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# Microbial and chemical analysis of independently produced batches of *Tenebrio molitor* larval powder

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A R T I C L E I N F O

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A B S T R A C T

Due to the continuous growth of the world population, European countries have recently declared *Tenebrio molitor* (mealworm) powder safe as an alternative protein source for humans. Despite the positive aspects raised about mealworm powder, its marketability faces challenges due to some safety concerns. There is a lack of profound insight into the microbial safety, chemical reactions during storage and shelf life of *Tenebrio molitor* larvae intended for human consumption in Zimbabwe. Mealworm powder can be contaminated by components such as heavy metals, mycotoxins and pesticides which usually occur during feeding and storage of finished products. The aim of this study was to investigate the presence of bacteria, fungi and aflatoxins in two independently produced batches of *Tenebrio molitor* larval powder quantitatively to confirm its safety. Culture dependent analyses were performed to determine the presence of fungi and bacteria. The potential spoilage bacteria and food pathogens found were then enumerated and characterised. Thin layer chromatography was used to analyse aflatoxins in the mealworm powder. Microbial analysis revealed the presence of *Aspergillus* species, *Rhizopus* species, *Rhodotorula* species, *Staphylococcus* species, *Enterobacteriaceae* and lactic acid bacteria (LAB) in the two independently produced batches of *Tenebrio molitor* larval powder. However, the microorganisms present were below levels that cause food poisoning. Aflatoxin analysis showed the presence of aflatoxin B1 at a lower value of 12 parts per billion in the sample compared to the FDA standards of 20 parts per billion. In conclusion, based on this study, *Tenebrio molitor* larval powder may be safe for human consumption as the microbial and aflatoxin concentrations were below poisonous levels.

## 1. Introduction

Several studies have established that entomophagy can be a viable substitute for traditional animal protein, as the rearing of insects is considered environmentally friendly. Insects also contribute to sustaining nature, adding diversity to human diets, and improving food security at the same time [6]. The consumption of insects is associated with minimal greenhouse gas emissions and low land usage. Additionally, edible insects like *Tenebrio molitor* can be consumed in their larvae or adult forms, with high feed conversion ratios, utilising less water, energy and land in their production, leading to a significant decrease in ammonia emissions [15].

However, consumers are concerned about the safety of edible insects, particularly regarding the presence of infectious microorganisms and harmful chemicals. A study on the risks related to entomophagy in the food and feed industries by the European Food Safety Authority (2015) found that the safety of edible insects depends on how they were reared and processed. Schabel. [23] concluded that factors such as the species of the insect, the feed they consume, their environment and production and processing methods play a crucial role in ensuring safety. Consumers are seeking assurance regarding the microbial safety of edible insects, especially in African countries where insects are often harvested from the wild [9].

One primary safety concern is allergies. Individuals who are insect intolerant may react to *Tenebrio molitor* larvae powder, especially those allergic to dust particles and shellfish [5]. Symptoms can include skin itching, swelling and breathing difficulties. *Tenebrio molitor* larvae also contain high concentrations of vitamin K which can interfere with medications like warfarin [24]. The high dietary fiber content in mealworm powder may cause digestive issues like diarrhea and bloating, so it is advisable to start with small amounts until the body adjusts [13].

Contamination is another concern with mealworm powder, as it can

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be affected by pesticides, heavy metals and mycotoxins [14]. Mycotoxins, toxic plant metabolites produced by fungi, such as aflatoxins and fumonisins, can contaminate mealworm powder in various ways [25]. Beetles feeding on contaminated agricultural commodities can transfer toxins to mealworm powder, and poor storage conditions can also lead to contaminations [26]. Microbial risks are significant safety issues with *Tenebrio molitor* as the sanitary conditions of the insects may not always be optimal due to lack of information, control and regulation [12]. Factors like cultivation, processing, storage conditions, and insects' characteristics all play a role in microbial safety. To ensure the safety of insects and their products, processing and storage conditions should adhere to health and sanitation guidelines like any other food product [2].

Therefore, the main objective of this study was to investigate the presence of pathogenic microorganisms and aflatoxins in two independently produced batches of dried *Tenebrio molitor* larval powder, intended for human consumption in the Zimbabwean market.

## 2. Materials and methods

### 2.1. Sample preparation

The Department of Biotechnology and Biochemistry at the University of Zimbabwe provided two independently produced batches of *Tenebrio molitor* larval powder labeled A and B. Each gram of samples labeled A and B was diluted with 9 ml of distilled water in sterile 15 ml Falcon tubes, then stored at 4 ºC until use.

### 2.2. Microbial determination

The presence of fungi in samples A and B of *Tenebrio molitor* larval powder was determined by aseptically inoculating 100 µl of each sample using the pour plate technique onto Sabouraud Dextrose Agar (SDA) plates, following the method by [10]. The inoculated agar plates were incubated at 37 ºC for 5 days to allow fungi to grow. *Staphylococcus* species presence was determined by aseptically inoculating 100 µl of each sample using the pour plate technique onto Mannitol salt agar (MSA) plates and incubating at 37 ºC for 24 hours as described by [19]. For *Enterobacteriaceae* species, 100 µl of samples A and B were aseptically transferred onto Eosin Methylene Blue (EMB) agar plates and incubated at 37 ºC for 24 hours following the method by [1]. DeMan, Rogosa and Sharpe (MRS) agar was used to determine the presence of lactic acid bacteria (LAB) as described by [20]. The MRS plates were incubated at 37 ºC for 24 hours.

### 2.3. Methylene blue staining

Methylene blue staining was used for further identification of the species detected in the *Tenebrio molitor* larval powder, following the method by [22]. Using a bacteriological loop, a loopful of culture was aseptically transferred onto a clean, grease-free and dry slide. The culture was mixed with a drop of aqueous methylene blue, then smear was made using a microscope glass cover. The smear was allowed to air dry slowly, the examined systematically under the 10 × objective lens, with photographs taken.

### 2.4. Aflatoxin determination

Aflatoxin analysis was carried out according to [21] using the TLC procedure consisting of seven steps: pre- treatment, pre- concentration, separation, evaporation, spotting, plate development, visualization and quantification.

### 2.5. Pre-treatment step

*Tenebrio molitor* larval powder samples A and B were thoroughly mixed, and 50 g of the mixture was weighed and placed in a console jar.

### 2.6. Pre-concentration step

Approximately 100 ml of 70 % methanol was added to the sample as the extracting solvent. Sodium chloride was then added to facilitate aflatoxin extraction from the sample. Defatting was done by adding a nonpolar reagents hexane and by petroleum ether (ACS grade). A high-speed blender was used to for sample homogenization to further facilitate aflatoxin extraction.

### 2.7. Separation

An aliquot of the sample was pipetted into a separating funnel. Chloroform was added to the sample solution, and shaking was done to mix them. Filtration was then done to separate the organic and inorganic portions. Anhydrous sodium sulphate was added to the organic portion to remove any remaining moisture.

### 2.8. Evaporation

The sample was evaporated on a rotary vapor until dry, then reconstituted using chloroform.

### 2.9. Spotting

The TLC plate was oven dried for 10 minutes to activate it. A micro syringe was used to spot aflatoxin standards against the sample. Different volumes of the standard solution were also spotted on the plate to help determine aflatoxin concentrations in the sample.

### 2.10. Plate development

Two plate development stages were done

#### 2.10.1. First development stage

The TLC plate was placed in a tank with petroleum ether as the first developing solvent, ensuring the markings were not dipped. The plate was then laid flat in the tank for even spot ascension, and air drying was done to remove impurities.

#### 2.10.2. Second development stage

The development solvent used was chloroform acetone. This allowed for migration of both the standard solutions and the sample up the TLC plate.

### 2.11. Visualization and quantification

After drying up, the plates were then viewed under UV light at 360 nm. Confirmatory tests were done by spraying the sample with dilute sulphuric acid. Results were recorded and calculations were done using the formula

$$C \times (S1/S2) \times (V/W) \times 1000$$

Where C is the concentration of the aflatoxin standard
S1 is the volume of the sample equivalent to the standard
S2 is volume of the sample spotted

V. is the dilution factors
W. is the effective weight

**Table 1**
Fungi counts obtained for samples A and B of Tenebrio molitor larval powder.

| Fungi | Amount detected in sample A (log colony-forming units/g) | Amount detected in sample B (log colony-forming units/g) | Legislated limit (log colony-forming units/g) |
|---|---|---|---|
| *Aspergillus* species 1 | 2.5 ± 0.4 | 2.0 ± 0.64 | 3.5 – 5.6 |
| *Aspergillus* species 2 | 2.2 ± 0.12 | 1.9 ± 0.2 | 3.5 – 5.6 |
| *Rhizopus* species | 2.0 ± 0.15 | 1.5 ± 0.22 | 3.5 – 5.6 |
| *Rhodotorula* species | 1.8 ± 0.06 | 1. 0 ± 0.18 | 3.5 – 5.6 |

**Table 2**
Bacterial counts obtained for samples A and B of the Tenebrio molitor larval powder.

| Bacteria | Amount detected in sample A (colony-forming units / g) | Amount detected in sample B (colony-forming units / g) | Legislated limit (colony-forming units / g) |
|---|---|---|---|
| *Staphylococcus* species 1 | $6.0 \times 10^2 \pm 2.0 \times 10^1$ | $5.0 \times 10^2 \pm 3.0 \times 10^1$ | $< 10^3$ |
| *Staphylococcus* species 2 | $2.7 \times 10^1 \pm 0.1 \times 10^1$ | $1.9 \times 10^2 \pm 0.7 \times 10^1$ | $< 10^3$ |
| *Enterobacteriaceae* | $1.5 \times 10^1 \pm 0.8 \times 10^0$ | $1.7 \times 10^1 \pm 0.4 \times 10^1$ | $10^2$ - $< 10^4$ |
| Lactic acid bacteria | $1.0 \times 10^2 \pm 5.0 \times 10^0$ | $1.2 \times 10^2 \pm 3.0 \times 10^1$ | $< 10^8$ |

## 3. Results

### 3.1. Enumeration of fungi and bacteria in samples A and B of the Tenebrio molitor larval powder

Based on the morphological characteristics observed on the plates, two different species of *Aspergillus*, *Rhizopus* and *Rhodotorula* were identified in the two samples of *Tenebrio molitor* larval powder. The microbial counts for the identified fungi are presented in Table 1. The counts obtained were compared with the legislated limit for minced meat or raw materials for meat preparation and were all found to be below the legislated limit.

Based on the morphological characteristics observed on the plates, two different species of *Staphylococcus*, *Enterobacteriaceae* and Lactic acid bacteria were identified in the two samples of *Tenebrio molitor* larval powder. The microbial counts for the identified bacteria are presented in Table 2. The counts obtained were compared with the legislated limit for minced meat or raw materials for meat preparation and were all found to be below the legislated limit.

## 4. Identification of the fungi and bacteria in samples A and B of the *Tenebrio molitor* larval powder

Further identification of fungi and bacteria was conducted using methylene blue reagent and microscopic images were captured. Figs. 1 and 2 display some of the microscopic images of both fungi and bacteria.

### 4.1. Aflatoxins detection

The TLC plate used for aflatoxin detection was visualized using UV light at 360 nm, and the results are shown in Fig. 3. A comparison of spot intensity between the standards and the sample was conducted, revealing the presence of aflatoxin B1. Following calculations, the total concentration of aflatoxin B1 in the *Tenebrio molitor* larval powder sample was determined to be 2 parts per billion (ppb)

360 nm. Key: line S represent the sample, line (1, 2, 3 and 4) represent aflatoxin standard of different volume (1, 2, 3 and 4 μ g/ml) 2)

## 5. Discussion

This study focused on the microbial and chemical analysis of independently produced batches of *Tenebrio molitor* larval powder to confirm its safety for human use as a novel food source.

For microbial analysis, the study aimed to determine the presence or absence of fungi and bacteria in two separate batches of *Tenebrio molitor* larval powder. Like other organisms, *Tenebrio molitor* larvae tend to harbor a complex variety of microbes, including bacteria, archaea, fungi, protozoa and viruses [17]. However, the counts obtained in this study for all the fungi species ranged from 1 to 2.5 log CFU/g in samples A and B of the *Tenebrio molitor* larval powder, which were found to be below the legislated limit for molds in minced meat or raw material for meat preparation. The legislated limit for molds in minced meat is between 3.5 and 5.6 log CFU/g [8]. Therefore, the amounts of fungi detected in *Tenebrio molitor* larvae do not pose threats for fungi-related health implications. It should also be acknowledged that edible insects such as *Tenebrio molitor* larvae are cooked or processed before consumption, which has been found to significantly decrease the microbial load [3]. For all the bacterial species identified in samples A and B of the *Tenebrio molitor* larval powder, the counts obtained were found to be below the legislated limit for bacteria in minced meat or raw material for meat preparation. The counts obtained in this study ranged from $1.5 \times 10^1$ to $6.0 \times 10^2$ CFU/g and the acceptable range for bacteria is less than$10^3$ CFU/g up to $10^8$ CFU/g. The counts obtained in this study are sufficiently low and do not present a hazard for bacterial food poisoning. These findings are in agreement with the study conducted by Banjo et al. [4] which established that insects can harbor various types of disease causing microorganisms.

For chemical analysis, the study aimed to determine the presence or absence of aflatoxins in two separate batches of *Tenebrio molitor* larval


**Fig. 1.** Microscopic images of fungi identified in the *Tenebrio molitor* larval powder.

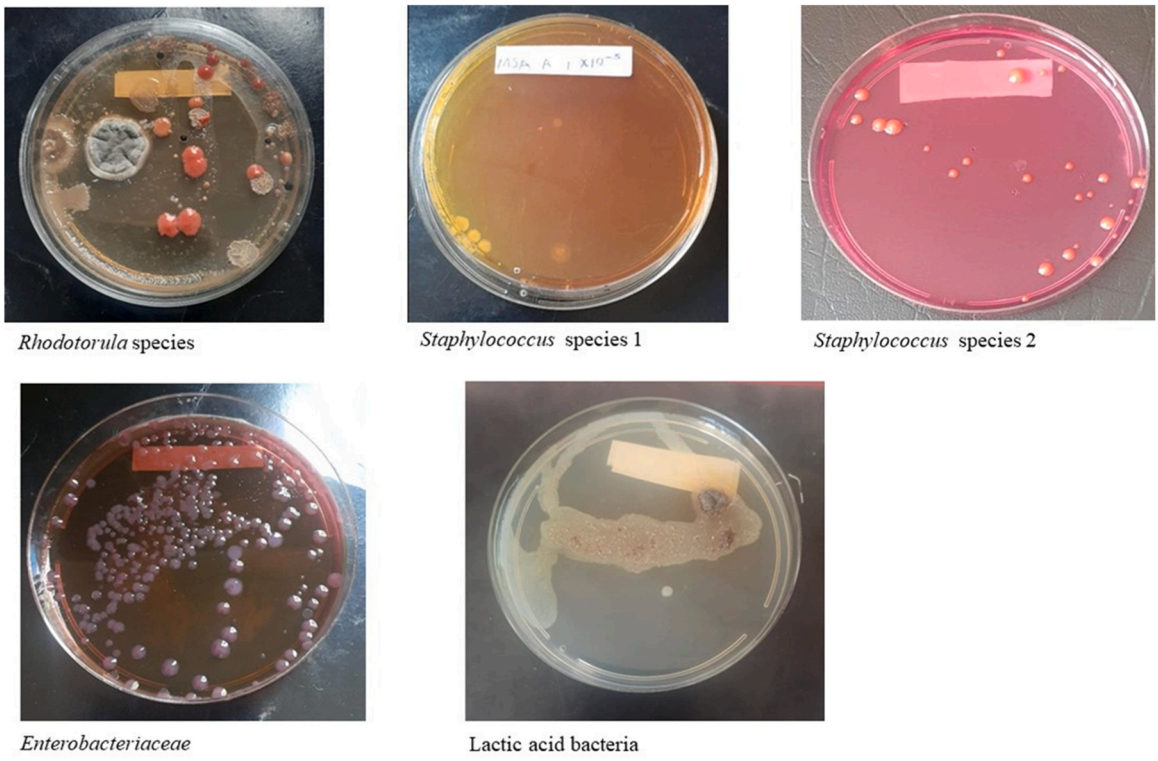


**Fig. 2.** Colony morphology images of bacteria and fungi identified in the *Tenebrio molitor* larval powder.

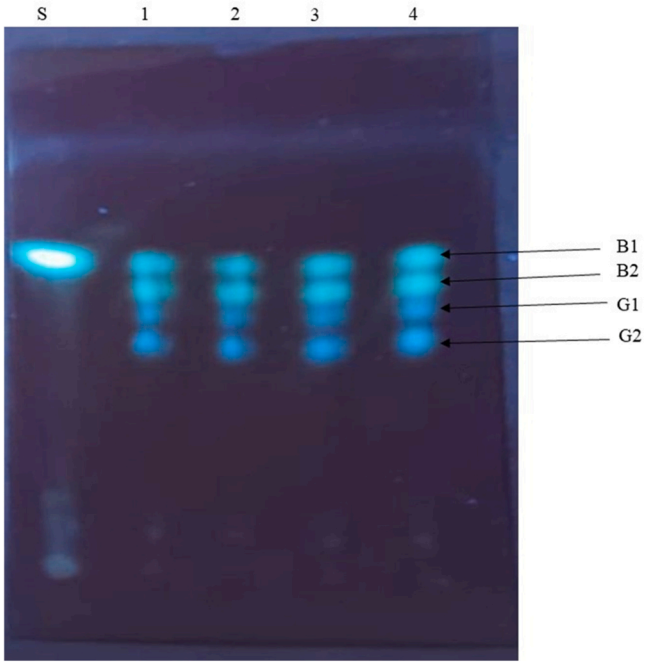


**Fig. 3.** TLC plate results after spraying with sulfuric acid and visualization using UV at.

powder. Aflatoxins are life threatening biochemicals produced by *Aspergillus flavus* that contaminate many food substrates. Aflatoxins have genotoxic, hepatotoxic, mutagenic, teratogenic and carcinogenic effects [18]. The occurrence of aflatoxins in infant foods results in growth impairment, stunting and underweight [11]. Aflatoxin analysis was carried out in this study using Thin Layer Chromatography (TLC). However, the *Tenebrio molitor* larval powder was found to contain aflatoxin B1 at a concentration of 12 ppb which according to the FDA is below the minimum limit of 20 ppb. Any level beyond that is considered toxic and unsafe for human consumption [27].

The safety of *Tenebrio molitor* has also previously been confirmed by determining the sub-chronic toxicity of powdered *Tenebrio molitor* larvae on Sprague-Dawley rats [16]. Findings in this study suggested *Tenebrio molitor* larvae to be safe for human consumption as it did not appear to affect the rats' normal physiological and metabolic processes. However, in another study, it has been reported that exposure to *Tenebrio molitor* larvae can induce primary sensitisation and may lead to food and inhalant allergy [7]

Thus, the combination of the current results and these previous results suggests that *Tenebrio molitor* larvae may be safe for human use. However, further studies such as clinical trials may be necessary to confirm these current results.

## 6. Conclusion

The *Tenebrio molitor* larval powder samples A and B that were analysed were found to contain insignificant amounts of fungi, bacteria and aflatoxin B1 when compared to the legislated limits. Therefore, when considering the sub-chronic toxicity effects of *Tenebrio molitor* larvae in the literature, these results suggest that it is safe for human consumption. However, further studies such as clinical trials may be necessary to confirm these current results.

## CRediT authorship contribution statement

**Rumbidzai Mangoyi:** Writing – review & editing, Writing – original draft, Validation, Supervision. **Oleen Machona:** Validation, Investigation. **Muzunze Christine Mitchell:** Investigation. **Maduviko Pepertual:** Investigation. **Farisai Chidzwondo:** Writing – review & editing, Validation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

No data was used for the research described in the article.

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