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Influence of a fermentation waste on rearing and growth of mealworm (*Tenebrio molitor* L.) (Coleoptera: Tenebrionidae): as a sustainable feed source

I.Idris, Y. Akeed, M. Harba, R. Alsafadi, R. Auda, M. Aljahjah. Y. Bakri

Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091 Damascus, Syria.

Abstract

Yellow mealworm (MY) (*Tenebrio molitor*) is a promising protein source for both human and animal food and feeding. However, the growth parameters and larvae mortality of mealworms are intricately linked to their feeding conditions. This study aimed to explore the improvements of the applications of waste fermented by *Aspergillus tubingensis* in the feeding course of MY larvae, particularly focusing on its impact on growth performance, and larvae mortality. The results indicate that introducing fermentation waste in the feeding significantly boosts mealworm growth while reducing larvae mortality. Additionally, the enzymes present in the fermented wastes may provide accessibility to crucial valuable nutrients by hydrolyzing the waste.

The study highlights the possible of employing fermentation waste in the mealworm feeding not only to enhance growth but to also provide a cost-effective diet.

Keywords: mealworm, fermentation, waste, growth, morality, *Aspergillus tubingensis*.

1.Introduction

Increasing global demand for sustainable sources of proteins have generated interest in alternative ways to meet nutritional needs [23, 13]. There are several emerging candidates for protein production, including yellow worms (MY)(*Tenebrio molitor*) (Coleoptera: Tenebrionidae), which have high protein content, while easy reproducibility, and low environmental impacts [39, 29]. Even so, optimizing mealworm rearing practices based on larvae feed for economic feasibility and ecological sustainability remains a challenge [17]. Studies have emphasized the benefit of wastes fermentation by-products in insect farming, emphasizing their ability to improve growth rates and nutrient profiles [40]. By converting organic waste into valuable resources, fermentation wastes provide a promising solution to waste management and sustainable protein production [22, 15].

Waste generated through fermentation, a byproduct of diverse industrial processes is enriched with valuable nutrients and organic matter, may make it an excellent medium for cultivating mealworms [30, 35, 40]. This byproduct constitutes a nutrient-rich source of proteins, carbohydrates, and lipids crucial for the growing and development of mealworms [2, 32]. The applications of fermentation waste for mealworm cultivation brings forth several benefits. Firstly, it provides a cost-effective additional for conventional feed sources, diminishing dependence on expensive and environmentally harmful ingredients [8]. Secondly, the utilization of fermentation waste aligns with circular economy principle, repurposing waste materials and reducing waste disposal [20]. Lastly, leveraging fermentation waste for mealworm cultivation can enhance growth and contribute to building a more sustainable and resilient food system [33].

* Corresponding author: I. Idris *, E-mail: ascientific23@aec.org.sy

This paper investigates a novel method to improve yellow worm cultivation by using a fermentation waste produce from *Aspergillus tubingensis* FSS117 [7] as a nutritional substrate to increase yellow worm production. In addition, we analyze how fermentation treatment impacts the growth parameters of yellow worms.

2. Material and methods

2.1. Mealworm stock culture establishment

Wheat-bran was sterilized in autoclave at 121 ºC for thirty min to make wheat bran appropriate for larval consumption, it must be sieved (mesh size 14), The mealworm colony was cultured in a growth chamber at a 25 ± 5 ºC temperature, with a relative humidity of 60~65%, and a 24- hour dark photoperiod.

2.2. Isolation and maintenance of fungal the strain

The fungal strain utilized in this investigation, *Aspergillus tubingensis* FSS117, was originally isolated from Syrian soil based on its demonstrated efficiency in xylanase production [7]. Identification was achieved through comprehensive analyses of 5.8S gene sequencing. To ensure the stability and availability of the strain, stock cultures were meticulously maintained. These cultures were preserved on potato dextrose-agar at 4ºC and stored in glycerol at -20ºC for prolonged periods, ready for utilization as needed. Furthermore, a fresh spore suspension was arranged from cultures grown on potato dextrose-agar medium, and an incubation period of 5 days at 30°C. The prepared solution consisted of peptone (3g/l), NaCl (5g/l), and Tritonx100 (1mL/l). The spore count in the resulting suspension was determined using a Neubauer counting chamber.

2.3. Fermentation process of Aspergillus tubingensis FSS117

BaoXing Bio-Engineering Equipment Co.,Ltd. and produced enzyme solutions; The fermentation substrate was prepared with the following ingredients in a 20L bioreactor provided by (BXBIO): 40.0g cotton cake, 5.0g yeast extract, 8.0g Na2HPO4·2H2O, 0.5g KCl, 20.0g. 0.15g MgSO4 7 H2O· the pH was carefully adjusted to 7 and heated at 120ºC for 30 minutes.

Fresh fungal spores (150 mL, 37×10^8 spores/mL) were added to the bioreactor for incubation. The fermenter temperature was maintained at 30ºC, with rousing speed of 200 rpm, for 8 days.

After fermentation, the solution was filtered and subsequently centrifuged at 6000rpm for 10 minutes. The supernatant gained was identified as the enzymatic solution, and the precipitate was gathered as a waste of fermentation, preserved at 4 °C for use in experiments. Tests were carried out on the fermentation waste to determine activating levels of different hydrolytic enzymes. This approach provides a solid basis for exploring the hydrolytic enzyme production capabilities of *Aspergillus tubingensis* FSS117.

3.Experiment design

In accordance with design of a completely randomized, an experiment was conducted with five distinct treatments, each replicated three times. The experimental setup involved the utilization of 10 petri dishes (12 cm x 2.5) allocated to each treatment, representing varied diet compositions: C (Diet before Fermentation), D (Wheat bran), W (Wheat bran + Fermentation diet 50%), F (Fermentation diet), and P (Poultry). Fifty larvae, aged 15 days, were assigned to each treatment for feeding.

Data collection encompassed durations (in days) of larval, pupal, and adult periods, in addition to measurements of weight (mg) and length (mm). A subset of N=25 individuals was randomly selected for each treatment, and larvae and adult specimens were subjected to a one-hour exposure at 4ºC to facilitate measurements. High-resolution images of larvae were captured using a HUAWEI- nova (11) camera with a resolution (2652 x 1200 pixels). Subsequent measurements of larval, pupal, and adult length were performed using Image J software (version 1.8.0). For the determination of mean larval duration, the calculation included the initial 15 days of larval age plus the time required for 50% of larvae to undergo pupation. For each diet, pupal stage duration was calculated from the time of pupal emergence to the time of adult emergence. Likewise, the duration of the stage of adult to each diet was computed from adult emergence to death. Measurements were conducted at the pre-pupal stage, characterized by larvae assuming a ºC shape and ceasing feeding. Pupal measurements were taken upon the manifestation of dark color spots, indicative of mature pupae. Adult size was measured on day 12 when the adult reached full growth, and the mean adult size was subsequently calculated.

3.1.Enzymatic hydrolysis activities assay in fermented waste of Aspergillus tubingensis FSS117

The enzymatic hydrolysis activities of xylanase, amylase, lipase, protease, pectinase, carboxymethyl cellulase (CMCase), and filter aperase were evaluated using colorimetric methods as described in several studies (Bakri et al., 2003; Bakri et al., 2014; Bakri et al., 2015; Murachi 1970; Bakri et al., 2022; Marđetko et al. 2021; Akeed et al., 2022). For each enzyme, substrate and standard curve chains were prepared. All enzymatic reactions were conducted under the conditions of 40°C and pH 7.

3.2 Statistical analysis

In all statistical analyses, a P value greater than 0.05 is considered statistically significant (Landau et al., 1999). Stat View statistical software (version 5.0; SAS Institute, Cary, North Carolina, USA) was used (Landau et al., 1999). To determine statistical significance of differences in means and percentages, the ANOVA-Tukey HSD test was used to analyze length, weight and mortality data. Larval mortality was calculated as a percentage of total dead larvae by counting dead larvae in each treatment: larval mortality % = (total dead larvae) ×100.

4.Results

4.1. Effects of various diet treatments on the larvae, pupae, and adult length of T. molitor

Figure 1 illustrations that there are a significants effect on measurement of the MY stages (larvae, pupae, adults) in all treatment diets ($df = 4$, $f = 252.717$, $p < 0.001$; $df = 4$, $f = 50.449$, $p < 0.001$; $df = 4$, $f = 100.704$, $p < 0.001$). There are no significands difference between poultry feed and feed fermentation for each insect phase. (Larval and pupa) lengths had the highest values in together treatments (19.7–20.3 mm, 18.4–19.2 mm, and 19.6–20.1 mm, respectively). In distinction, pupae and larvae exposed to the treatment diet exhibited least length in adults (11.7 mm, 14 mm, and 14 mm, respectively). In contrast, pupae and larvae exposed to the treatment diet (11.7 mm, 14 mm, and 14 mm, respectively) exhibited minimum length. Significant differences were observed between the wheat-bran and fermentation diet (50%) with wheat-bran and compared to other treatments, impacting larval, pupal and adult length (12.8-16.4 mm, 14.9-16.7 mm, 15.7-17.8 mm, respectively).

4.2.Effects of various diet treatments on the larvae, pupae, and adult weight of MY T. molitor

In response to different diet treatments, larvae, pupae, and adult *T molitor* weight significantly changed ($df = 4$, $f = 214.610$, $p < 0.001$; $df = 4$, $F = 330.365$, $p < 0.001$; $df = 4$, $f = 126.804$, $p < 0.001$). Figure 2 shows that the highest significant of mean weights for larvae, pupae, and adults were reported in fermented feed and poultry feed treatments (63.6-66.6 mg, 105.6-109.2 mg, 84.4-88.5 mg, respectively); and then brunt wheat and fermentation diet (50%) treatments decreased at (56-52.5 mg, 85.8-91 mg, 66.7-74.4 mg, respectively). Though, the lowest levels were detected for the diet before the fermentation method (51.6 mg- 84.6 mg- and 64.6 mg, respectively). The highest weights of larval , pupal and adults were observed in fermentation and poultry feed (63.6-66.6 mg, 105.6-109.2 mg, 84.4-88.5 mg, respectively) and then, the wheat-bran and (50%) fermentation diet treatments (56-52.5 mg, 85.8-91 mg, 66.7-74.4 mg, respectively), and the diet before fermentation treatment (95.6 mg, 84.6 mg, 64.6 mg, respectively).

4.3.Effects of various diet treatments on the larvae, pupae, and adult duration times of MY T. molitor

Its notables that there is a differed significantly in the duration times of mealworm stages (larvae, pupae and adults) between all diet treatments ($df = 4$, $f = 145.608$, $p < 0.001$; $df = 4$, $f = 109.609$, $p < 0.001$; $df = 4$, $f = 193.017$, $p < 0.001$) (Figure 3). In the feed treatments of the fermentations waste and poultry feed, shorter feeding times for larval and pupal were reported (70.8–66.9 days, 14.3–13.4 days, respectively). In contrast, the wheat bran and the fermentation diet (50%) treatments showed longer but noteworthy durations at 84.8-88.4 days and 22.5-19 days, respectively, compared to the diet before fermentation treatment (92.9-24.2 days, respectively). Conversely, the longest duration for adults was in the fermentation diet and poultry feed treatments at 125.3-130.4 days, followed by the wheat-bran and fermentation diet (50%) with wheat bran treatments (102.8-115.6 days). The shortest duration was observed in the diet before fermentation treatment (98.8 days).

4.4.The effect of different treatments on the larvae mortality

Figure 4 demonstrated that there were significant differences between the diet before fermentation

treatment and other treatments which did not exhibit significant differences (df = 4, f = 168.659, $p < 0.001$). It is also noteworthy that no larval mortality was observed in wheat-bran, which means that wheat bran was completely safe, and did not negatively impact the larvae stage of *T molitor*.

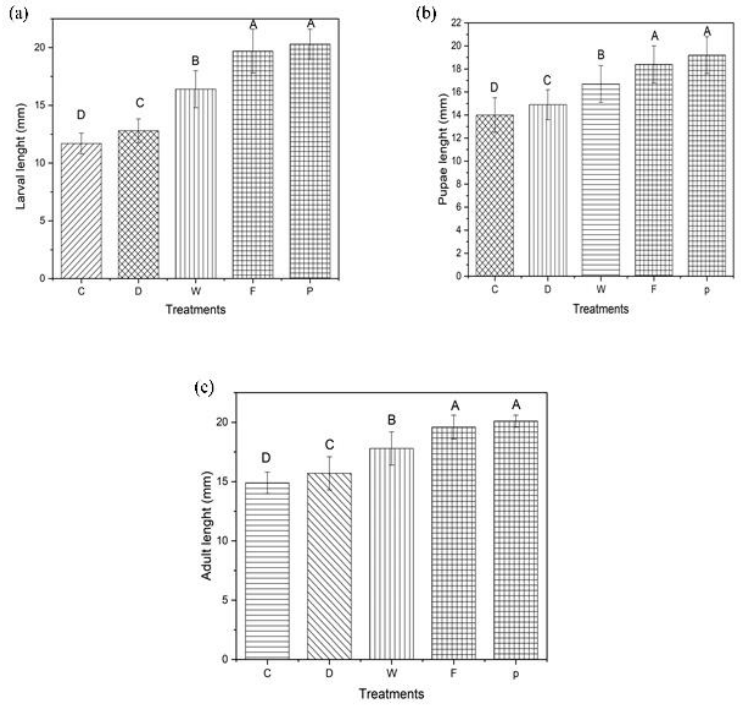

Figure 1. The effect of different treatments on the length (mm) of (a) larvae, (b) pupae, and (c) adults of *T. molitor*. C: Diet before Fermentation**;** D: Wheat bran; W: Wheat bran+ Fermentation diet 50%; F: Fermentation diet;P: Poultry feed**.** Means followed by different capital letters above the bar graph are significantly different at P <0.05 (Tukey HSD test).

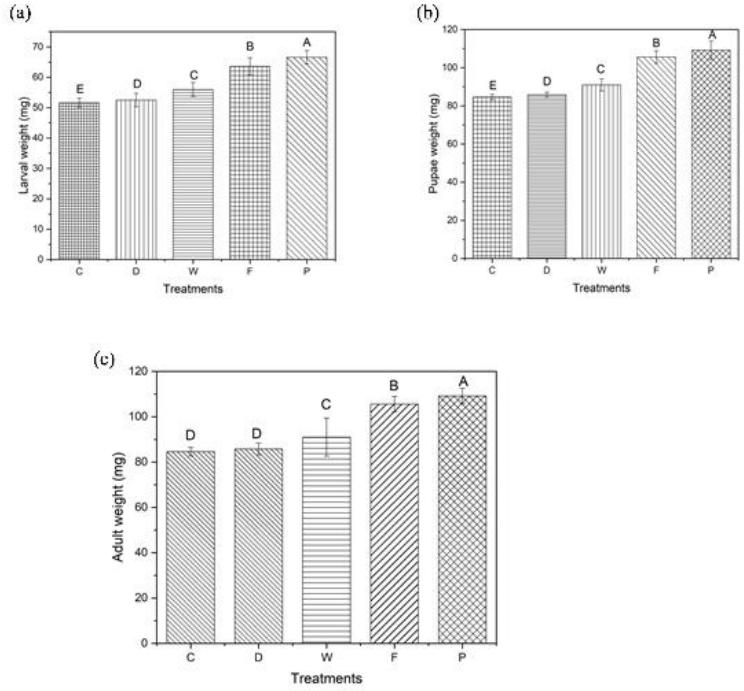

Figure 2. The effect of different treatments on the weight of larvae, pupae, and adults of *T. molitor*. C**:** Diet before Fermentation; D: Wheat bran; W: Wheat bran+ Fermentation diet 50%; F: Fermentation diet;P: Poultry feed**.** Means followed by different capital letters above the bar graph are significantly different at P <0.05 (Tukey HSD test).

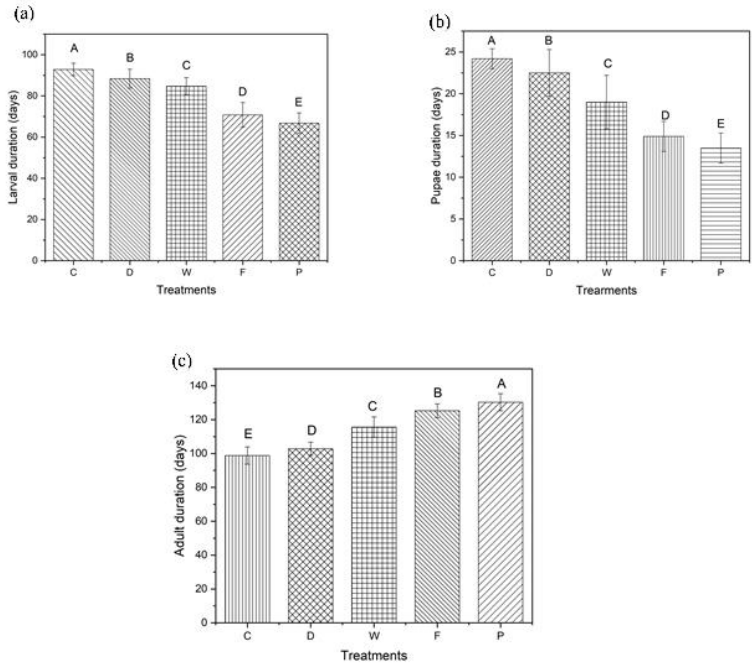

Figure 3. The effect of different treatments on the duration of larvae, pupae, and adults of *T. molitor*.C: Diet before Fermentation; D: Wheat bran; W: Wheat bran+ Fermentation diet 50%; F: Fermentation diet; P: Poultry feed. Means followed by different capital letters above the bar graph are significantly different at P 0.05 (Tukey HSD test)

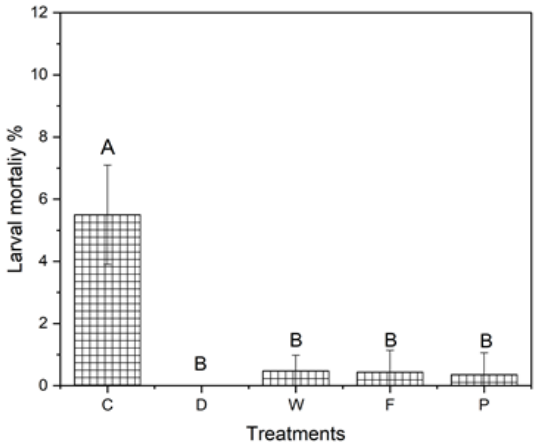

Figure 4. The effect of different treatments on the larvae mortality of T. molitor.C: Diet before Fermentation; D: Wheat bran; W: Wheat bran+ Fermentation diet 50%; F: Fermentation diet;P: Poultry feed. Means of percentages followed by different capital letters above the bar graph are significantly different at P <0.05 (Tukey HSD test)

4.5.Enzyme's activities in the fermented waste of Aspergillus tubingensis FSS117

Seven enzymatic assays were done to know how many enzymes the fermentation waste has. These enzymes were: lipase, carboxymethyl-cellulase, filter-paperase, pectinase, phytase, xylanase and amylase (Table 1).

Table 1. Enzyme's activities in the fermented waste of *Aspergillus tubingensis* FSS117

Enzyme	Activity (U/g)
Xylanase	215.3
CMCase	4.5
Filterpase	1.13
Pectenase	1
Amylase	1.3
Lipase	10.5
Protease	0.19

5.Discussion

YM (*Tenebrio molitor*) is not only commonly used but also economically important feed source for livestock and aquaculture [16]. However, the traditional diet for mealworms, consisting of cereal grains and oilseed products, is being challenged by the need for more sustainable and cost-effective alternatives [18, 36]. As a result, the exploration of alternative feed sources has grown in popularity that can capture more nutrients in waste products; organic wastes can therefore be a valuable source of nutrients for mealworms [28, 17]. It was found that the growth of mealworm in relations of length and weight was considerably higher when they feed on fermented cotton cake by *Aspergillus tubingensis* FSS117 comparing to non-fermented of same substrate (Figs 1, 2). The period duration of larvae and pupae in F treatment were significantly briefer than D treatment; however, adults' period was significantly longer in F treatment comparing to D treatment (Fig 3). These findings are in line with the reported studies that showed when a different amount of nutrient diet such as proteins, fatty acids, and carbohydrates applied to larvae contributed to significant changes in insect growth parameters, an increase in length and weight, for example for all insect stages, along with a reduction in larvae and pupae duration [33, 37]. As stated by Riaz et al., 2023, mealworm larvae that feed on yeast diet have shorter adult period despite the fact that it has a higher protein ratio (49%), resulting in increased lengths and weights throughout all stages. However, as opposed to what we found for treatment F that showed significantly longer adult period. Even though the yeast diet includes high protein, essential amino-acids, vitamins, and minerals, it contains very little fat, which may combine with other essential ingredients to build the adult mealworm's body structure [12, 10]. Nutrient-rich substrate can be provided for larvae after fermentation of cotton cake (the substrate for Strain *Aspergillus tubingensis* FSS117), just like other wastes from various fermentation processes. A variety of compounds, including proteins, carbohydrates and fats, can supply mealworms with energy besides essential nutrients [25, 30]. The experiment's findings demonstrate a notably higher larvae mortality rate in treatment D (cotton cake) compared to other treatments, as outlined in Fig 4. This increased larval mortality is associated with elevated level of Gossypol in the diet of treatment D (cotton cake).

Previous studies have reported that species within the Aspergillus genus, such as *A. niger* and *A. oryzae*, can effectively diminish free Gossypol concentrations in cotton-seed meal [34,41, 42]. Consequently, it is hypothesized that the strain *A. tubingensis* FSS117 may reduce the Gossypol concentration in treatment F (cotton cake) through the proces of fermentation. Furthermore, the study illustrated that the fermentation conducted by Strain *A. tubingensis* FSS117 on cotton cake led to the appearance of diverse enzymes, offering potential advantages for mealworm nutrition. The addition of enzymes has the capacity to augment protein content, amino acid profiles, and energy availability within the mealworm diet. This optimization, in consequence, has the potential to result in heightened biomass, decreased feed intake, and enhanced conversion efficiency. Our results reveled that, through the cotton cake fermentation process by strain FSS117, various enzyme types founded in the substant diet (Table 1). It is known that enzymes cleaving and digest the numerous bonds within cell walls are complex, protein and fat in the diet [9], these can be attributed to affect consuming and growth of larvae after the fermentation on the diet of cotton cake [11, 19,34]. These enzymes play a critical role in breaking down and digesting complex bonds within cell walls, proteins, and fats, thereby influencing larval consumption and growth. the Studies have shown that the addition of enzymes to the mealworm diet can increase the accessibility to the protein content, amino acid, and energy of the feed [24, 38]. Additionally, enzymes supplementations have been found to improve the conversions efficiency of feed into biomass by mealworms, resulting in increased body weight and reduced feed intake [14, 24]. Furthermore, the use of enzymes in MY farming can also contribute to sustainable and environmentally friendly livestock production. By optimizing the nutrient content and reducing the amounts of feed mandatory for mealworm production, the environmental footprint of animal feed production can also be reduced [39].

6.Conclusion

The current study delved into the uses of alternative feed sources, with a specific focus on fermenting cotton cake as a substrate for the strain *Aspergillus tubingensis* FSS117 in the nutritional context of MY (*Tenebrio molitor*). The findings demonstrated improved growth parameters and highlighted how variations in nutrient content influence insect growth.

Emphasizing the significance of the fermentations proces, the study highlighted its role in creating a nutrient-rich substrate for mealworms. The observed effects could be attributed to the diverse spectrum of crude enzymes present in the fermented cotton cake diet. Therefore, utilizing fermentations waste may provide a viable solution for optimizing the growth and cultivation of mealworms; In addition to providing a cost-effective and sustainable method for mass producing mealworms, it is also suitable for bioconversion, animal feed, and food production.

Compliance with Ethics Requirements. The authors declare that they comply with the Ethics requirements of the journal. The authors declare that they have no conflicts of interest and that all procedures involving human or animal subjects (if any) comply with specific regulations and standards.

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