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Harnessing black soldier fly (*Hermetia illucens*) prepupae against *Aeromonas hydrophila*: Fermentation-based fatty acids production and its bioinformatic assessment

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Abstract

Background: *Aeromonas hydrophila* (*A. hydrophila*) is a bacterium with zoonotic potential and is multidrug-resistant. It utilizes hemolysin and aerolysin to spread infection. Black soldier flies (BSFs) can be antibacterial because of the fatty acids it contains.

Aims: This study aimed to investigate and compare the fatty acid profiles of BSF prepupae grown in fermented and nonfermented media using bioinformatics tools and assess their potential as antibacterial agents against *A. hydrophila*.

Methods: The study used BSF prepupae reared on various organic substrates. BSF prepupae grown in fermented or nonfermented substrate were observed against fatty acid. The fatty acid analysis was performed using GC-MS. Fatty acids were analyzed statistically using the one-way ANOVA test with a 95% confidence level. Fatty acid bioactivity was predicted using the online PASS-two-way drug program. Molecular docking on BSF fatty acid compounds was analyzed with PyMol 2.2 and discovery Studio version 21.1.1.

Results: The molecular docking test showed the strongest bond was oleic acid with aerolysin and linoleic acid with hemolysin. BSF prepupae grown on fermented media showed higher crude fat and saturated fatty acids (SFAs) but lower unsaturated fatty acids than nonfermented media.

Conclusion: Black soldier fly prepupae, particularly those grown on fermented media, possess antibacterial activity against *A. hydrophila* through potential fatty acid-mediated inhibition of crucial virulence factors.

Keywords: Zoonotic, Fermentation, Bioinformatics, Prepupae BSF, Bacteria.

Introduction

As a maritime country, Indonesia has the most expansive sea territory and approximately 17,000 islands across the archipelago. Indonesia's rich biodiversity marine sector has economic potential fish provide vitamins and minerals as food supplements for human health.

Aeromonas hydrophila (*A. hydrophila*) bacteria are opportunistic (Jiang *et al.,* 2020) and zoonotic infections (Kim *et al.,* 2021). These bacteria can infect animals (fish, amphibians, birds, and reptiles) and humans (Krovacek *et al*., 1995), causing foodborne illnesses. These bacteria are transmitted to humans through contact with infected fish, water, or seafood contaminated with bacteria (Dong *et al*., 2021). The infection can also be transmitted to humans by reptiles through bites and touch (Kwon *et al*., 2019). Nosocomial infections in humans are associated with contaminated medical equipment (Hilt *et al.,* 2020). In humans, these bacteria cause acute gastroenteritis in children and adults and extraintestinal septicemia in individuals with organ dysfunction. In fish, transmission is through contact between healthy fish and sick fish. In fish, bacteria cause acute and chronic infections (Bhattacharya *et al*., 2020; Duarte *et al*., 2021), economic losses (Chandravanshi *et al*., 2020), and high mortality (Delafont *et al*., 2019).

Aeromonas hydrophila bacteria secrete virulence factors such as extracellular toxins, namely aerolysin, and hemolysin, which are involved in the development of hemorrhagic septicemia and contribute to the pathogenicity of the host. This toxin has cytotoxic and hemolytic activity, which causes diarrhea and anemia (Yadav *et al*., 2022). These bacteria can transfer antibiotic-resistance genes or take advantage of the antibiotic-resistance properties of their outer membrane proteins, thereby triggering antibiotic-resistant isolates (Cao *et al*., 2020). The ability of bacteria to form biofilms can trigger resistance. *Aeromonas hydrophila* bacteria are resistant to the antibiotic clindamycin through target site modification, ribosomal methylation, and mutations, which prevent the binding of the antibiotic

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to its ribosomal target. So, to deal with this problem, proper handling of *Aeromonas* infection is needed.

Insects are potential future human food/feed sources (Mancini *et al.,* 2018). Black soldier fly larva (BSFL) is currently widely used by the community because it can degrade organic waste into a source of high nutrition, protein, and lipid that can be used as animal feed, as well as bioactive ingredients (such as chitin and antimicrobial peptide/AMP) as antibacterial compound (Nardiello *et al*., 2022). Antimicrobial peptides work against broad-spectrum bacteria and bacteria resistant to multidrug antibiotics (Alvarez *et al.*, 2019). Crude fat content is closely related to the percentage of fatty acids identified in the larvae. Black soldier flies (BSF) larvae contain % fat content of 40% and are rich in saturated fatty acids (SFA), especially lauric acid, which acts as an antimicrobial (Hilt *et al*., 2020). BSF prepupae have higher protein and fat content than larvae.

There are many studies on optimizing the maintenance conditions of BSF prepupae. However, there is still no scientific research that leads to the potential of fatty acids from BSF prepupae as antibacterial based on bioinformatics, so this study aimed to investigate and compare the fatty acid profiles of BSF prepupae grown in fermented and nonfermented media, using bioinformatics tools, and to assess their potential as antibacterial agents against *A. hydrophila*.

Materials and Methods

Molecular docking test of fatty acids against hemolysin and aerolysin

Capturing the 3D structure of the target compound and predicting bioactivity

The structure of the fatty acids contained in BSF was downloaded from the PubChem NCBI database (lauric Acid, CID: 3893), (oleic Acid, CID: 445639), and (linoleic acid, CID: 5280450). Fatty acid bioactivity was predicted using the online PASS-Two-way drug program (Filimonov *et al*., 2014).

Docking and data analysis

Ligands in the form of fatty acids and antibacterial target proteins were imported into the Molegro virtual Docker program version 5.0. Aerolysin and hemolysin target proteins were predicted to be active sites (binding cavity) with the Molegro virtual Docker program version 5 with van der Waals parameters 5 (Bitencourt-Ferreira and De Azevedo, 2019). Fatty acid docking with aerolysin was carried out on specific grids $X = 17.74$, $Y = 53.13$, $Z = 31.37$, and radius 24. The hemolysin-specific grids were $X = -11.97$; $Y = -8.61$; $Z = 17.23$, and radius 12. Other docking parameters are Moldock Grid 0.3A, R.M.S.D. less than 2, Binding pose 5, and ten run repetitions. Docking results were analyzed with PyMol 2.2 and Discovery Studio version 21.1.1.

Preparation of BSFL growth media

The study used 7-day-old BSF larvae. Larvae were harvested during the prepupae period at the age of 25 days. Larvae were reared at a temperature of 33°C–40°C, with a relative humidity of 60%–70% (Lin *et al*., 2019). BSF prepupae were dried using a microwave at 1,000 watts for 5 minutes, then aired for 1 minute, and then in the microwave again for 5 minutes (Qosimah et al., 2023).

Treatment of BSF prepupae

BSF larvae were grown on organic media, namely T1 (fruit waste), T2 (fermented fruit waste), T3 (tofu waste), T4 (fermented tofu waste), and T5 (fermented fruit waste and tofu waste, with a ratio of 1:1), respectively, as much 300 g each. Larvae of as many as 150 g were put in a container containing organic substrate and 100 g of coconut pulp. The substrate was placed in a plastic barrel and then fermented using a mixture of EM4, molasses, and water. The fermentation process is for 48 hours.

Crude fat content analysis

Crude fat content was analyzed using the goldfish method (Burina et al., 2022). A glass beaker was filled with 2–3 boiling stones and then placed in the oven at 105°C for 1 hour. Beaker glass was put into the desiccator. The filter paper was weighed (A), and 3–5 g of sample was put into a porcelain apparatus (B). The beaker glass (C) was filled with 50 ml of n-hexane. The glass beaker and porcelain apparatus were attached to the Goldfish apparatus and extracted for 4 hours. The beaker glass containing fat was placed in a vacuum oven at 80°C for 1.5 hours. The beaker glass was then put in a desiccator for 1 hour and weighed (D). Fat content was calculated with the following equation: D-C/B-A × 100%.

Analysis of BSF prepupa fatty acid levels using GC-mass spectrometry methods

Analysis of FAME (Fatty Acid Methyl Ester) was performed on a G.C. Agilent 7890B (Shimadzu, Japan) equipped with a split/splitless inlet. Separations were achieved using a fused silica Zebron ZB-FFAP capillary column (30 m × 0.25 mm ID, 0.25 m film thickness). The sample was injected using the split mode; the carrier gas, helium, was passed with a flow rate of 85,583 ml/min, and the temperature started from 250°C (Abbas, 2019).

Statistical analysis

Data of crude fat was analyzed descriptively, and fatty acids were statistically analyzed (SPSS Statistic 21.0 software) using the One-way ANOVA test to determine the difference between treatments ($p < 0.05$).

Ethical approval

The study was conducted under the approval of the Universitas Brawijaya ethical committee (Approval no. 035-KEP-UB-2022).

Results

Molecular docking of BSF fatty acids with aerolysin and hemolysin

Fat is composed of fatty acids. The fatty acids found in BSF are oleic acid, lauric acid, and linoleic acid.

The 3D views of oleic acid, linoleic acid, and lauric acid compounds showed the same binding area to aerolysin protein (Fig. 1). These three fatty acids bind to the amino groups of HIS186, PHE184, LYS185, and VAL250 residues (Table 1). The bonds formed on the three fatty acid compounds are hydrogen bonds and hydrophobic interactions, with the number of bonds being oleic acid > linoleic acid > lauric acid. The 2D view also shows the presence of van der Waals forces on some of the aerolysin residues.

The 3D view of fatty acid compounds against hemolysin showed inhibition in the same area with the bonds formed, namely hydrogen bonds, hydrophobic interactions, and van der Waals forces (Fig. 2). The same amino acid active site residue was bound by three fatty acids, namely ARG73, PRO485, ALA523, and HIS484 (Table 2). The highest bond energy was formed from the interaction of fatty acids with hemolysin, sequentially mentioned as linoleic acid, oleic acid, and lauric acid. The lowest binding energies for oleic acid to aerolysin protein complex and linoleic acid to hemolysin complex were −268.2 and −343.4 (kJ/mol, respectively).

The three BSF fatty acids showed potential as immunostimulants, immunosuppressants, and immunomodulators in 42%–56%. BSF fatty acids have a relatively high potential, above 52%, as an anti-inflammatory but relatively low as an antioxidant (22%–31%) and antibacterial (below 50%). Linoleic acid had the highest function as an immunostimulator, immunomodulator, and anti-inflammatory (>45%) but not as an antibacterial (0%–16%) (Fig. 3).

Fat content of prepupa BSF

The results of the average percentage of fat content in T.I., T2, T3, T4, and T5 treatments, respectively, were 31.78, 34.47, 29.64, 30.00, and 31.83. The highest fat content was found in the T2 treatment, while the lowest was in the T3 treatment, significantly different from the other treatment groups ($p < 0.05$). The fat content of the

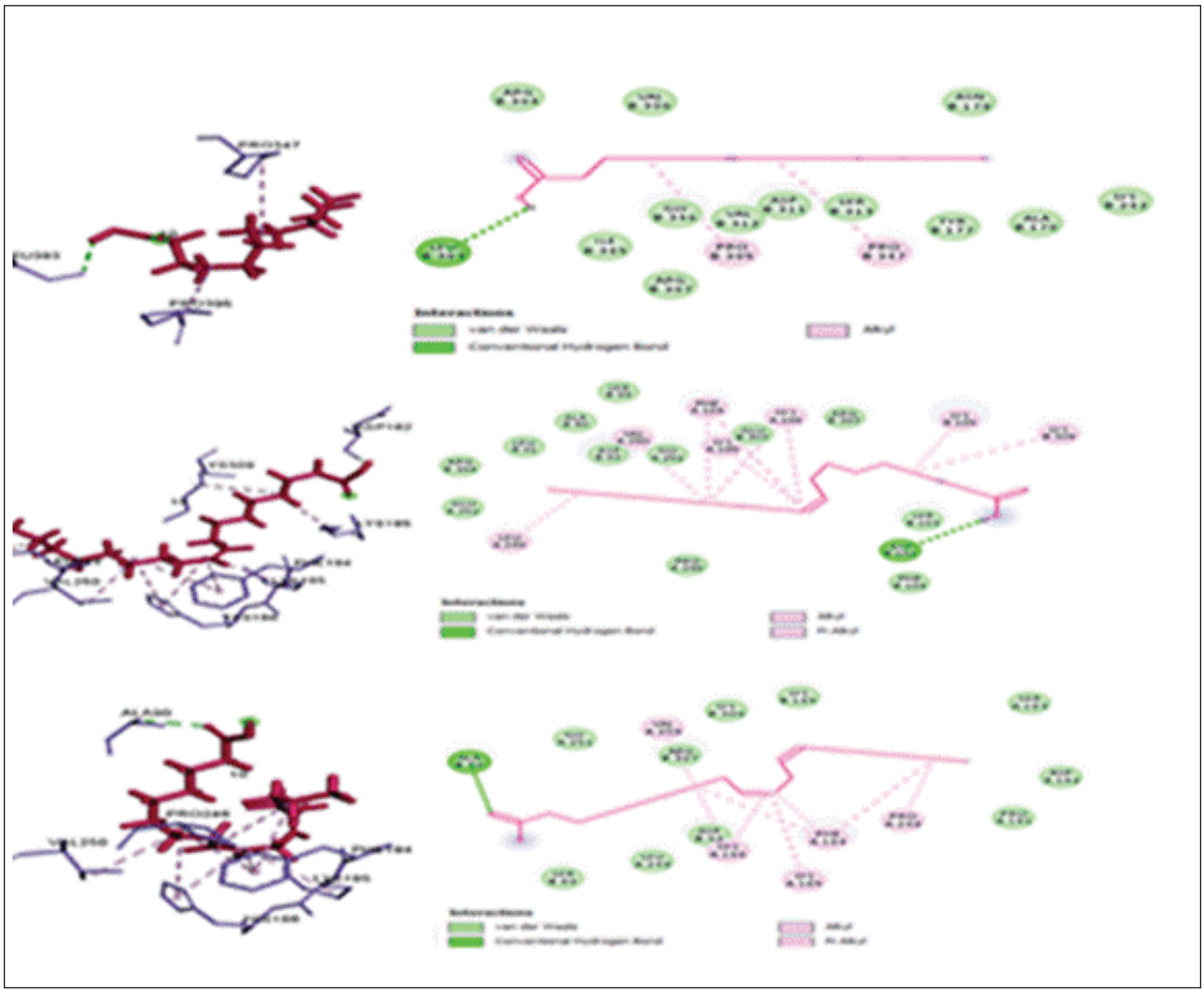

Fig. 1. Interaction between fatty acids from BSF and aerolysin.

Table 1. Interaction between fatty acids and proaerolysin.

Complex	Binding energy (kJ/mol)	Interaction	Distance (A)	Category	Type
Lauric Acid—Proaerolysin	−225.4	:10:H24-B:LEU393:O	2.38614	Hydrogen bond	Conventional hydrogen bond
		B:PRO347-:10	5.15165	Hydrophobic	Alkyl
		B:PRO395-:10	4.86777	Hydrophobic	Alkyl
Oleic Acid—Proaerolysin	−268.2	:10:H34-B:ASP182:O	2.81099	Hydrogen Bond	Conventional hydrogen bond
		A:LYS185-:10	4.66343	Hydrophobic	Alkyl
		A:VAL250-:10	4.16004	Hydrophobic	Alkyl
		B:LYS185-:10	5.22809	Hydrophobic	Alkyl
		B:LYS309-:10	5.4141	Hydrophobic	Alkyl
		:10:C13-A:LEU249	4.97205	Hydrophobic	Alkyl
		A:PHE184-:10	5.37157	Hydrophobic	Pi-Alkyl
		A:PHE184-:10	5.11633	Hydrophobic	Pi-Alkyl
		A:HIS186-:10	4.68784	Hydrophobic	Pi-Alkyl
		A:HIS186-:10	4.66454	Hydrophobic	Pi-Alkyl
Linoleic Acid—Proaerolysin	−258	B:ALA90:N-:10:O2	2.6715	Hydrogen Bond	Conventional hydrogen bond
		A:LYS185-:10	4.64715	Hydrophobic	Alkyl
		A:VAL250-:10	4.52114	Hydrophobic	Alkyl
		:10:C12-A:PRO248	4.90323	Hydrophobic	Alkyl
		A:PHE184-:10	5.06693	Hydrophobic	Pi-Alkyl
		A:PHE184-:10	5.44973	Hydrophobic	Pi-Alkyl
		A:PHE184-:10:C12	4.86639	Hydrophobic	Pi-Alkyl
		A:HIS186-:10	4.85102	Hydrophobic	Pi-Alkyl
		A:HIS186-:10	4.40295	Hydrophobic	Pi-Alkyl

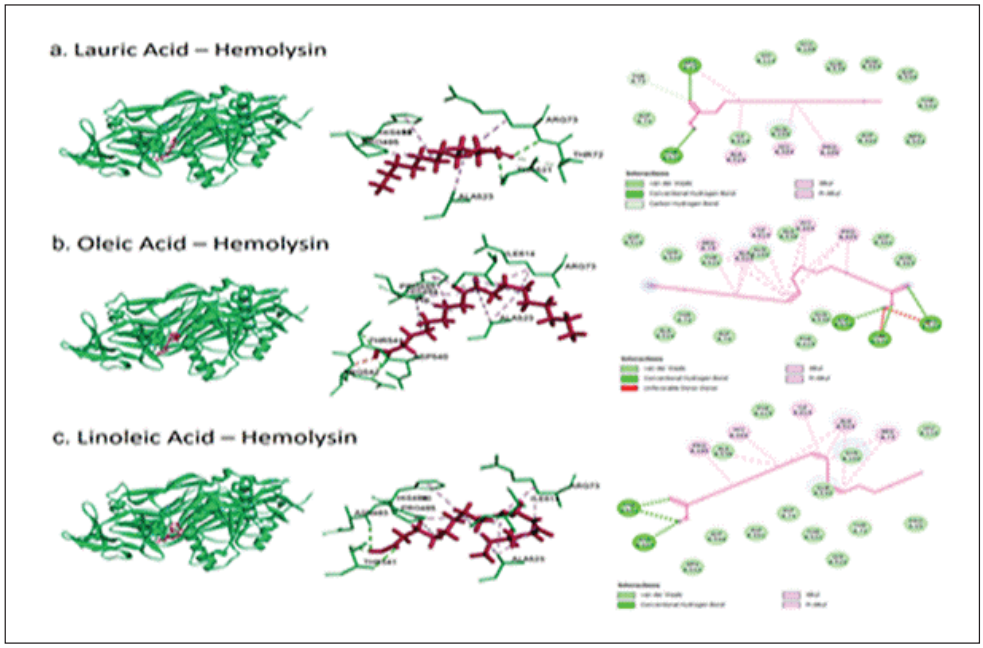

Fig. 2. 3D and 2D structures of the complex interaction between fatty acids and hemolysin.

Table 2. Interaction between fatty acids and hemolysin.

Complex	Binding energy (kJ/mol)	Interaction	Distance (A)	Category	Type
Lauric Acid—Hemolysin	−266	A:ARG73:N-:10:O2	3.13989	Hydrogen bond	Conventional hydrogen bond
		:10:H24-A:THR521:O	2.05482	Hydrogen bond	Conventional hydrogen bond
		A:THR72:CB-:10:O2	3.41481	Hydrogen bond	Carbon hydrogen bond
		A:ARG73-:10	4.57277	Hydrophobic	Alkyl
		A:PRO485-:10	4.43975	Hydrophobic	Alkyl
		A:ALA523-:10	4.35448	Hydrophobic	Alkyl
		A:HIS484-:10	4.41799	Hydrophobic	Pi-Alkyl
Oleic Acid—Hemolysin	−326	A:THR541:OG1-:10:O1	2.54259	Hydrogen bond	Conventional hydrogen bond
		A:ARG542:NE-:10:O2	2.98123	Hydrogen bond	Conventional hydrogen bond
		:10:H34-A:ASP540:OD1	1.96745	Hydrogen bond	Conventional hydrogen bond
		A:ARG73-:10	4.36548	Hydrophobic	Alkyl
		A:ARG73-:10	5.1846	Hydrophobic	Alkyl
		A:PRO485-:10	3.61079	Hydrophobic	Alkyl
		A:PRO485-:10	5.23348	Hydrophobic	Alkyl
		A:PRO485-:10	4.91669	Hydrophobic	Alkyl
		A:ALA523-:10	3.98085	Hydrophobic	Alkyl
		A:ALA523-:10	4.46798	Hydrophobic	Alkyl
		:10-A:ILE614	4.61747	Hydrophobic	Alkyl
		:10-A:ILE614	4.61723	Hydrophobic	Alkyl
		A:HIS484-:10	4.37957	Hydrophobic	Pi-Alkyl
		A:HIS484-:10	5.21603	Hydrophobic	Pi-Alkyl
Linoleic Acid—Hemolysin	−343.4	A:THR541:N-:10:O2	2.86913	Hydrogen bond	Conventional hydrogen bond
		:10:H32-A:ASN483:O	3.06691	Hydrogen bond	Conventional hydrogen bond
		:10:H32-A:THR541:OG1	2.32322	Hydrogen bond	Conventional hydrogen bond
		A:ARG73-:10	4.4688	Hydrophobic	Alkyl
		A:ARG73-:10	4.45861	Hydrophobic	Alkyl
		A:PRO485-:10	4.07407	Hydrophobic	Alkyl
		A:PRO485-:10	4.38261	Hydrophobic	Alkyl
		A:ALA523-:10	5.12741	Hydrophobic	Alkyl
		A:ALA523-:10	4.13492	Hydrophobic	Alkyl
		A:ALA523-:10	4.23502	Hydrophobic	Alkyl
		:10-A:ILE614	3.97664	Hydrophobic	Alkyl
		A:HIS484-:10	4.463	Hydrophobic	Pi-Alkyl

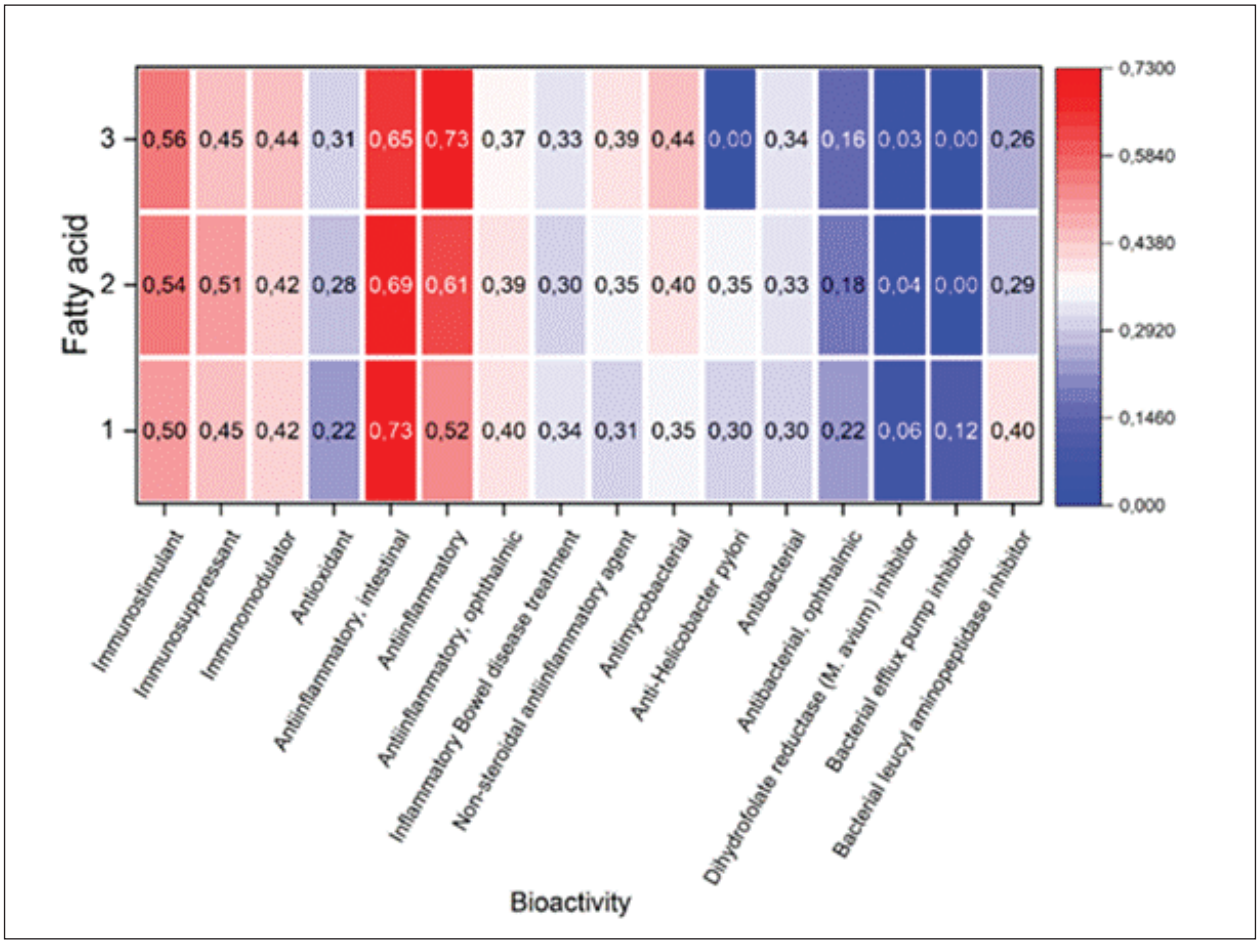

Fig. 3. Prediction of fatty acid bioactivity from BSF. (1) Lauric acid. (2) Oleic acid. (3) Linoleic acid.

BSF prepupa reared on the fermented media (T2 and T4) was higher than that of the nonfermented media (T1 and T3).

The fatty acid content of BSF pre prepupa

Based on the examination of fat content, it showed that the fatty acid content in the BSF prepupa was high, namely SFA, mono unsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) see Table 3. The highest average content of the 34 fatty acids tested was lauric acid in all groups—the highest level found in the T5 treatment (53.66%). BSF prepupa fermented growth media can increase fatty acid levels compared to nonfermented media. Fermentation media in groups T2 and T4 can increase unsaturated fatty acids for both MUFA (26.7% and 28.18%) and PUFA (11.58% and 13.11%) but decrease saturated fat (SFA) (53.79% and 48.71%, respectively) compared to prepupa grown on nonfermented media in group T1 and T3.

In this study, fatty acids found in relatively low levels (<1%) are myristate acid, stearate acid, tridecanoate acid, cis-10-pentadecenoate acid, cis-10-pentadecenoate acid, cis-9-oleic acid + trans-9-elaidate acid, arachidic acid, cis-10-heptadecenoic acid, erucate acid, cis-11-eicosenoate acid, linolenate acid, cis-13,16-docosadienoic acid, and cis-5,8,11,14-eicosatetraenoic acid. Meanwhile, fatty acids found in relatively high levels (>1%) are lauric acid, Myristoleic acid, palmitoleic acid, linoleate + linolelaidate acid, Gamma-Linolenic acid, palmitic acid, and decanoic acid.

BSF prepupae had higher n-6 PUFA fatty acid levels in all treatment groups than n-3 PUFA The results showed that the T4 group had significantly higher levels of n-3 PUFA, comprised of linolenate and gamma-Linolenic acid methyl ester (0.93% and 8.8%, respectively), and n-6 PUFA, composed of docosadienoic acid and Linoleic acid (0.29% and 12.81%, respectively), compared to the other treatment groups.

Discussion

Molecular docking interactions between BSF fatty acids and target proteins

Hemolysin protein from *A. hydrophila* plays a role in transmitting bacteria from fish to humans, so it can be used as a diagnosis of *A. hydrophila* infection in fish as an effort to prevent and control the spread

Table 3. The fatty acid of BSF prepupae in all group treatments.

Fatty acid	Fruit waste	Fermented fruit waste	Tofu waste	Fermented tofu waste	Fermented fruit waste and tofu waste
a. SFA					
Myristate acid (C14:0)	0.46 ± 0.01^{c}	0.42 ± 0.02^{b}	0.12 ± 0.00^{a}	0.58 ± 0.01^{d}	0.47 ± 0.00^{c}
Stearate acid (C18:0)	1.47 ± 0.07^{d}	0.00^{a}	1.32 ± 0.02^{c}	0.00^{a}	0.76 ± 0.00^{b}
Palmitate acid (C16:0)	4.22 ± 0.00^{e}	3.95 ± 0.1^{d}	3.68 ± 0.01^{c}	3.46 ± 0.00^{b}	3.14 ± 0.02^{a}
Decanoate acid	1.19 ± 0.00^{b}	1.11 ± 0.08^{ab}	1.18 ± 0.00^{ab}	1.09 ± 0.00^{a}	1.32 ± 0.00^{c}
Tridecanoate acid	0.00a	0.00a	0.47 ± 0.05^{c}	0.18 ± 0.00^{b}	0.00^{a}
Pentadecanoate acid (C15:0)	0.14 ± 0.00^{a}	0.25 ± 0.00^{a}	0.23 ± 0.00^{a}	0.16 ± 0.00^{a}	0.46 ± 0.01^{a}
Cis-10-pentadecenoate acid	0.11 ± 0.00	0.08 ± 0.57	0.13 ± 0.00	0.11 ± 0.00	0.00
Lauric acid (C12:0)	49.01 ± 0.04^{c}	47.98 ± 1.21^{ab}	47.03 ± 0.03^{b}	43.13 ± 0.06^{a}	53.66 ± 0.02^{d}
Total SFA	56.6 ± 0.92^{a}	53.79 ± 2.75^{a}	54.16 ± 0.16^{a}	48.71 ± 0.07^{a}	59.81 ± 0.05^{a}
b. Unsaturated fatty acid					
MUFA					
Cis-9-oleate acid+trans-9-elaidate acid	0.65 ± 0.02^{a}	0.94 ± 1.00^{a}	0.50 ± 0.01^{a}	0.25 ± 0.01^{a}	0.41 ± 0.00^{a}
Palmitoleate acid (C16:1)	11.76 ± 0.00^{b}	12.01 ± 0.57^{b}	12.36 ± 0.01^{b}	12.04 ± 0.04^{b}	10.1 ± 0.04^{b}
Arachidate acid	0.45 ± 0.00^{b}	0.37 ± 0.06^{a}	0.56 ± 0.00^{c}	1.24 ± 0.00^{d}	0.60 ± 0.00^{c}
Cis-10-heptadecanoic acid	0.00^{a}	0.11 ± 0.01^{b}	0.00^{a}	0.16 ± 0.00^{c}	0.11 ± 0.00^{b}
Cis-11-eicosenoate acid	0.34 ± 0..03^{a}	0.23 ± 0.15^{a}	0.37 ± 0.04^{a}	0.19 ± 0.08^{a}	0.39 ± 0.00^{a}
Erucate acid	0.24 ± 0.00^{c}	0.23 ± 0.09^{bc}	0.23 ± 0.00^{bc}	0.13 ± 0.00^{b}	0.00^{a}
Myristoleic acid methyl ester	12.37 ± 0.02^{a}	12.81 ± 0.76^{a}	12.66 ± 0.04^{a}	14.17 ± 0.02^{b}	12.57 ± 0.05^{a}
Total MUFA	25.81 ± 0.07^{a}	26.7 ± 2.64^{a}	26.68 ± 0.1^{a}	28.18 ± 0.15^{a}	14.18 ± 0.09^{a}
PUFA					
Linolenate acid	0.57 ± 0.00^{a}	0.70 ± 0.06^{b}	0.66 ± 0.00^{b}	0.93 ± 0.00^{c}	0.66 ± 0.00^{b}
Gamma-Linolenic acid methyl ester	5.60 ± 0.01^{a}	6.38 ± 0.18^{c}	6.93 ± 0.00^{d}	8.8 ± 0.00^{e}	6.03 ± 0.01^{b}
Cis-5,8,11,14-eicosatetraenoic acid	0.15 ± 0.00^{c}	0.00^{a}	0.17 ± 0.00^{d}	0.13 ± 0.00^{c}	0.11 ± 0.00^{b}
Σn-3 PUFA	6.32 ± 0.01^{a}	7.08 ± 0.24^{a}	7.76 ± 0.00^{a}	9.86 ± 0.00^{a}	6.8 ± 0.01^{a}
Cis-13,16-docosadienoic acid (C22:2n-6)	0.46 ± 0.00^{a}	0.39 ± 0.09^{c}	0.39 ± 0.01^{c}	0.29 ± 0.00^{b}	0.29 ± 0.00^{b}
Linoleate acid + linolelaidate acid	10.81 ± 0.03^{b}	11.19 ± 0.56^{b}	11.06 ± 0.00^{b}	12.81 ± 0.04^{a}	9.08 ± 0.00^{a}
Σn-6 PUFA	11.27 ± 0.03^{a}	11.58 ± 0.55^{a}	11.45 ± 0.01^{a}	13.11 ± 0.04^{a}	9.37 ± 0.00^{a}
Total PUFA	17.59 ± 0.07^{a}	18.66 ± 0.17^{a}	19.21 ± 0.02^{a}	22.97 ± 0.08^{a}	16.17 ± 0.02^{a}
n-6/n-3	1.78	1.6	1.48	1.33	2.38
14 other fatty acids	<0.1	<0.1	<0.1	<0.1	<0.1

$^{a, b, c, d, e}$ Different letters indicate significant differences ($p < 0.05$). Mean ± SD (Standard deviation).

of disease to new areas (Singh *et al*., 2017). Docking results show binding energy affinity (kcal/mol). The more negative the binding affinity, the better the ligand docking at the binding site. The results of the in silico test showed that the strongest binding affinity of BSF fatty acids was oleic acid for aerolysin and linoleic acid for hemolysin. Oleic acid and linoleic acid act as an antibacterial against Gram-positive and Gram-negative bacteria (Ali *et al*., 2017) by changing the membrane profile, causing bacteria to lose membrane integrity and resulting in bacterial death (Prasad *et al*., 2019).

Fat content of prepupa BSF

The study showed that BSF prepupa grown on fermented media showed higher crude fat content

than without fermentation. This study found that the crude fat content in BSF larvae growth on fruit waste substrate was higher than that of tofu waste. This phenomenon happens because the substrate's carbohydrate content affects the larvae's natural fat content (Ewald *et al*., 2020). The results of the study showed that the fat content of BSF prepupae aged 25 days in fermented fruit waste media (34.47%) was higher than the study conducted by Nyakeri et al. (2017), which showed BSF prepupae aged 23 days grown on media a mixture of fruit and vegetable waste that is equal to 32.62%. The crude fat content in this study is higher compared to the research conducted by Zulkifli *et al*. (2022), who reared BSF larvae on organic waste media and subsequently dried the larvae into powder using a combination of a sprayer and an oven at 50°C for 24–36 hours. The resulting crude fat content was 25.69% and 28.43%, respectively. However, the crude fat content of larvae grown on food waste media and subsequently dried using an oven at 60°C overnight showed a crude fat content of 38.36%. The obtained results are higher than those of this study; however, it should be noted that their research required a longer drying time compared to the approach adopted by the current researchers. In this study, BSFL drying was achieved using a microwave at 1,000 watts for 5 minutes.

The fatty acid content of BSF pre prepupa

The study results showed that the BSF prepupa grown on different substrates showed different levels of fatty acids. The results of this study agreed with the research stated that BSFL grown on different substrates such as dairy cow dung, fruits, vegetables, and fish could change the fatty acid composition. BSF prepupae grown on fermented media had higher levels of fat and unsaturated fatty acids but lower levels of SFAs compared to BSF prepupa grown on nonfermented media (Mai *et al*., 2019). This result is in line with the research conducted by Hadj Saadoun *et al*. (2020), which showed that the fermentation process can significantly change the molecular composition of biomass, including fat and fatty acid profiles. During fermentation, lactic acid bacteria hydrolyze fats into fatty acids and produce secondary metabolites such as AMP The study also showed that BSF prepupae had higher levels of SFAs than unsaturated fatty acids (MUFA and PUFA). These results are consistent with a study conducted by Ewald *et al*. (2020), who grew BSF prepupae on four different media (bread, food scraps, fish, and shellfish), which produced higher levels of SFAs than unsaturated fatty acids in prepupae. In general, SCFA in fish helps provide energy for intestinal cells, which will then activate cells to produce mucin and tight junction proteins while also regulating the diversity of gut microbiota by activating beneficial microorganisms, suppressing pathogenic microbes, and increasing the absorption area of villi to improve feed digestibility (Dawood, 2021).

PUFA fatty acids (n-3 and n-6) in the body play a role in cellular signaling pathways and regulate the physiological and pathophysiological processes of the body (Chen *et al*., 2021). BSF prepupa's PUFA fatty acid content in all treatments showed that n-3 was lower than n-6. PUFAs affect the inflammatory response, where n-6 PUFAs are associated with pro-inflammatory effects. In contrast, n-3 PUFAs are associated with anti-inflammatory effects, so it is necessary to balance n-3 and n-6 PUFAs to reduce inflammation (Myles *et al*., 2014). The results of the study in all treatments showed that the ratio of omega-6:omega-3 was 1.33:1 to 2.38:1. The recommended consumption ratio of omega-6:omega-3 in animals and humans is 1:1 to 4:1 (Enos *et al*., 2014). The study results showed that BSF prepupa contained docosadienoic acid, which has a better antioxidant effect than docosahexaenoic acid (Chen *et al*., 2021). Thus, it can reduce free radicals caused by bacteria.

The BSF prepupa in this study had very high lauric acid. Lauric acid is a SFA that has antimicrobial potential. It can be used as a natural supplement to prevent diarrhea in human food and animal feed. Lauric acid is converted into monolaurin, which acts as an antiviral, antibacterial, and antiprotozoal glyceride. As an antibacterial, lauric acid lowers the pH, allowing acidic conditions to kill bacteria (Rabani *et al*., 2019). In this study, the lauric acid content across all treatments ranged from 43.13% to 53.66%, exceeding the levels found in Zulkifli *et al*. (2022) study on organic media, which were between 17.89% and 37.18%. Notably, the T5 group exhibited a lauric acid level of 53.66%, and the palmitoleic acid content in BSF prepupae reared in all groups was higher (10.1%–11.76%) compared to those grown in BSFL using chicken and cow feces, which ranged from 23.4% to 50.7% and 2.3% to 7.6%, respectively. Palmitoleic acid is known for its anti-apoptotic and anti-inflammatory properties (Mai *et al.,* 2019). This contrasts with Mohamed *et al*. (2021), who found the most effective antibacterial agents against *A. hydrophila in vitro* to be rich in cis-oleic acid (22.65%–26.28%), palmitoleic acid (3.03%–3.15%), and myristic acid, but with lower levels of lauric acid (17.66%–19.32%). However, the in silico study in this research reveals that oleic acid and linoleic acid exhibit elevated docking scores and robust interactions, including hydrogen bonding, Van der Waals forces, and hydrophobic interactions, with amino acid residues in aerolysin and hemolysin found in the cell membrane of *A. hydrophila*, as opposed to lauric acid. The mechanism of action of lauric acid involves damaging the bacterial cell membrane, leading to bacterial death, rather than affecting the contents within the bacterial cell membrane.

BSF prepupa grown in fermented media can produce high levels of fat and unsaturated fatty acids compared to nonfermented media. Unsaturated fatty acids such as PUFA and MUFA are higher, and SFA is lower in

fermented substrate when compared to nonfermented media. Fatty acids from BSF have more potential as an anti-inflammatory than antibacterial based on the in silico test, so developing a rich acid fractionation test from BSF prepupae, especially lauric acid as a potent antibacterial, is necessary.

The protein profile detected in the BSF prepupa grown in various test media showed the same molecular weight (MW), namely 13–14 kDa, leading to AMP (antimicrobial peptide), but the type of protein was unknown, but it acted as an antibacterial, while 17 kDa led to myeloid differentiation factor protein. 2 (MD-2)-related lipid-recognition related to pattern recognition of microbes by host receptors (unpublished data). In bioconversion, the insect immune system plays an important role. Insects rely on innate humoral immunity to produce antibacterial compounds because they do not have specific immune organs (Lu *et al*., 2019). BSF larvae are easy to maintain in substrates such as fruit waste and tofu dregs, either by fermentation or without using EM4. BSF larvae rearing uses substrates that are easily obtained from the environment. BSF larvae are easily reared on low-cost substrates such as manure or by-products (such as vegetable or fish waste) (Mancini *et al*., 2018). BSF larvae have AMP to eliminate pathogens. AMP is an endogenous peptide with a MW of ~2–22 kDa and is released locally in the various surface epithelium or is secreted systemically by hemocytes and fat bodies (liver analogs in mammals) into the hemolymph to clear the microbial infection. AMP is positively charged and can interact with bacterial cell walls, including lipopolysaccharides (LPS) and phospholipids (Elhag *et al*., 2022). It can also bind to the bacterial cell membrane, which then causes lysis and death of bacterial cells. Peptide release in hemolymph is triggered by pathogen recognition via the pattern recognition receptor AMP, which is produced from a relatively short protein containing 50–300 amino acids with MW between 5 and 30 kDa (Buonocore *et al*., 2021).

BSFL peptides range from 20 to 50 amino acids (1.6 to 7.3 kDa). BSF larval peptide with a MW of 2.0–4.5 kDa acts as an antibacterial against bacteria such as Helicobacter pylori (Alvarez *et al*., 2019). BSFL has AMP-type defensin-like peptides (DLP), which consist of 34–43 amino acids, have a MW of 3–4 kDa, is a cationic peptide, and has three pairs of disulfide bridges (Park *et al*., 2018). Defensins are innate immunity in insect species that are overexpressed when insects are infected with microorganisms (Elhag *et al.,* 2022). According to a study conducted by Park *et al*. (2015), AMP protein from BSFL with a MW of 10–30 kDa showed inhibition zones of 25.6, 15.1, and 19.8 mm against *Pseudomonas* bacteria, namely *Pseudomonas marginalis*, *Pseudomonas viridiflava*, and *Pseudomonas syringae*. However, the type of protein with this MW has not been identified. The MW of another protein from BSF prepupae is 17 kDa. This 17 kDa protein is associated with myeloid differentiation factor 2 (MD-2)-related lipid-recognition, which is linked to the pattern recognition of microbes by host receptors.

The fermentation process also produces AMP, but it is unknown how effective AMP is as an antibacterial made from fermented media compared to nonfermented media. Protein profiles in BSF prepupa grown in various test media showed the same MW, namely 13–14 kDa leading to AMP, but the type of protein was unknown, and 17 kDa leading to myeloid differentiation factor 2 (MD-2)-related lipid recognition protein. Further research is needed to analyze the myeloid differentiation factor 2-related lipid-recognition protein characterization from BSF prepupa against *A. hydrophila*. BSF prepupa grown on fermented and nonfermented substrates in this study can be used as a theoretical basis for further studies on the innate immunity antimicrobial peptide of BSF prepupa against *A. hydrophila* infection.

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Conflict of interest

No conflict of interest in research and publication.

Authors' contributions

DQ: The design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript. LA, DS, CM: The writing of the manuscript.

Data availability

All data are provided in the manuscript.

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