treatment on resistance, reproductive potential, microbiota, and the expression of antimicrobial peptides in the BSF. This will provide deeper insights into BSF physiology and immunity, contributing to a more comprehensive understanding of the potential benefits of incorporating rosemary into the BSF diet.

## Supplementary material

Supplementary material is available online at:
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### Author contributions

The study was conceptualised by Mani Kannan and Itai Opatovsky. The original draft of the manuscript was written, edited and reviewed by Mani Kannan and Itai Opatovsky. Mani Kannan and Tzach Vitenberg performed the experiments, while Ron Schweitzer and Soliman Khatib performed metabolomics analysis using HR-LC-MS/MS. We have all read and agreed to the published version of the manuscript.

### Conflict of interest

The authors have no conflict of interest to declare.

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