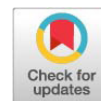


Research Article



Optimization of Molasses Concentration and Incubation Time for the Production of Fermented Microbial Feed Additive from Black Soldier Fly Larvae (*Hermetia illucens*)

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Abstract | The restriction on the use of antibiotic growth promoters (AGPs) in animal production has encouraged the development of sustainable and environmentally friendly feed additives. This study evaluated the optimization of molasses concentration and incubation time for producing fermented microbial feed additive derived from black soldier fly larvae (BSF, *Hermetia illucens*). The novelty of this study lies in the simultaneous evaluation of molasses concentration and incubation time as key fermentation factors for improving physicochemical stability and beneficial microbial populations in BSF larvae-based microbial feed additives. A completely randomized factorial design with two factors was applied, consisting of molasses concentration (0, 75, and 150 mL per 150 g BSF larval paste and 150 mL distilled water) and incubation time (5, 10, and 15 days), with three replications for each treatment. The observed parameters included pH, total titratable acidity (TTA), and microbial populations of lactic acid bacteria (LAB), *Actinomyces*, *Bacillus* sp., and yeast. The results showed a significant interaction ($P < 0.05$) between molasses concentration and incubation time on all observed parameters. In general, increasing molasses concentration and extending incubation time reduced pH values while increasing TTA and microbial populations, indicating improved fermentation activity. The combination of 50% molasses concentration and 10 days of incubation produced the most favorable fermentation characteristics, with a pH value of 3.91, TTA of 2.43%, LAB population of 21.17×10^7 CFU/mL, *Actinomyces* population of 3.90×10^7 CFU/mL, *Bacillus* sp. population of 2.83×10^7 CFU/mL, and yeast population of 3.83×10^7 CFU/mL. In conclusion, the use of 50% molasses combined with 10 days of incubation provided optimal conditions to produce a fermented microbial feed additive from black soldier fly larvae by improving fermentation characteristics and supporting the growth of beneficial microorganisms. The resulting product has potential application as a sustainable microbial-based feed additive for livestock production systems.

Keywords | Black soldier fly larvae, Fermentation, Microbial, Feed additive, Molasses, Incubation time

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INTRODUCTION

Global challenges related to food security and sustainable livestock production have encouraged the development of innovative feed technologies that are

economically feasible and environmentally sustainable. Feed costs remain the largest component of livestock production expenses, accounting for approximately 70% of total production costs (Amran *et al.*, 2021). In addition to economic concerns, the livestock sector is currently facing

increasing pressure to reduce the use of antibiotic growth promoters (AGPs) due to their negative impacts on public health and the environment. Long-term application of AGPs has been associated with the emergence of antimicrobial-resistant bacteria and antibiotic residues in animal-derived food products (Coniglio *et al.*, 2023; Peng *et al.*, 2024). In Indonesia, the use of antibiotics as feed additives has been prohibited through the Regulation of the Minister of Agriculture No. 14/PERMENTAN/PK.350/5/2017 since January 2018 (Kementerian, 2017). Consequently, there is growing interest in developing sustainable microbial-based feed additives as alternatives to AGPs in livestock production systems.

Fermented microbial feed additives have attracted considerable attention because they may improve feed quality, support beneficial microbial activity, and enhance feed utilization efficiency. Fermentation processes can also increase the stability and functionality of feed materials through the production of organic acids and beneficial metabolites (Mokhtar *et al.*, 2024). The effectiveness of microbial fermentation is strongly influenced by substrate composition and fermentation conditions, particularly the availability of fermentable carbohydrates and incubation time. Black soldier fly (BSF; *Hermetia illucens*) larvae have recently gained significant attention as a sustainable biological resource for animal feed development. BSF larvae contain high protein levels and various bioactive compounds, including lauric acid and antimicrobial peptides, which contribute to their antimicrobial properties and potential application in livestock production (Moretta *et al.*, 2020; Fitri *et al.*, 2023). Despite these advantages, the direct use of BSF larvae as feed material may be limited by several factors, including variation in nutrient composition, the presence of chitin in the exoskeleton, and the need to improve microbial stability and functional properties before application as a feed additive (Aminuddin *et al.*, 2023). Fermentation can be used as a bioprocessing strategy to improve the functional quality of BSF larvae-based materials by promoting beneficial microbial growth, increasing organic acid production, and enhancing product stability (Mokhtar *et al.*, 2024). Therefore, fermenting BSF larvae into microbial feed additives may provide added value compared with their direct use as conventional feed ingredients. In addition, BSF larvae harbor diverse microbial communities, including *Bacillus* spp., *Enterobacter* spp., *Microbacterium* spp., and *Actinomyces*, which may contribute to fermentation activity and microbial product development (Zheng *et al.*, 2013; Utari *et al.*, 2024). Therefore, BSF larvae have promising potential as raw material for producing fermented microbial feed additives.

The success of fermentation is closely related to the availability of carbon sources that support microbial growth.

Molasses, a by-product of the sugar industry, is widely used in fermentation processes because it contains readily fermentable sugars such as sucrose, glucose, and fructose (Dewi and Rasmiyana, 2025). Appropriate molasses concentration can stimulate microbial proliferation and organic acid production, whereas incubation time influences microbial adaptation and metabolite accumulation during fermentation (Van de Loo *et al.*, 2025). Optimization of these factors is important to produce stable fermentation characteristics and desirable microbial populations. Several previous studies have reported the presence of beneficial microorganisms in BSF larvae. Fitri *et al.* (2023) observed that fermented products derived from BSF larvae were dominated by lactic acid bacteria, while Fassah *et al.* (2024) successfully isolated several LAB strains from BSF larvae. Utari *et al.* (2024) also reported that BSF larvae juice contained Gram-positive bacteria, particularly *Bacillus* spp. and *Actinomyces*, which may support microbial-based feed applications. However, information regarding the optimization of fermentation conditions for producing fermented microbial feed additives from BSF larvae remains limited, particularly concerning the combined effects of molasses concentration and incubation time on fermentation characteristics and microbial populations.

Although previous studies have reported the microbial potential of black soldier fly larvae and the isolation of beneficial microorganisms from BSF-derived materials, most available studies have mainly focused on microbial identification or general fermentation outcomes (Gorrens *et al.*, 2021; Cifuentes *et al.*, 2022; Fitri *et al.*, 2023; Utari *et al.*, 2024). However, the optimization of fermentation conditions for producing BSF larvae-based microbial feed additives remains limited, particularly regarding the combined effects of molasses concentration and incubation time on fermentation characteristics and beneficial microbial populations. Therefore, the novelty of this study lies in optimizing these two key fermentation factors to determine favorable conditions that simultaneously improve acidity profile and support the growth of lactic acid bacteria, *Actinomyces*, *Bacillus* sp., and yeast. It was hypothesized that molasses concentration and incubation time, as well as their interaction, would significantly affect the fermentation characteristics and beneficial microbial populations of BSF larvae-based microbial feed additives. Based on this research gap, this study aimed to evaluate the effects of molasses concentration and incubation time on pH, total titratable acidity, and microbial populations, including lactic acid bacteria, *Bacillus* spp., *Actinomyces*, and yeast, in fermented microbial feed additive derived from BSF larvae (*Hermetia illucens*). The findings of this study are expected to contribute to the development of sustainable microbial-based feed additives for livestock production systems.

LOCATION AND DURATION OF THE STUDY

This research was conducted at the Laboratory of Feed Nutrition and Technology and the Animal Production Laboratory, Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara. The study was carried out over a period of four months, from July to October 2025.

MATERIALS

The equipment used in this study included fermentation bottles, fermentation tubes, BSF larval rearing containers, analytical balances, autoclave, laminar airflow cabinet, microbiological analysis equipment, digital pH meter, centrifuge, vortex mixer, and titration apparatus. The materials used consisted of BSF eggs, tofu dregs as larval growth substrate, molasses, distilled water, media for microbial enumeration, and reagents for total titratable acidity analysis.

EXPERIMENTAL DESIGN

A factorial completely randomized design (CRD) was used with two factors: molasses concentration (A) and incubation time (B), each consisting of three levels. Each treatment combination was replicated three times, resulting in a total of 27 experimental units. Factor A consisted of three molasses concentration levels: A1 = 0% molasses (0 mL molasses), A2 = 50% molasses (75 mL molasses), and A3 = 100% molasses (150 mL molasses). While, factor B consisted of three incubation times: B1= 5 days, B2= 10 days, and B3= 15 days. Molasses concentration was defined based on the volume of molasses added relative to the volume of distilled water used in the fermentation mixture. Each experimental unit contained 150 g of BSF larval paste and 150 mL of distilled water. Accordingly, the 50% molasses treatment consisted of 75 mL of molasses, whereas the 100% molasses treatment consisted of 150 mL of molasses.

PREPARATION OF FERMENTED MICROBIAL FEED ADDITIVE FROM BLACK SOLDIER FLY LARVAE

Black soldier fly larvae used in this study were obtained from larvae reared under controlled laboratory conditions at the Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara. Tofu dregs were first fermented using a commercial microbial starter (Probio-7) for seven days and used as the primary growth substrate for BSF larvae. During larval rearing, substrate moisture was maintained by spraying distilled water daily, while manual mixing was performed every two days to prevent excessive heat accumulation. The larvae were harvested at 18 days of age before entering the prepupal stage to ensure relatively uniform physiological conditions

(Amran *et al.*, 2021). Harvested larvae were separated from the remaining substrate, washed thoroughly under running water, and air-dried to remove excess surface moisture. The cleaned larvae were then homogenized using a chopper until a uniform paste was obtained.

Prior to fermentation, all fermentation equipment, including bottles, tubing, spatulas, and glassware, was sterilized using an autoclave at 121°C for 15 minutes. Approximately 150 g of BSF larval paste was transferred into sterile fermentation bottles and mixed with molasses according to the assigned treatment level. Distilled water (150 mL) was subsequently added to each bottle. The fermentation mixture in each experimental unit therefore consisted of 150 g of BSF larval paste, 150 mL of distilled water, and molasses according to the treatment level, resulting in different final fermentation volumes depending on molasses addition. The fermentation containers were tightly sealed and connected to water-filled airlocks to maintain anaerobic conditions, facilitate gas release, and prevent oxygen entry. Fermentation was carried out at room temperature for 5, 10, or 15 days according to the respective treatment combinations. Samples were collected at the end of each incubation period, namely on days 5, 10, and 15. Before analysis, each fermented sample was homogenized to obtain a representative sample for pH, total titratable acidity, and microbial population analyses. The overall preparation procedure is presented in Figure 1.

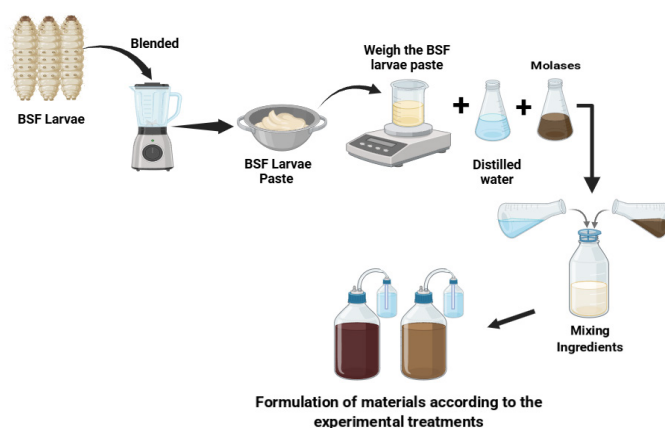


Figure 1: Preparation process of fermented microbial feed additive derived from *Hermetia illucens* (Black Soldier Fly) larvae, illustrating the main stages of larval homogenization, molasses addition, anaerobic fermentation, incubation, and microbial analysis.

MEASURED PARAMETERS

pH MEASUREMENT

The pH value of the probiotic samples was measured using a calibrated digital pH meter (AOAC, 2005). The instrument was standardized at pH 4 and 7 before use. The electrode was immersed in the sample until a stable reading was obtained.

TOTAL TITRATABLE ACIDITY (TTA) (%)

TTA was determined according to AOAC (2005). The supernatant was obtained by centrifuging the probiotic sample, and 10 mL of the supernatant was diluted with sterile distilled water (1:10 ratio). The diluted sample was titrated with 0.1 N NaOH using phenolphthalein as an indicator until a faint pink color appeared. The TTA value, expressed as a percentage of lactic acid equivalent, was calculated using the formula:

$$TTA (\%) = \frac{N \times V_1 \times Eq.Wt}{V_2 \times 10}$$

Where; N = Normality of titrant (mol L⁻¹), V₁ = Volume of titrant (mL), V₂ = Volume of sample (mL), Eq.Wt = Equivalent weight of lactic acid (90.08)

MICROBIAL POPULATION COUNT

The total microbial population was determined using the total plate count (TPC) method. The media used for microbial enumeration included de Man, Rogosa, and Sharpe agar (MRS) for lactic acid bacteria (LAB), starch casein agar (SCA) for *Actinomycetes*, tryptic soy agar (TSA) for *Bacillus* spp., and potato dextrose agar (PDA) for yeasts. All tools and media were sterilized using an autoclave at 121°C for 15 minutes. One milliliter of sample was added to 9 mL of 0.1% Buffered Peptone Water (BPW) and vortexed for 1 minute to prepare the 10⁻¹ dilution. Serial dilutions were made up to 10⁻⁷. From each dilution, 1 mL was poured into sterile Petri dishes (in triplicate) and mixed with molten agar. After solidification, plates were incubated at 37°C for 24 hours in an inverted position. The colony forming units (CFU/mL) were calculated using the formula (Rum *et al.*, 2025).

$$\text{Colony Forming Units} \left(\frac{CFU}{ml} \right) = \text{Number of colonies} \times \frac{1}{\text{dilution factor}}$$

DATA ANALYSIS

Data were analyzed using Analysis of Variance (ANOVA) based on a completely randomized factorial design. When significant differences (P < 0.05) were observed, means were further compared using Duncan's Multiple Range Test (DMRT). The optimum treatment was determined based on an integrated evaluation of physicochemical and microbiological responses, including low pH, appropriate total titratable acidity, and high populations of beneficial microorganisms. Although this study used factorial ANOVA and DMRT to identify the most favorable treatment combination, further studies may apply more advanced optimization methodologies to improve predictive accuracy and strengthen model-based treatment optimization.

RESULT AND DISCUSSION

EFFECT OF MOLASSES CONCENTRATION AND INCUBATION TIME ON pH AND TTA OF FERMENTED MICROBIAL FEED ADDITIVE DERIVED FROM BSF LARVAE

The analysis of variance showed a significant interaction (P < 0.05) between molasses concentration and incubation time on pH value and TTA of fermented microbial feed additive derived from *Hermetia illucens* larvae (Table 1). The results of DMRT indicated that the lowest pH value was observed in treatment A2B2 (3.91±0.07), although it was not significantly different (P > 0.05) from treatments A3B2 (3.92±0.03) and A3B3 (4.01±0.06). However, these treatments showed significantly lower pH values (P < 0.05) compared to A3B1, A2B3, A2B1, A1B2, A1B1, and A1B3. In contrast, the highest pH value was recorded in treatment A1B3 (6.28±0.11), indicating weak fermentation activity under conditions without molasses supplementation. The reduction in pH observed in treatments supplemented with molasses indicates that fermentation proceeded

Table 1: Interaction effect of molasses concentration and incubation time on pH and total titratable acidity of fermented microbial feed additive derived from Black Soldier Fly larvae.

pH					Titratable acidity (%)				
Factor A (Molasses level)	Factor B (Incubation time)			Mean	Factor A (Molasses level)	Factor B (Incubation time)			Mean
	B1	B2	B3			B1	B2	B3	
A1	6.05 ± 0.02 ^b	5.91 ± 0.07 ^c	6.28 ± 0.11 ^a	6.08 ± 0.18	A1	0.71 ± 0.06 ^d	0.75 ± 0.06 ^d	0.81 ± 0.14 ^d	0.76 ± 0.09
A2	4.12 ± 0.08 ^d	3.91 ± 0.06 ^c	4.11 ± 0.03 ^d	4.04 ± 0.11	A2	2.41 ± 0.26 ^c	2.43 ± 0.06 ^c	3.74 ± 0.08 ^a	2.86 ± 0.70
A3	4.07 ± 0.02 ^d	3.92 ± 0.03 ^c	4.01 ± 0.06 ^{dc}	4.00 ± 0.07	A3	2.60 ± 0.20 ^c	3.37 ± 0.45 ^{ab}	3.29 ± 0.16 ^b	3.09 ± 0.45
Mean	4.74 ± 0.98	4.58 ± 1.00	4.80 ± 1.11		Mean	1.91 ± 0.91	2.18 ± 1.17	2.61 ± 1.38	

Note: A1 = 0% molasses, A2 = 50% molasses, A3 = 100% molasses; B1 = 5 days of incubation, B2 = 10 days of incubation, and B3 = 15 days of incubation. Values are presented as mean ± SD. Different superscript letters within the interaction means indicate significant differences (P < 0.05). Because a significant interaction between molasses concentration and incubation time was observed, the interpretation was primarily based on interaction means. Marginal means are presented descriptively to show the general trends of each factor.

effectively due to increased microbial metabolic activity. Molasses contains readily fermentable sugars such as sucrose, glucose, and fructose that can be utilized as energy sources by fermentative microorganisms during the fermentation process (El-Asri and Faraq, 2023; Huang *et al.*, 2023; Wang *et al.*, 2024). The availability of these simple carbohydrates stimulates microbial metabolism and promotes the production of organic acids, which subsequently lowers the pH of the fermentation substrate. Similar findings were reported by Vera *et al.* (2022), who stated that carbohydrate-rich substrates enhance microbial fermentation activity and accelerate acidification during fermentation. The present study demonstrated that treatments without molasses supplementation (A1B1–A1B3) exhibited relatively high pH values ranging from 5.91 to 6.28. This condition indicates limited fermentation activity due to insufficient fermentable carbon sources. In addition, the increase in pH observed in treatment A1B3 suggests that prolonged incubation without adequate carbon availability may trigger protein degradation and the formation of alkaline metabolites such as ammonia, which contribute to pH elevation during fermentation (Muhamad *et al.*, 2021). Conversely, treatments containing 50% and 100% molasses showed substantially lower pH values, indicating improved fermentation efficiency and greater acid production. Incubation time also influenced fermentation characteristics. The 10-day incubation period appeared to represent the optimal phase of microbial activity, as indicated by the lowest pH values observed in A2B2 and A3B2. During this period, fermentative microorganisms were likely in the exponential growth phase, actively converting simple sugars into organic acids. However, prolonged incubation for 15 days tended to slightly increase pH values in several treatments, possibly due to nutrient depletion and reduced microbial activity caused by excessive acid accumulation during fermentation (Ghosh, 2020; Huang *et al.*, 2021).

A similar interaction pattern was also observed for TTA. The highest TTA value was obtained in treatment A2B3 ($3.74 \pm 0.08\%$), although it was not significantly different ($P > 0.05$) from A3B2 ($3.37 \pm 0.45\%$). Both treatments exhibited significantly higher TTA values ($P < 0.05$) compared to A3B3, A3B1, A2B2, A2B1, A1B3, A1B2, and A1B1. In contrast, treatments without molasses supplementation produced the lowest TTA values, ranging from 0.71 to 0.81%. The increase in TTA values reflects the accumulation of organic acids produced during microbial fermentation. Higher molasses concentrations provided sufficient fermentable substrates for microbial metabolism, thereby increasing acid production during fermentation (Oliveira *et al.*, 2016; Mokhtar *et al.*, 2024). Extended incubation time also supported continued acid synthesis, particularly under conditions with adequate carbon

availability. Nevertheless, excessive fermentation duration combined with high sugar concentration may eventually suppress microbial activity due to acid accumulation and osmotic stress (Guan and Liu, 2020; Hawaz *et al.*, 2023). An inverse relationship between pH and TTA was clearly observed in this study. Treatments showing low pH values generally exhibited high TTA values, indicating that acid accumulation was the main factor responsible for pH reduction during fermentation (Shi *et al.*, 2022). This relationship reflects active microbial fermentation and stable fermentation conditions within the fermented microbial feed additive. Overall, the combination of 50% molasses and 10–15 days of incubation produced favorable fermentation characteristics, characterized by low pH values and high TTA. These findings indicate that optimization of molasses concentration and incubation time is essential for improving fermentation efficiency and physicochemical quality of fermented microbial feed additive derived from black soldier fly larvae (Memon *et al.*, 2025). From an optimization perspective, treatment A2B2 was considered the most favorable condition because it provided a balanced fermentation profile, characterized by the lowest pH value and adequate organic acid production without excessive acidity. Although several treatments produced comparable or higher TTA values, excessive acid accumulation may not always indicate better fermentation quality because it can suppress certain beneficial microorganisms during prolonged incubation. The availability of fermentable carbohydrates from molasses may stimulate lactic acid bacteria activity and organic acid production, leading to pH reduction and improved fermentation stability (Luo *et al.*, 2021; Peng *et al.*, 2024). Therefore, the selection of 50% molasses and 10 days of incubation was based not only on acidification response, but also on the ability of this treatment to maintain favorable microbial populations and fermentation stability.

EFFECT OF MOLASSES CONCENTRATION AND INCUBATION TIME ON THE POPULATIONS OF LAB, ACTINOMYCETES, BACILLUS SP., AND YEAST IN FERMENTED MICROBIAL FEED ADDITIVE DERIVED FROM BSF LARVAE

The analysis of variance revealed a significant interaction ($P < 0.05$) between molasses concentration and incubation time on the populations of LAB, *Actinomyces*, *Bacillus* sp., and yeast in fermented microbial feed additive derived from *Hermetia illucens* larvae (Tables 2 and 3). The results of DMRT indicated that the highest LAB population was observed in treatment A3B2 ($21.80 \pm 0.62 \times 10^7$ CFU/mL), although it was not significantly different ($P > 0.05$) from treatment A2B2 ($21.17 \pm 1.67 \times 10^7$ CFU/mL). Both treatments showed significantly higher LAB populations ($P < 0.05$) compared to A3B1, A2B3, A3B3, A2B1, A1B2, A1B1, and A1B3. In contrast, the lowest

Table 2: Interaction effect of molasses concentration and incubation time on lactic acid bacteria and *Actinomycetes* populations of fermented microbial feed additive derived from Black Soldier Fly larvae.

Total Lactic Acid Bacteria x 10 ⁷ (CFU/ml)					Total Actinomycetes x 10 ⁷ (CFU/ml)				
Factor A (Molasses level)	Factor B (Incubation time)			Mean	Factor A (Molasses level)	Factor B (Incubation time)			Mean
	B1	B2	B3			B1	B2	B3	
A1	2.60 ± 0.40 ^{ef}	3.13±0.85 ^c	0.60 ± 0.50 ^f	2.11 ± 1.27	A1	0.87 ± 0.65 ^d	1.27±0.25 ^{bcd}	1.20±0.75 ^{cd}	1.11 ± 0.55
A2	6.13 ± 1.85 ^d	21.17±1.67 ^a	12.57±2.10 ^{bc}	13.29±6.73	A2	2.13 ± 0.06 ^b	3.90±0.35 ^a	3.10±0.40 ^a	3.04 ± 0.81
A3	13.83 ± 1.44 ^b	21.80±0.62 ^a	10.70±0.61 ^{dc}	15.44 ± 5.03	A3	3.07 ± 0.25 ^a	3.73± 0.38 ^a	1.97±0.85 ^{bc}	2.92 ± 0.91
Mean	7.52 ± 5.11	15.37±9.23	7.96 ± 5.69		Mean	2.02 ± 1.02	2.97±1.31	2.09±1.02	

Note: A1 = 0% molasses, A2 = 50% molasses, A3 = 100% molasses; B1 = 5 days of incubation, B2 = 10 days of incubation, and B3 = 15 days of incubation. Values are presented as mean ± SD. Different superscript letters within the interaction means indicate significant differences (P < 0.05). Because a significant interaction between molasses concentration and incubation time was observed, the interpretation was primarily based on interaction means. Marginal means are presented descriptively to show the general trends of each factor.

Table 3: Interaction effect of molasses concentration and incubation time on *Bacillus* sp. and yeast populations of fermented microbial feed additive derived from Black Soldier Fly larvae.

Total Bacillus sp. x 10 ⁷ (CFU/ml)					Total Yeast x 10 ⁷ (CFU/ml)				
Factor A (Molasses level)	Factor B (Incubation time)			Mean	Factor A (Molasses level)	Factor B (Incubation time)			Mean
	B1	B2	B3			B1	B2	B3	
A1	0.40 ± 0.10 ^{cd}	0.33 ± 0.15 ^{cd}	0.17 ± 0.06 ^d	0.30 ± 0.14	A1	0.17 ± 0.06 ^c	0.30 ± 0.20 ^c	0.20 ± 0.10 ^c	0.22 ± 0.13
A2	1.90 ± 0.60 ^{ab}	2.83 ± 0.47 ^a	1.23 ± 0.45 ^{bc}	1.99 ± 0.83	A2	1.43 ± 1.06 ^b	3.83 ± 0.58 ^a	1.47 ± 0.25 ^b	2.24 ± 1.34
A3	0.57 ± 0.35 ^{cd}	2.73 ± 1.10 ^a	1.73 ± 0.51 ^b	1.68 ± 1.13	A3	1.30 ± 0.53 ^b	3.30 ± 0.72 ^a	1.00 ± 0.17 ^{bc}	1.87 ± 1.17
Mean	0.96 ± 0.79	1.97 ± 1.37	1.04 ± 0.77		Mean	0.97 ± 0.85	2.48 ± 1.72	0.89 ± 0.58	

Note: A1 = 0% molasses, A2 = 50% molasses, A3= 100% molasses; B1= 5 days of incubation, B2= 10 days of incubation, and B3= 15 days of incubation. Values are presented as mean ± SD. Different superscript letters within the interaction means indicate significant differences (P < 0.05). Because a significant interaction between molasses concentration and incubation time was observed, the interpretation was primarily based on interaction means. Marginal means are presented descriptively to show the general trends of each factor.

LAB population was recorded in treatment A1B3 (0.60 ± 0.50 × 10⁷ CFU/mL). The increase in LAB populations in treatments supplemented with molasses indicates that the availability of simple carbohydrates strongly supported microbial fermentation activity. Molasses contains fermentable sugars such as sucrose, glucose, and fructose that are readily utilized by LAB as energy sources during fermentation (Zi *et al.*, 2022; El-Asri and Faraq, 2023). The high LAB populations observed in A2B2 and A3B2 coincided with low pH values (3.91–3.92) and relatively high TTA values (2.43–3.37%), indicating active organic acid production during fermentation. These findings confirm that LAB played a dominant role in acidification and stabilization of the fermentation system. Similar observations were reported by Peralta *et al.* (2021), who stated that optimal carbohydrate availability promotes rapid LAB proliferation and enhances organic acid synthesis during feed fermentation. The present study also demonstrated that the 10-day incubation period represented the optimum phase for LAB growth. During

this phase, microorganisms were likely in the exponential growth stage, actively converting carbohydrates into organic acids through fermentative metabolic pathways (Vera *et al.*, 2022). However, prolonged incubation for 15 days reduced LAB populations, particularly in treatment A3B3 (10.70 ± 0.61 × 10⁷ CFU/mL). This decline was likely associated with excessive acid accumulation, nutrient depletion, and osmotic stress resulting from high sugar concentration during prolonged fermentation (Huang *et al.*, 2021). In contrast, treatments without molasses supplementation (A1B1–A1B3) produced substantially lower LAB populations due to limited fermentable carbon availability. Black soldier fly larvae primarily contain complex organic compounds such as chitin and protein that are less readily utilized by LAB without additional carbohydrate supplementation (Aminuddin *et al.*, 2023).

A similar interaction pattern was observed in the *Actinomycetes* population (Table 2). The highest *Actinomycetes* count was recorded in treatment A2B2 (3.90

$\pm 0.35 \times 10^7$ CFU/mL), although it was not significantly different ($P > 0.05$) from treatments A3B2, A2B3, and A3B1. Nevertheless, these treatments exhibited significantly higher populations ($P < 0.05$) compared to A2B1, A3B3, A1B2, A1B3, and A1B1. The increase in *Actinomyces* population under moderate fermentation conditions indicates that this microbial group adapted well to the acidic fermentation environment. *Actinomyces* are known to produce extracellular enzymes and bioactive metabolites that contribute to microbial balance and fermentation stability (El-Tarabily and Sivasithamparam, 2021). The optimum growth of *Actinomyces* observed in A2B2 suggests that fermentation conditions characterized by moderate acidity and adequate carbon availability were favorable for their development. However, prolonged fermentation combined with high molasses concentration tended to reduce *Actinomyces* populations, as observed in A3B3. Excessive acidity and osmotic pressure may inhibit *Actinomyces* growth and suppress metabolic activity during fermentation (Bhattacharjee *et al.*, 2018). The relatively low populations observed in treatments without molasses supplementation further indicate that fermentable carbohydrate availability is essential for supporting microbial growth during fermentation.

The population of *Bacillus* sp. was also significantly affected ($P < 0.05$) by the interaction between molasses concentration and incubation time (Table 3). Treatment A2B2 produced the highest *Bacillus* sp. population ($2.83 \pm 0.47 \times 10^7$ CFU/mL), although it was not significantly different ($P > 0.05$) from A3B2 ($2.73 \pm 1.10 \times 10^7$ CFU/mL). Both treatments showed significantly higher populations compared to A3B3, A2B3, A2B1, A3B1, A1B1, A1B2, and A1B3. The increase in *Bacillus* populations suggests that moderate molasses supplementation and optimum incubation time created favorable environmental conditions for microbial growth. *Bacillus* species are known for their ability to produce extracellular enzymes such as proteases and amylases that contribute to substrate degradation during fermentation (Li *et al.*, 2023). The high *Bacillus* populations observed in A2B2 and A3B2 likely contributed to improved fermentation efficiency and nutrient transformation within the substrate. However, the decline in *Bacillus* populations after prolonged fermentation suggests that nutrient depletion and excessive acid accumulation may suppress microbial activity during the later stages of fermentation (Li *et al.*, 2023; Holanda *et al.*, 2025).

The total yeast population also showed a significant interaction ($P < 0.05$) between molasses concentration and incubation time (Table 3). The highest yeast population was observed in treatment A2B2 ($3.83 \pm 0.58 \times 10^7$ CFU/mL), although it was not significantly different ($P > 0.05$)

from A3B2 ($3.30 \pm 0.72 \times 10^7$ CFU/mL). Both treatments showed significantly higher yeast populations compared to A2B3, A2B1, A3B1, A3B3, A1B2, A1B3, and A1B1. The increase in yeast populations indicates that molasses effectively supplied fermentable carbohydrates required for yeast metabolism during fermentation. Yeasts are capable of converting simple sugars into ethanol, carbon dioxide, and various organic metabolites that contribute to fermentation dynamics (Kasmiarti *et al.*, 2023). The optimum yeast growth observed during the 10-day incubation period indicates that this stage provided suitable environmental conditions, including sufficient nutrients and moderate acidity. However, prolonged fermentation reduced yeast populations, likely due to ethanol accumulation, acid stress, and nutrient depletion during the later stages of fermentation (Santos *et al.*, 2024). Treatments with high yeast populations also showed elevated LAB and *Bacillus* populations, suggesting synergistic interactions among microorganisms during fermentation. LAB contribute to environmental acidification, while yeast and *Bacillus* species produce metabolites and enzymes that support microbial stability and substrate degradation. This microbial interaction likely contributed to the improved physicochemical characteristics observed in treatments A2B2 and A3B2. The favorable microbial profile observed in A2B2 suggests that moderate molasses supplementation and 10 days of incubation created a balanced ecological condition for microbial growth. In this condition, LAB may contribute primarily to acid production and pH reduction, while *Bacillus* sp. may support substrate degradation through extracellular enzyme production and probiotic-related functions (Elshaghabee *et al.*, 2017; Peng *et al.*, 2024). *Actinomyces* may contribute to microbial stability and bioactive metabolite production, whereas yeast may support fermentation dynamics through the utilization of simple sugars and production of growth-supporting metabolites (De Simeis *et al.*, 2021; Pang *et al.*, 2022). This microbial balance is important because the quality of fermented microbial feed additives is not determined only by a single microbial group, but by the combined activity and stability of several beneficial microorganisms. Therefore, A2B2 was selected as the optimum treatment because it provided the most balanced response among pH, TTA, LAB, *Actinomyces*, *Bacillus* sp., and yeast populations.

CONCLUSION

The present study demonstrated that molasses concentration and incubation time significantly affected the physicochemical characteristics and microbial populations of fermented microbial feed additive produced from *Hermetia illucens* larvae. Among all treatments, the combination of 50% molasses and 10

GENERATIVE AI AND AI-ASSISTED TECHNOLOGY STATEMENT

During the preparation of this manuscript, the authors used AI-assisted technology solely to improve language clarity, grammar, and readability. All scientific content, data analysis, interpretation of results, and conclusions were developed and verified entirely by the authors. The authors take full responsibility for the content of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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days of incubation (A2B2) provided the most favorable fermentation condition, characterized by a low pH value (3.91), moderate total titratable acidity (2.43%), and high populations of beneficial microorganisms, including lactic acid bacteria (21.17×10^7 CFU/mL), *Actinomycetes* (3.90×10^7 CFU/mL), *Bacillus* sp. (2.83×10^7 CFU/mL), and yeast (3.83×10^7 CFU/mL). These results indicate that appropriate molasses supplementation and controlled fermentation duration are essential to support microbial growth and improve fermentation quality. Overall, fermented black soldier fly larvae produced under optimum conditions showed considerable potential as a locally sourced microbial feed additive for sustainable livestock production systems. These findings highlight the novelty and practical relevance of optimizing molasses concentration and incubation time as key fermentation factors for developing BSF larvae-based microbial feed additives.

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NOVELTY STATEMENT

This study introduces a new approach to developing fermented microbial feed additives from *Hermetia illucens* larvae through the optimization of molasses concentration and incubation time. Unlike previous studies that mainly focused on microbial isolation, this research demonstrates how fermentation conditions influence physicochemical characteristics and the growth of beneficial microorganisms, including lactic acid bacteria, *Actinomycetes*, *Bacillus* sp., and yeast. The findings provide new insight into the potential use of black soldier fly larvae as a sustainable locally sourced microbial feed additive.

AUTHORS' CONTRIBUTION

MA and WFS conceived and designed the study and supervised the overall research activities. MA, VOA, AT, Y, and MAP conducted the field and laboratory experiments and collected the research data. MA and WFS performed the data analysis and prepared, revised, and finalized the manuscript. All authors read, reviewed, and approved the final version of the manuscript.

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