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Fishmeal-Based Media Supports Growth and Endospore Production of Locally-Isolated *Lysinnibacillus sphaericus* and Induces its Toxicity to 3rd Instar *Aedes aegypti* Larvae in Laboratory Conditions

Bambang Fajar Suryadi[1*], Ika Mustika[1], Zuriatun Annisa[1], Sarkono[1], Galuh Tresnani[1]

[1] Program Studi Biologi, FMIPA, Universitas Mataram, Mataram, Indonesia.

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Corresponding Author:
Bambang Fajar Suryadi
bambangfajar@unram.ac.id

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Abstract: The aim of this study was to determine whether fishmeal-based media could be used to grow *L. sphaericus* and induce its toxicity against *Aedes aegypti* larvae. Three concentrations (10, 20 and 30%) of fishmeal-based media were used to grow *L. sphaericus* isolate Bs2-1-2. Cell growth and endospore production were observed every 12 hours for 72 hours. The lethal concentration was measured every 24 hours for 72 hours of fermentation. The highest cell concentration was found in L. sphaericus grown on the media with 30% fishmeal concentration ($3.03x10^{08}$ cells/mL), followed by 20% concentration ($2.43x10^{08}$ cells/mL) and the lowest at 10% concentration ($2.20x10^{08}$ cells/mL). At the end of fermentation, the highest concentration was found in L. sphaericus grown on 30% fishmeal-based media ($1.51x10^{08}$ cells/mL), followed by 20% media ($6.95x10^{07}$ cells/mL) and 10% media ($3.21x10^{07}$ cells/mL). After 72-hour incubation, the highest endospore concentration was achieved by *L. sphaericus* grown on 20% ($2.51x10^{08}$ cells/mL) and 10% ($2.19x10^{08}$ cells/mL) fishmeal-based media. Initial larval toxicity of *L. sphaericus* showed the highest mortality on 20 and 30% fishmeal-based media (both reaching 53.33%), while 10% fishmeal-based media gave only 26.67% larval mortality. The LC50 value at 72 hours was achieved by *L. sphaericus* cultured on 30% fishmeal-based media (2.47 x 10*08* cells/mL), followed by 20% concentration (4.82 x 10^{08} cells/mL) and 10% concentration (9.01 x 10^{09} cells/mL). The conclusion of this study was all concentrations of fishmeal-based media could support cell growth, endospore production and larval toxicity induction of *L. sphaericus*.

Keywords: Fishmeal-based media; *Lysinibacillus sphaericus*; 3rd-instar *Aedes aegypti* larvae

Introduction

The eradication of diseases transmitted by mosquitoes, despite the availability of effective control measures, remains a challenging objective, as evidenced by the continued prevalence of dengue fever and malaria in Indonesia (Dalilah et al., 2022; Agustina, 2015).

A variety of mosquito control methods have been employed globally, including in Indonesia. The most effective approach is to reduce the breeding environment, which can be achieved through the removal of rubbish and the elimination of stagnant

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water. Additionally, the use of environmentally safe biological insecticides, such as fish, bacteria and fungi, can be employed. Finally, public education plays a crucial role in the prevention and control of mosquito-borne diseases (The US Environmental Protection Agency, 2023; Astuti et al., 2023; Judijanti et al., 2024).

One new method in suppressing the spread of mosquitoes in environment is by using entomopathogenic bacteria. The use of entomopathogenic bacteria not only avoids the problems of chemical contamination of the environment, but also avoids the killing of non-target insects (with the exception of mosquitoes) in the environment (Benelli et al., 2016; Da Silva et al., 2020). One bacteria species that has ability to kill larvae is *Lysinnibacillus sphaericus* (formerly known as *Bacillus sphaericus* (Ahmed et al., 2007; Couret et al., 2020). This bacterium was first discovered in 1964 near Fresno, California (Kellen et al., 1965) and several researchers have isolated and tested its toxicity characteristics from many places in the world (Park et al., 2010; Rahman et al., 2012).

L. sphaericus was initially reported kill only 2 genera of mosquito larvae, namely Culex spp. and Anopheles spp., but several reports have shown its toxicity to *Aedes aegypti*. Because of this ability, *L. sphaericus* has also been used as a biopesticide to complement existing mosquito eradication methods (Berry, 2012; Santana-Martinez et al., 2019).

A unique characteristic found in this bacterium is its inability to metabolize carbohydrates in its growth media. This bacterium uses protein and other substances to support its growth and metabolism. Therefore, high protein content material is significant for designing natural/alternative media for *L. sphaericus* (Russell et al., 1989; El-Bendary, 2010).

One natural ingredient that contains high levels of protein is fishmeal. The protein content of fishmeal can reach 60-72%. In addition to protein, fishmeal also contains fat (4-20%) and minerals (10-12%) (Cho & Kim, 2011). Fishmeal is an abundant fish by-product affordable in price and widely available in the market (Mathai et al., 1985; Gamit et al., 2023).

To the best of our knowledge, fishmeal (derived from both marine and freshwater fish) has not been documented as a component in the growth of *L. sphaericus*, which is employed as a biopesticide. Hence, fishmeal can be potentially utilized as an alternative media to grow bacteria including *L. sphaericus*.

The aim of the study is to observe whether fishmeal-based media can be used to grow *L. sphaericus* and stimulate its toxicity against 3rd instar *Ae. aegypti* in laboratory condition.

Method

Bacterial Preparation. **T**he isolate of *L. sphaericus* bacteria used in this study was *L. sphaericus* isolate Bs2-1-2 isolated by Dewi et al. (2022) in the sewage in one of the housing complexes in Mataram City. *L. sphaericus* was grown on Nutrient Agar solid media (Oxoid, UK) with standardized concentration supplemented with Streptomycin antibiotic (30 µg/mL) at 33°C. Before further use in this study, the *L. sphaericus* isolate Bs2-1-2 was characterized for colony and cell morphology, as well as physiological and biochemical characteristics to ensure there was no contamination on it.

Media Preparation. In this study, a natural media was used in the form of fishmeal mixed with water in several concentrations, namely 10%, 20% and 30%. The mixture of media components is presented in Table 1. Control was made using Nutrient Broth liquid media (Oxoid, UK)

Table 1. Natural Growth Media Components Based on Fishmeal

Percentage (%)	Amount of fishmeal	Amount of water (added until)
10	50 grams	500 mL
20	100 grams	500 mL
30	150 grams	500 mL

After mixed with water evenly, this mixture was heated and allowed to boil for 3-5 minutes. During heating, the final volume was kept at 500 mL. The test media was transferred into an erlenmeyer tube and sterilized with an autoclave at 121°C 2 atm for 30 minutes. For control media, 500 mL of Nutrient Broth liquid media (Oxoid, UK) with standard concentration was prepared.

Mosquito Larvae Preparation. *Ae. aegypti* eggs were immersed in sterile distilled water for 24 hours under 12 dark and 12 light conditions at 25°C (Dulmage et al., 1990; Imam et al., 2014). After hatching, larvae were fed with mashed sterile dry dog/cat food. The larvae of *Ae. aegypti* are then reared until they reach the third instar (larvae reach a length of 4-5 mm). Upon reaching instar III, *Ae. aegypti* larvae are ready to be used for testing.

Bacterial Fermentation and Endospore Production. This method was based on the standard method (Doğan et al., 2020) with slight modifications. One full loop of bacterial colony was taken from a single colony of *L. sphaericus* that had been grown for 24 hours and put into 5 mL of sterile distilled water to calculate the concentration using the Improved Neubauer counting chamber. The initial concentration was determined at 10^6 cells/mL before fermentation. Dilution or concentration can be done as necessary. Fermentation was carried out for 72 hours at 33°C with 200 rpm shaking. Observations

of cell and endospore concentrations were made every 12 hour for 72 hours.

Quantitative Bioassay. The Bioassay was carried out to obtain LC value. This procedure was applied using WHO standardized procedure (Dulmage et al., 1990; EFSA Panel on Additives and Products or Substances used in Animal Feed/FEEDAP, 2014). For each combination of test media, 15 testing containers were prepared, consisting of 5 concentrations of testing media with 3 replicates. Each container was filled with 200 mL of test media with 10 larvae. The same was done for 4 other test media and 1 standardized media (as a comparison). In addition to the test larvae, three containers (as negative control) containing sterile distilled water and 10 larvae in each container were also prepared. Observation of mortality was observed for 3 days of testing (24, 48 and 72 hours).

The mortality rate of tested larvae at each concentration of testing media and standard media were calculated using this formulation (Abbott, 1925; Dulmage et al., 1990; Rahardianingtyas & Wianto, 2014).

$$Observed\ mortality = \frac{Total\ number\ of\ dead\ larvae}{Total\ sample\ size}\ X\ 100\% \quad (1)$$

If larval death in the control group exceeds 20%, the experiment should be repeated. However, if larval death in control is less than 20%, the larval mortality value should be corrected using corrected mortality formulation (Abbot, 1925; Dulmage et al., 1990).

$$Corrected\ mortality = \frac{\%\ observed\ mortality - \%\ control\ mortality}{(100\% - \%\ control\ mortality}\ X\ 100\% \quad (2)$$

The mortality rate of larvae in the test media and standard media was used to find LC (lethal concentration) 50 and 90. LC calculations were carried out using Probit Analysis (Finney, 1971; Dulmage et al., 1990). Larval mortality from 3 different concentration of fishmeal-based media in 72 hours (the end of the incubation) were subjected to two-way ANOVA with α = 0.05. Post-hoc tests were performed using the Tukey (HSD) method. Flowchart of the procedure is presented in Figure 1.

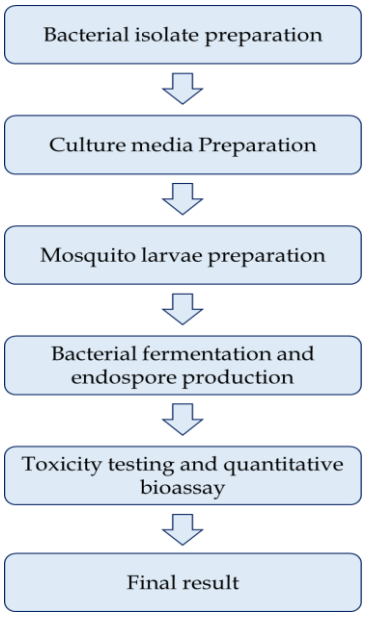

Figure 1. Flowchart of the procedure used in this study

Result and Discussion

L. sphaericus isolate Bs2-1-2 was isolated by Dewi et al. (2022) in a drain in one of the housing complexes in Mataram, West Nusa Tenggara. This isolate was the most toxic isolate when tested on A. *aegypti* instar 3 larvae in the laboratory. The Bs2-1-2 isolate characteristic is presented in Table 2 as follows.

Table 2. Colony, Cell, Physiological and Biochemical Characteristics of Isolate Bs2-1-2 Used in this Study (Source: Personal Research Data)

Characteristics	Isolate Bs2-1-2
Colony	
Form	Circular
Margin	Entire
Surface	Smooth
Color	Cream
Translucency	Non-translucent
Single colony diameter in 24-hour culture	2-4 mm
Cell	
Form	
Gram staining	Bacil (Rod)
Endospore	Positive
Endospore position	+ (Exist)
Swollen sporangia	Terminal
	+ (Exist)

Characteristics	Isolate Bs2-1-2
Physiological	
Growth on	
30°C	positive
40°C	positive
50°C	negative
Growth on NaCl concentration	
3%	positive
5%	positive
10%	negative
Biochemical	
Glucose	negative
Sucrose	negative
Lactose	negative
Maltose	negative
Manitol	negative
Catalase	positive
Urease	positive
Oxidase	positive
Indole	negative
Methyl Red	negative
Voges Proskauer	negative
Motility	positive

The cell morphology of *L. sphaericus* Bs2-1-2 cell is presented in Figure 2.

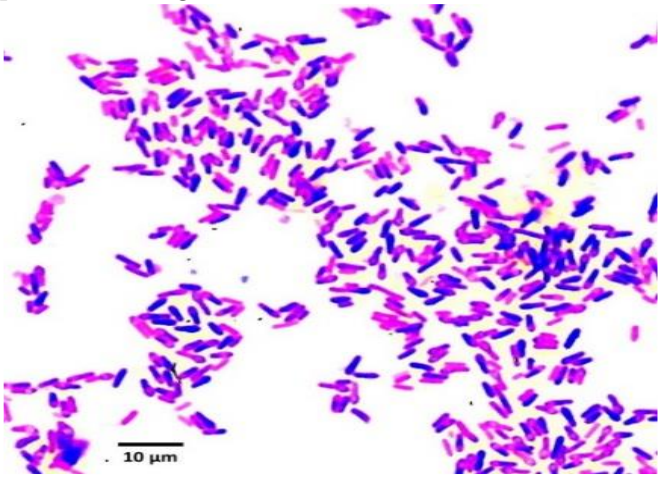

Figure 2. Cell morphology of *L. sphaericus* isolate Bs2-1-2 in endospore staining used in this study in Nutrient Agar + Streptomycin media (24-hour incubation) (source: personal research data)

Bacterial cell morphology observed in the first 24-hour incubation showed a short rod/bacil form (almost 95% of the cell population), with a length of 2.5-3.5 µm. In 48-hour (day 2) incubation, 20-30% of cell population showed vegetative cell form with swollen sporangia structure at terminal/the cell's end. Cell population with 90% vegetative and swollen sporangia was seen at 72-hour (day 3) incubation. At 96-hour (day 4) incubation, no vegetative cells and only rounded endospores (lysed endospore) were found. From this observation, it can be concluded that the isolate used in this study was not contaminated with other unwanted bacteria.

Curve of *L. sphaericus* fermentation and endospore production in 3 different concentrations of fishmeal-based media are presented in Figure 3 and Figure 4 as follows.

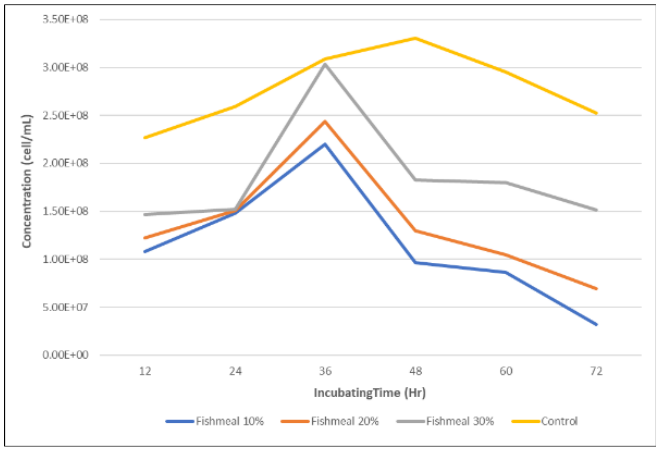

Figure 3. Bacterial cell growth of *L. sphaericus* isolate Bs2-1-2 using 10%, 20%, 30% fishmeal-based and control (Nutrient Broth) media within 72-hour incubation (source: personal research data)

All cultures of *L. sphaericus* grown on fishmeal-based media with 3 different concentrations (10, 20 and 30%) showed a relatively sloping trend of increasing cell concentration in the first 24 hours of fermentation.

The peak of cell concentration of *L. sphaericus* grown in all concentrations (10, 20 and 30%) of the media was reached at 36 hours. The highest cell concentration was shown in *L. sphaericus* grown on the media with 30% fishmeal concentration ($3.03x10^{08}$ cells/mL), followed by 20% concentration ($2.43x10^{08}$ cells/mL) and the lowest at 10% concentration ($2.20x10^{08}$ cells/mL). After 36 hours of fermentation, the concentration of *L. sphaericus* decreased until the end of fermentation. At the end of fermentation, the highest concentration was shown in *L. sphaericus* grown on 30% fishmeal-based media ($1.51x10^{08}$ cells/mL), followed by 20% media ($6.95x10^{07}$ cells/mL) and 10% media ($3.21x10^{07}$ cells/mL). This decrease in bacterial concentration was most likely caused by the decrease in nutrient concentration in the media after 36 hours of incubation. The concentration of bacterial cells continued to decrease until the end of the fermentation.

L. sphaericus grown on Nutrient Broth media which was used as a control in this study showed a similar growth pattern to *L. sphaericus* isolate Bs2-1-2 grown on 3 concentrations of fishmeal-based media. However, the growth peak was reached at the 48th hour ($3.30x10^{08}$ cells/mL), 24 hours later than those grown on fishmeal-based media.

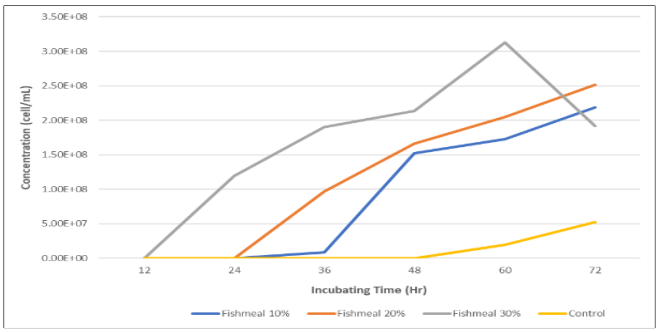

Figure 4. Bacterial endospore production of *L. sphaericus* isolate Bs2-1-2 using 10%, 20%, 30% fishmeal-based and control (Nutrient Broth) media within 72-hour incubation (source: personal research data)

The chart of bascterial endospore production using 3 different concentrations of fishmeal-based media can be seen in Figure 4. The onset of endospore production was shown at different times in *L. sphaericus* grown in the various concentrations of fishmeal-based media in this study. The earliest endospore production was shown by *L. sphaericus* reared on 30% fishmeal-based media (at the first 12 hours), followed by 20% (at 24 hours) and 10% fishmeal-based media (at 36 hours).

An increase in endospore production occurred in *L. sphaericus* grown in all concentrations of fishmeal-based media until the end of fermentation (72 hours), except in 30% fishmeal-based media (which dropped at hour 60). At the end of fermentation (72nd hour), the highest endospore concentration was achieved by *L. sphaericus* grown on 20% ($2.51x10^{08}$ cells/mL) and 10% ($2.19x10^{08}$ cells/mL) fishmeal-based media.

L. sphaericus grown on Nutrient Broth media (control) only showed a significant increase in endospore production at 48 hours. At the end of fermentation (72nd hour), endospore production continued to increase although the concentration was not as high as that obtained in fishmeal-based media ($5.25x10^{07}$ cells/mL).

Larval mortality rate graph in 3 different concentrations of fishmeal-based and control media is presented in Figure 5.

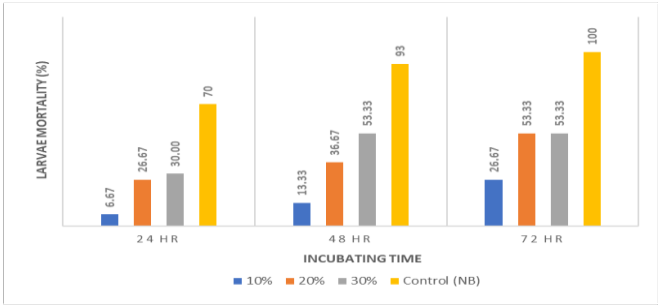

Figure 5. Larval mortality plotted for 3 different concentrations of fishmeal-based and control media within 72 hours of incubation (source: personal research data)

Within 72-hour incubation, the highest larval mortality rate was showed by *L. sphaericus* grown in 20% and 30% fishmeal-based media (both reached 53.33%), whereas *L. sphaericus* grown in 10% only reached 26.67% mortality rate. *L. sphaericus* grown in control media reached 100% larval mortality in 72-hour incubation. The ANOVA results showed significance differences in both larval mortality due to the three different fish meal concentrations (p value = 0.004; α = 0.05) and larval mortality due to the different incubating times (p value = 0.014; α = 0.05).

LC values from 3 days of incubation are presented in table 3. From quantitative bioassay, the most toxic *L. sphaericus* culture was the culture that used 30% concentration of fishmeal-based media (2.47×10^{08} cell/mL), followed by 20% (4.82×10^{08} cell/mL) and 10% concentration (9.01×10^{09} cell/mL). All toxicity values were measured in 72 hours. *L. sphaericus* grown in NB media (control media) showed the highest larval toxicity (2.85×10^{06}cell/mL), 100 times more toxic than those grown in fishmeal-based media.

Table 3. LC Values Obtained from *L. sphaericus* Cultured in 10, 20 and 30% Fishmeal-based and Control Media (Source: Personal Research Data)

Media percentage		Lc values
10%	LC_{50} 24 hrs	1.48×10^{10} cell/mL
	LC_{50} 48 hrs	1.20×10^{10} cell/mL
	LC_{50} 72 hrs	9.01×10^{09} cell/mL
20%	LC_{50} 24 hrs	1.73×10^{09} cell/mL
	LC_{50} 48 hrs	7.23×10^{08} cell/mL
	LC_{50} 72 hrs	4.82×10^{08} cell/mL
30%	LC_{50} 24 hrs	2.72×10^{08} cell/mL
	LC_{50} 48 hrs	2.48×10^{08} cell/mL
	LC_{50} 72 hrs	2.47×10^{08} cell/mL
NB (control)	LC_{50} 24 hrs	7.17×10^{06} cell/mL
	LC_{50} 48 hrs	3.23×10^{06} cell/mL
	LC_{50} 72 hrs	2.85×10^{06} cell/mL

In this study, only the main component (fishmeal alone) was tested without the addition of other ingredients (except distilled water) to see if fishmeal alone could be used to grow cells and endospores of *L. sphaericus*, taking into account the characteristics of *L. sphaericus*, which is unable to use carbohydrates as its main energy and carbon source. Based on the consideration of these characteristics, fishmeal-based media were able to demonstrate the ability to support the growth of cells and endospores of *L. sphaericus* isolate Bs2-1-2.

The cell growth of *L. sphaericus* in the three concentrations of fishmeal-based media was still below that of *L. sphaericus* grown in nutrient broth (control media) (see Figure 3). Although fishmeal can be used by *L. sphaericus* to increase its cell concentration, even at the

highest concentration (30%) it could not exceed that of *L. sphaericus* grown on control media. Interestingly, the endospore growth of *L. sphaericus* on fishmeal-based media progressed earlier than that of *L. sphaericus* grown on nutrient broth (control media) (see Figure 4).

When the curves of cell growth and endospore production were observed, the cell growth of *L. sphaericus* grown on fishmeal media (at all concentrations) showed a gentle trend until the first 24 hours and then a rapid growth until the 36th hour. Similarly, the process of endospore formation was observed at 12, 24 and 36 hours for each concentration of fishmeal. Compared to *L. sphaericus* grown on control media, the onset of sporulation was observed earlier in *L. sphaericus* grown on fishmeal media. The onset of sporulation in *L. sphaericus* reared on control media was observed at 48 hours. This shows that the components in fishmeal were able to rapidly promote cell growth and endospore production. Shevtsov et al. (1990) reported that *L. sphaericus* is able to utilize arginine, glutamic acid, methionine, threonine, serine, alanine and lysine for cell and endospore growth. He also reported that arginine, methionine and glutamic acid could have the greatest effect on cell and endospore growth. These amino acids were found in varying concentrations in fish meal (Mathai et al., 1985; Mohanty et al., 2014).

The various amino acids utilized by *L. sphaericus* serve as carbon and energy sources (as *L. sphaericus* is unable to use carbohydrates as a carbon source), as well as nitrogen sources used to synthesize its proteins (including toxin proteins) (Klein et al., 1989). In addition to different types of amino acids, some minerals contained in fishmeal (such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+} and Cu^{2+}) have been reported to have a positive effect on the production of *L. sphaericus* toxin protein (Ahmed, 1990; El-Bendary, 1999; Zhang et al., 2018).

It has been reported that several natural materials have been used by various researchers to cultivate and induce toxicity in *L. sphaericus*. Plant protein-based natural materials, such as soybean and peanut, have been reported to yield satisfactory results in the growth and induction of toxicity in these bacteria (Prabakaran et al., 2007).

In addition to plant protein-based media, animal proteins have also been reported to facilitate the growth of *B. sphaericus*. This has been demonstrated in the form of egg yolk (Prabakaran & Hoti, 2008) and cow blood (Obeta & Okafor, 1983), with high toxicity results. It is anticipated that fishmeal-based natural media will prove to be a plentiful and cost-effective alternative in Indonesia, offering a viable option for the growth and induction of toxicity in *L. sphaericus*.

The toxicity of *L. sphaericus* is derived from several types of toxin proteins it possesses. There are two types of toxin proteins, the binary toxin (abbreviated as Bin/Btx) and the mosquitocidal toxin (abbreviated as Mtx). The binary toxin is composed of two subunits, BinA (41.9 kDa in size) and BinB (51 kDa in size). These subunits are synthesized in equal molecular amounts and form parasporal crystals that are produced at stage III during sporulation (Baumann et al., 1991; Opota et al., 2011).

The mosquitocidal toxin is composed of 3 subunits, namely Mtx1 (100 kDa), Mtx2 (31.8 kDa) and Mtx3 (35.8 kDa). These proteins are synthesised by *L. sphaericus* during vegetative growth. Compared to the binary toxin, the Mtx toxin has a lower larval toxicity (Thanabalu et al., 1991; Wirth et al., 2007).

In addition to the poisons stated above, the presence of a-glucosidase receptors in the brush border microvillar area of the larvae's midgut affects *L. sphaericus*' capacity to kill mosquito larvae. This receptor is present in Culex and Anopheles mosquitoes. In Aedes mosquitoes, these receptors are present at low levels or missing (Delécluse et al., 2000; Surya et al., 2016).

L. sphaericus' toxin works in the following way to kill mosquito larvae. Once eaten by the larvae, the protein crystal is solubilized and activated by the mosquito midgut's high pH and protease. The active toxins subsequently adhere to the brush border membranes of the midgut cells. A part of the toxin enters the membrane lipid bilayer, generating an ion-selective channel or pore. This permits water to enter the cell while ions and other bigger molecules escape. This condition causes cell enlargement and lysis (Poopathi & Tyagi, 2004; Khachatourians, 2019).

The investigation of the utilisation of fishmeal-based media to stimulate the generation of cells and endospores in L. sphaericus is in its preliminary stages, as no analogous studies have been conducted utilising this material. The application of fishmeal-based media to enhance cell and endospore production can still be employed to achieve optimal outcomes.

Conclusion

The results of this study indicate that the use of fishmeal can stimulate the growth of cells and endospores, as well as the production of toxins that can suppress the development of *Aedes aegypti* mosquito larvae in laboratory settings.

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Author Contributions

Conceptualization: B.F.S., G.T.; methodology: B.F.S., S.S; software: I.M. and Z.A.; validation: B.F.S, S.S., G.T.; formal analysis: B.F.S., I.M. and Z.A; investigation: B.F.S., I.M. and Z.A; resources: B.F.S, I.M., Z.A.; data curation: B.F.S., I.M., Z.A.; writing--original draft preparation: B.F.S.; writing--review and editing: G.T., S.S; visualization: I.M., Z. A; supervision: B.F.S.; project administration: B.F.S.; funding acquisition: B.F.S.. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

All authors declare that there were no conflicts of interest. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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