



Cost-Effective Artificial Media as Substrates for Mass Cultivation of Entomopathogenic Nematodes (EPNs)

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Abstract: Entomopathogenic nematodes (EPNs) are eco-friendly alternatives to chemical pesticides in controlling agricultural insect pests, yet their mass cultivation remains constrained by high costs and ingredient availability. This study evaluated three cost-effective artificial semi-solid media for cultivating *Heterorhabditis* spp.: (A) egg yolk + mung bean, (B) egg yolk + fish meal, and (C) egg yolk + dog food. Each medium was inoculated with infective juveniles (IJs) and monitored weekly for 4 weeks, during which population density and physicochemical parameters (color, aroma, shape, and pH) were assessed. By week one, all media supported rapid multiplication, with no significant differences observed among treatments (A: 6,071.60 IJs/mL; B: 6,911.80 IJs/mL; and C: 7,546.20 IJs/mL, $p = 0.279$). From week two onward, significant differences emerged, with fish meal and dog food supporting higher population densities (9,221.60 $\pm$ 1,170.95 and 8,711.60 $\pm$ 1,303.25 IJs/mL, respectively) than mung bean (4,231.00 IJs/mL) ($p < 0.001$). Dog food medium sustained the highest populations through week three (4,682.20 IJs/mL) and retained the largest residual density by week four (287.00 $\pm$ 238.00 IJs/mL). Fish meal peaked highest (9,221.60 $\pm$ 1,170.95 IJs/mL) on the second week but declined thereafter. Mung bean, the sole plant-based medium, showed descriptive premature deterioration in shape by week two and significant aroma decline by week three, leading to population collapse by week four. These findings establish dog food-supplemented medium as the most cost-effective substrate for sustained EPN mass cultivation.

Keywords: Entomopathogenic nematodes; *Heterorhabditis* spp.; semi-solid media; mass cultivation; physicochemical properties

1. Introduction

Insect pest attacks are among the limiting factors for production losses in the Philippine agricultural sector. The injudicious use of pesticides and their degradation products in the environment has been associated with toxic effects on non-target organisms, including bees, aquatic organisms, and humans [1]. To address the environmental and health problems associated with this, entomopathogenic nematodes (EPNs), which are microorganisms that parasitize, cause disease, and kill insect pests [2], have been used by Pascual et al. [3] as ecologically acceptable pesticides. EPNs, primarily from the genera *Steinernema* and *Heterorhabditis*, are natural enemies of many insect species and

are characterized by their ability to infect and kill hosts [4 - 5] around 24 - 48 hours [6 - 7] by invading their natural openings, such as the mouth, anus, tracheae, or directly through the cuticle [8].

Mass production of microbial control agents (MCAs), such as entomopathogenic nematodes (EPNs), with reliable quality is the backbone of the biopesticide industry [9]. The use of nematodes for biological control of insect pests requires mass-culture techniques that produce large numbers of nematodes quickly, cheaply, and simply [10]. These nematodes can be produced in vivo by baiting insects and commercially by in vitro solid/liquid culturing [11]. In-vivo mass culture of EPNs uses various insects, such as Hong Kong caterpillar (*Tenebrio molitor*) [12, 8], Red flour beetle (*Tribolium castaneum*) (Herbst) larvae, pupae, and adults [13], and wax moth larvae (*Galleria mellonella*) [14], among others. On the other hand, in vitro mass culture of EPNs started when House et al. [10] utilized dog food as a medium (semi-solid) for *S. carpocapsae* [15]. Indriyanti and Muharromah [8] reported the use of egg yolk as a base – a protein source in nematode diets [16] – combined with other protein-rich supplementary ingredients such as dog food, soybean powder, and chicken liver as media for EPN mass cultivation. Thiruchchelvan et al. [13] also reported the use of soy flour, palmyra tuber flour, corn flour, and black gram flour (dhal flour) as media for in vitro mass cultivation of the nematode *Acrobelloides longiuterus*.

However, both in vivo and in vitro mass production techniques have many limitations, especially regarding their cost-effectiveness and the availability of the ingredients. In-vivo production requires less capital but lacks economies of scale due to labor and insect costs, whereas in-vitro liquid culture (which comprises the bulk of commercial production) generally offers the greatest economic efficiency but requires the largest capital [17]. Locally, soybeans, palmyra tuber flour, and black gram flour or dhal flour are not common, and chicken liver is too expensive to be just fed to nematodes, leaving dog food as the only cheap and available ingredient in the market. In 2020, Mathew et al. [18] reported a modified semi-solid dog food media (utilizing Pedigree Gravy Adult Chicken and Liver Chunks), which has been proven to contain essential nutrients such as crude protein (8%), crude fat (4%), and crude fiber (3%) – found to be safe and cheap for the mass production of EPNs.

Many locally available ingredients that provide the same nutritional sources, which are much cheaper than the more expensive ones, could be used without compromising the nutrients nematodes need to multiply. Fish meal, aside from being locally available and cheap, is a naturally balanced feed ingredient that is high in protein (60% - 72%), energy, and minerals such as calcium and phosphorus [19], making it a potential substitute for chicken liver. Mung bean, on the other hand, could also serve as an effective substitute for soybeans and various flours, as it is becoming another legume crop that could provide high-quality plant protein – ranging from 20–30% [20] - after soybean and pea [21]. Additionally, its availability in the local market is year-round. Although gelatin is commonly available in the market, agar-agar – a versatile hydrocolloid extracted from red algae that primarily serves as a gelling agent [22] – is a constant necessity in various microbiological activities, not only as a carbon source but also to minimize errors, especially in medium-based experiments.

Entomopathogenic nematodes (EPNs) are widely recognized as effective biological control agents, but their practical application is often limited by challenges in formulation stability, survival during storage [23], and the availability and cost of the ingredients. This study investigated how EPNs thrive when mass-cultivated in alternative artificial semi-solid media using combinations of egg yolk and mung bean, egg yolk and fish meal, and egg yolk and dog food. The main goal of this study was to assess which artificial media using protein-rich nutrient sources extended the longevity and shelf-life of EPNs, thereby addressing many critical gaps in EPN utilization.

2. Materials and Methods

2.1 Location of the Study

The study was conducted at the Center for Biotechnology Studies – Cebu Technological University – Barili Campus (10° 7' 54" N, 123° 32' 41" E) under controlled conditions (temperature: 25-28°C; relative humidity: ~85–95%; light period: no light).

2.2 Identification of Entomopathogenic Nematodes (EPNs)

Heterorhabditis spp. were identified to the family and genus levels using morphological characterization and keys from Nguyen and Hunt [24]. Representative nematodes were teased out from the clear suspension, killed, and fixed by adding hot 4% formalin (65–70°C), and then stained. EPNs were mounted on slides and examined under a compound light microscope for identification.

2.3 Production of Artificial Media and EPN Multiplication

EPN cultivation media were prepared as described by Indriyanti and Muharromah [8] with slight modifications. The artificial media were made in three formulae, each having five replications. They were coded as A, B, and C. The compositions of each medium are listed in Table 1 below. All media were pulverized except for the yolk. Mung bean and dog food were ground using a blender. The fish meal was procured from a local market. These supplementary ingredients were then meshed. Meanwhile, the yolk was sourced from a liquid chicken egg. Mixed media in each treatment were added with 0.20 g agar-agar and 30 mL distilled water. All components in each medium were mixed continuously and placed in a glass bottle (14 cm high and 9 cm in diameter), with a white sponge measuring 6 cm high and 1 cm wide placed to absorb the medium. The closed bottles were sterilized in an autoclave for 30 minutes at 121°C. After sterilization, the media were allowed to cool. No post-sterilization sterility verification (e.g., plating on nutrient agar) was performed, as the study was designed to simulate practical, low-cost production conditions; however, all treatments were prepared and handled uniformly to minimize variability. Each medium was then inoculated with infective juveniles (IJs). Initial populations per treatment were listed in Table 2. The bottles were then sealed and placed at room temperature in the dark.

Table 1. Artificial media formulations.

Media Composition	Yolk (g)	Mung Bean Powder (g)	Fish Meal (g)	Dog Food (g)	Agar-agar (g)	Distilled water (mL)
(A) Yolk + Mung Bean	1	1	-	-	0.20	30
(B) Yolk + Fish Meal	1	-	1	-	0.20	30
(C) Yolk + Dog Food	1	-	-	1	0.20	30

Table 2. Initial populations of infective juveniles (IJs) per treatment.

Media Composition	IJs/mL
(A) Yolk + Mung Bean	1,258.00 ± 283.23
(B) Yolk + Fish Meal	1,378.00 ± 405.12
(C) Yolk + Dog Food	1,544.00 ± 306.07

Although the dog food treatment had a slightly higher initial inoculum density, statistical analysis in Table 7 ($F(2,12) = 0.915$, $p = 0.427$) showed no significant differences among treatments. This indicates comparable starting conditions, allowing subsequent differences in population dynamics to be attributed primarily to media composition.

2.4 EPN Multiplication

EPN population (*Heterorhabditis* spp.) originated from Leyte, Philippines. EPNs were multiplied using wax moth larvae (*Galleria mellonella*) as an insect host in the White trap method. The modified white trap consisted of filter paper on an inverted petri dish lid (5 cm diameter) onto which the cadavers were placed. The dish was placed into a larger petri dish (12 cm diameter) and filled with distilled water. The traps were checked daily for infective juvenile (IJ) emergence from the cadavers. For rapid EPN multiplication, IJs from infected cadavers were applied to fresh larvae as described by Pascual et al. [25]. EPN isolates were maintained in distilled water suspensions in Falcon tubes and stored at 10–15°C until inoculated into the media.

2.5 Assessment of Culture Media Properties and EPN Population Dynamics

EPNs were observed weekly over 4 weeks to assess changes in population density among the different artificial media. Observation of its abundance was conducted by taking 100 µL of EPN culture from the same

area in the bottle using a sterilized micropipette. The EPN culture was then dropped onto a glass slide and observed using a binocular compound light microscope. Alive EPNs showing vigor were counted using a hand counter. Each sample was counted three times to obtain the average. EPN populations were counted using the equation:

$$\text{EPN population (IJs/mL)} = \text{Mean IJ count} \times (1000 / \text{sample volume in } \mu\text{L})$$

Average EPN abundance data from the 1st to 4th week were statistically analyzed using a one-way ANOVA (F test) at a significance level of 5% and a Post hoc test (Tukey test). Data analysis was conducted using SPSS 25. Other measured parameters were color, aroma, shape, and pH of the different media. Changes in color, aroma, and shape were analyzed using a scale of 5 (desirable, excellent) to 1 (undesirable). The scoring criteria for color, aroma, and shape were developed for this study and applied consistently across treatments. Assessments were conducted without blinding, and inter-rater reliability was not evaluated. Finally, parameters exhibiting zero variance in one or more groups were excluded from inferential statistical analysis, and results were interpreted descriptively.

2.5.1 Color of Media

The changes in color of insects when infected with entomopathogenic nematodes (EPNs) are mainly caused by the metabolic activity of the nematodes and their symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*), which produce pigments, toxins, and enzymes that alter the appearance of the insect host [26]. Similarly, in artificial media as a substrate for nematodes, discolorations indicate interaction between actively feeding nematodes and their symbionts and signify that the medium is utilized, thereby being favorable.

Color progression from initial preparation through the end of the culture period was assessed based on the following criteria:

- 5 – consistent color changes from the initial setup to the end, having similar hue; no signs of contamination or precipitation; discoloration from light (initial setup) to dark (termination) are constant and uniform; 100% discoloration
- 4 – distinct and uniform changes, but with slight unevenness; no significant contamination; up to 75% discoloration
- 3 – moderate color changes; few areas are less pronounced; up to 50% discoloration
- 2 – unclear color changes, possibly due to the color of the ingredients; possible early contamination or separation; up to 25% discoloration
- 1 – no observable changes

2.5.2 Aroma of Media

Indriyanti and Muharromah [8] observed that aroma changed from fresh to slightly pungent or stinging over time in various artificial media due to nutrient breakdown and nematode metabolic progression, reflecting culture stages and sometimes posing contamination risk. In addition, the metabolic activity of nematodes, along with their symbionts, which are released by the nematodes [27], contributes to the aroma of nematode cultures, linked to enzymatic protein and lipid degradation. Under unfavorable culture conditions, these processes lead to the development of distinctly unpleasant or foul odors, which can indicate excessive microbial decomposition, contamination, or suboptimal media quality.

Smell changes indicating spoilage or fermentation were assessed based on the following criteria:

- 5 – pleasant aroma is maintained or developed along the culture period; no foul odors or signs of contamination
- 4 – changes in aroma into a neutral smell, having a mild background odor
- 3 – changes in aroma are noticeable; some samples start to smell unpleasant
- 2 – clear presence of unpleasant odors, indicating early contamination or degradation of media
- 1 – very strong, unpleasant, foul odors are dominating, indicating contamination and decay

2.5.3 Shape of Media

Somwong and Petcharat [28] reported that a good medium maintains nematode morphology and supports high yields, reflecting a stable semi-solid medium; thus, any deviations in shape or media integrity indicate culture issues (e.g., contamination). Shape alteration is also affected by the composition and waste products of cultivated EPNs in closed conditions [8].

Assessment for changes in shape (physical and structural) was based on the following criteria:

- 5 – Media is maintained at a stable semi-solid state and has a firm consistency throughout the culture period
- 4 – media has few surface irregularities (minimal unevenness & shrinking) and mostly retains its semi-solid integrity
- 3 – media starts to exhibit minimal liquefaction, or surface cracks
- 2 – media integrity is compromised with significant liquefaction, collapse, or irregular shape (e.g., watery zones)
- 1 – media structure is collapsed; highly contaminated with surface irregularities, pockets of liquefaction, or visible fungal/bacterial overgrowth

2.5.4 pH of Media

The pH of each medium was measured at the initial setup and at the termination of the experiment to provide an overall indication of media condition. Weekly monitoring focused on population density and observable physicochemical characteristics, including color, aroma, and shape. Intermediate pH measurements were not performed to minimize disturbance of the culture system and reduce the risk of contamination, as repeated sampling could alter media composition and compromise experimental consistency. Thus, initial and final pH values were considered sufficient to capture the overall direction and magnitude of pH change across treatments.

3. Results and Discussion

3.1 Entomopathogenic Nematode Identification

The entomopathogenic nematodes (EPNs) used in the study were identified as *Heterorhabditis* (Figures 1 & 2), a genus in the family Heterorhabditidae. When infecting suitable hosts, these nematodes release *Photorhabdus*, its bacterial symbiont – turning the cadaver into red due to the production of anthraquinone pigments [29] (Figure 3B). Additionally, Booyesen et al. [30] reported that different strains of *Photorhabdus* also cause various pigmentations to cadavers, turning them into steel grey-blue (blue), red, and green (yellow with a green tint). In artificial media, the bacterial symbionts would produce varying color shades of the red pigment [31] (Figure 3A).



Figure 1. *Heterorhabditis* showing both (a) anterior and (b) posterior body segments. (10x/0.25 oil)



Figure 2. *Heterorhabditis* (A) anterior; (B) metacarpus; (C) posterior. (100x/1.25 Oil)



Figure 3. Discolorations on (A) media and (B) cadavers, indicating the presence of *Photorhabdus*.

In vitro mass cultivation of *Heterorhabditis* yielded varying results across treatments. At the start, initial nematode populations were statistically comparable across all media, with means of $1,258 \pm 283.23$ IJs/mL for mung bean, $1,378 \pm 405.12$ IJs/mL for fish meal, and $1,544 \pm 306.07$ IJs/mL for dog food, as shown in Table 2. One-way ANOVA confirmed no significant difference ($F(2,12) = 0.915$, $p = 0.427$) (Table 7), and Tukey's test placed all treatments in the same subset, indicating that later divergences could be attributed to the diets rather than unequal inocula. Similarly, the physicochemical parameters of the media (color, aroma, shape, and pH), as shown in Table 9, were indistinguishable at the time of inoculation. Color was uniform across all treatments (Mean = 1.00 ± 0.00), with no measurable variance. Because all values were identical, inferential statistics could not be applied; results are reported descriptively only. Aroma and shape likewise showed homogeneity (Means = 5.00 ± 0.00 each). Finally, initial pH values were descriptively comparable across all treatments (mung bean: 6.60, fish meal: 6.49, dog food: 6.38), indicating similar starting acidity conditions. The absence of initial differences across both biological inocula and physicochemical parameters ensures that subsequent divergences observed during culture can be attributed to dietary and substrate effects rather than initial inequalities. This is consistent with Shapiro-Ilan et al. [32], who emphasized that optimized inoculation parameters (i.e., starting populations, culture conditions, etc.) are critical for interpreting dietary effects on entomopathogenic nematode (EPN) reproduction. Similarly, Koppenhöfer et al. [33] emphasized that consistent starting parameters, including media properties and inoculum size, are critical for reproducible mass production systems.

By the first week, nematode populations increased across all culture media as shown in Table 3, with means of $6,071.60 \pm 1,586.16$ IJs/mL (mung bean), $6,911.80 \pm 1,236.50$ IJs/mL (fish meal), and $7,546.20 \pm 1,315.04$ IJs/mL (dog food), but no significant differences were detected among treatments ($F(2,12) = 1.421$, $p = 0.279$).

Tukey's test confirmed a single statistical subset, indicating comparable early reproductive performance across all media. The observed variability likely reflects inherent biological heterogeneity of entomopathogenic nematodes and minor micro-environmental differences under non-axenic, low-intervention conditions, which do not obscure the overall similarity in early culture performance. This aligns with Shapiro-Ilan et al. [32], who reported that EPNs often perform similarly across substrates during initial reproductive cycles before diet-specific effects emerge, and McMullen II and Stock [34], who emphasized that early production is primarily driven by general nutrient support for both nematodes and their symbiotic bacteria. For physicochemical parameters, color and shape exhibited zero variance in one or more treatments (mung bean and dog food for color; fish meal and dog food for shape), indicating complete within-treatment uniformity. Because ANOVA assumptions were violated, these parameters were not subjected to inferential statistical analysis and are interpreted descriptively. Fish meal showed more pronounced color development, suggesting earlier metabolic activity of symbiotic bacteria, while mung bean displayed reduced shape compared to fish meal and dog food, indicating early signs of media instability. In contrast, aroma did not differ significantly among treatments ($F(2,12) = 0.167$, $p = 0.848$), and all media maintained generally acceptable odor profiles. This suggests that, during the first week, microbial activity and nutrient degradation were still at an early stage and relatively uniform across substrates.

Table 3. Mean population and physicochemical changes for the 1st week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	6,071.60 ± 1,586.16a	6,911.80 ± 1,236.50a	7,546.20 ± 1,315.04a	1.421 (2,12)	0.279	Same subset
Color	1.00 ± 0.00	3.20 ± 1.10	1.00 ± 0.00	—	—	Descriptive only
Aroma	4.20 ± 0.84	4.20 ± 0.45	4.40 ± 0.55	0.167 (2,12)	0.848	NS
Shape	3.80 ± 0.45	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the second week, a clear divergence in nematode production was observed (Table 4). Mung bean declined to $4,231.00 \pm 1,607.65$ IJs/mL, while fish meal ($9,221.60 \pm 1,170.95$) and dog food ($8,711.60 \pm 1,303.25$) maintained significantly higher populations. This difference was statistically significant ($F(2,12) = 20.004$, $p < 0.001$), with Tukey's test grouping fish meal and dog food together and separating mung bean as the lower-performing substrate. These results are consistent with those of Widiyaningrum et al. [26], who reported that animal-based substrates better support EPN reproduction due to higher nutrient availability. Physicochemical traits also diverged. Color values showed no variance across treatments and were therefore interpreted descriptively. Fish meal showed more advanced color progression, likely reflecting increased metabolic activity of symbiotic bacteria compared to the other media. Shape values in fish meal and dog food exhibited zero variance (5.00 ± 0.00), indicating consistent structural stability. Because variance assumptions were violated, inferential statistical analysis was not applicable; however, descriptively, both media maintained their semi-solid integrity, whereas the mung bean showed noticeable degradation. Aroma remained statistically similar across treatments ($F(2,12) = 0.222$, $p = 0.804$), indicating that odor-related changes were still not strongly differentiated despite emerging differences in population density and structural stability.

Table 4. Mean population and physicochemical changes for the 2nd week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	4,231.00 ± 1,607.65a	9,221.60 ± 1,170.95b	8,711.60 ± 1,303.25b	20.004 (2,12)	<0.001	Fish meal = Dog food > Mung bean
Color	1.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Aroma	3.40 ± 0.55	3.60 ± 0.55	3.60 ± 0.55	0.222 (2,12)	0.804	NS
Shape	2.60 ± 0.55	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the third week, nematode production showed strong divergence (Table 5). Dog food showed significantly higher population ($4,682.20 \pm 987.64$ IJs/mL) compared to both fish meal ($1,715.00 \pm 835.65$) and mung bean (799.80 ± 309.63). These differences were highly significant ($F(2,12) = 34.916$, $p < 0.001$), and Tukey's test indicated a graded response (dog food > fish meal = mung bean), consistent with improved performance in nutrient-rich animal-based media as reported by Zhen et al. [27] and Koppenhöfer et al. [33]. For sensory and physical traits, color exhibited zero variance in one or more treatments (fish meal and dog food) and was therefore not subjected to inferential statistical analysis. Descriptively, fish meal maintained the highest color intensity, indicating sustained metabolic activity, while mung bean and dog food showed less pronounced changes. Aroma differed significantly among treatments ($F(2,12) = 10.750$, $p = 0.002$), with mung bean exhibiting the lowest scores, indicating greater spoilage or degradation. Fish meal and dog food maintained relatively better odor profiles, suggesting more stable biochemical conditions. Shape values showed zero variance across all treatments, preventing inferential analysis. Descriptively, mung bean exhibited structural collapse, while fish meal and dog food exhibited minimal liquefaction, yet retained sufficient semi-solid integrity to continue supporting nematode survival at this stage.

Table 5. Mean population and physicochemical changes for the 3rd week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	799.80 ± 309.63a	1,715.00 ± 835.65a	4,682.20 ± 987.64b	34.916 (2,12)	<0.001	Dog food > Fish meal = Mung bean
Color	1.40 ± 0.55	5.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Aroma	1.40 ± 0.55	2.60 ± 0.55	2.80 ± 0.45	10.750 (2,12)	0.002	Mung bean < Fish meal = Dog food
Shape	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the fourth week, nematode numbers declined across all diets (Table 6), but dog food supported the highest residual populations despite late-stage declines, retaining the largest population (287.00 ± 238.00), followed by fish meal (69.80 ± 60.34), while mung bean dropped almost completely (7.20 ± 14.48). ANOVA still detected significant differences ($F(2, 12) = 5.347$, $p = 0.022$), with Tukey's post hoc test confirming that dog food was the highest, fish meal an intermediate, and mung bean the lowest. Physicochemical and sensory traits also showed divergence. Color and shape exhibited zero variance across treatments, indicating uniform

measurements within groups. As a result, these parameters were not subjected to inferential statistical analysis and are interpreted descriptively. Fish meal maintained the highest color intensity, while both fish meal and dog food retained partial structural integrity compared to the complete collapse observed in the mung bean. Aroma differed significantly among treatments ($F(2,12) = 16.333$, $p < 0.001$), with dog food showing higher scores, indicating less degradation than fish meal and mung bean. pH also differed descriptively, with dog food exhibiting a lower pH than the other treatments. This suggests greater accumulation of acidic metabolic by-products, likely associated with microbial activity. However, since pH was only measured at the beginning and end of the experiment, these results should be interpreted as indicative of overall changes rather than continuous trends. The observed pH differences among media suggest substrate-dependent variation in microbial metabolism, particularly the activity of symbiotic bacteria (*Photorhabdus* spp.), which drive the production of organic acids and nitrogenous compounds. These metabolic by-products influence the culture's physicochemical environment and may affect late-stage infective juvenile (IJ) survival. Such late-stage declines are common in closed culture systems due to food depletion and waste accumulation [8].

Table 6. Mean population and physicochemical changes for the 4th week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	7.20 ± 14.48a	69.80 ± 60.34ab	287.00 ± 238.00b	5.347 (2,12)	0.022	Dog food > Mung bean; Fish meal: ns
Color	3.00 ± 0.00	5.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
Aroma	1.00 ± 0.00	1.60 ± 0.55	2.60 ± 0.55	16.333 (2,12)	<0.001	Dog food > Fish meal > Mung bean
Shape	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
pH	6.85	6.94	5.91	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively. pH values represent single measurements per treatment.

Mean population of entomopathogenic nematodes (EPNs) during mass cultivation across four weeks (Table 7) showed no significant differences among treatments in Week 1 ($F = 1.421$, $p = 0.279$), indicating comparable early establishment across mung bean ($6,071.60 \pm 1,586.16$ IJs/mL), fish meal ($6,911.80 \pm 1,236.50$ IJs/mL), and dog food ($7,546.20 \pm 1,315.04$ IJs/mL). From Week 2 onward, significant differences emerged ($F = 20.004$, $p < 0.001$), where fish meal ($9,221.60 \pm 1,170.95$ IJs/mL) and dog food ($8,711.60 \pm 1,303.25$ IJs/mL) were significantly higher than mung bean ($4,231.00 \pm 1,607.65$ IJs/mL), indicating superior early infective juvenile (IJ) proliferation in animal-based media. By Week 3, divergence became more pronounced ($F = 34.916$, $p < 0.001$), with dog food ($4,682.20 \pm 987.64$ IJs/mL) significantly outperforming fish meal ($1,715.00 \pm 835.65$ IJs/mL) and mung bean (799.80 ± 309.63 IJs/mL), demonstrating a clear shift in sustained productivity favoring dog food. Week 4 still showed significant differences ($F = 5.347$, $p = 0.022$), where dog food (287.00 ± 238.00 IJs/mL) retained higher residual populations than fish meal (69.80 ± 60.34 IJs/mL) and mung bean (7.20 ± 14.48 IJs/mL), both of which declined sharply. Despite the low terminal counts, no zero-variance conditions were observed in any treatment group, indicating that variability remained estimable across all weeks. However, Week 4 values exhibited lower means with greater dispersion (notably in mung bean and dog food), reflecting increased heterogeneity at low population levels without affecting ANOVA validity. Overall, fish meal supported rapid early proliferation but showed reduced persistence compared to dog food, which retained higher residual populations across later weeks despite fluctuations. This aligns with previous findings that animal-derived, nutrient-rich media can enhance EPN production; however, culture stability is substrate-dependent and varies among different animal-based formulations [8,11].

Table 7. Weekly mean population of EPNs in various artificial media in four weeks.

Week	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 0	1,258.00 ± 283.23a	1,378.00 ± 405.12a	1,544.00 ± 306.07a	0.915 (2,12)	0.427	Same subset
Week 1	6,071.60 ± 1,586.16a	6,911.80 ± 1,236.50a	7,546.20 ± 1,315.04a	1.421 (2,12)	0.279	Same subset
Week 2	4,231.00 ± 1,607.65a	9,221.60 ± 1,170.95b	8,711.60 ± 1,303.25b	20.004 (2,12)	<0.001	Fish meal = Dog food > Mung bean
Week 3	799.80 ± 309.63a	1,715.00 ± 835.65a	4,682.20 ± 987.64b	34.916 (2,12)	<0.001	Dog food > Fish meal = Mung bean
Week 4	7.20 ± 14.48a	69.80 ± 60.34ab	287.00 ± 238.00b	5.347 (2,12)	0.022	Dog food > Mung bean; Fish meal: ns

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

Despite slight differences in initial inoculum densities among treatments (Table 2), the weekly population changes of entomopathogenic nematodes (EPNs) did not consistently follow these initial variations (Table 8). In Week 1, all treatments exhibited positive population growth—mung bean ($4,813.60 \pm 1,320.81$ IJs/mL), fish meal ($5,533.80 \pm 2,679.36$ IJs/mL), and dog food ($6,002.20 \pm 1,019.55$ IJs/mL)—with no significant differences among media ($F = 1.531$, $p = 0.256$), indicating comparable early establishment across treatments. By Week 2, mung bean showed a population decline ($-1,840.60 \pm 736.94$ IJs/mL), while fish meal ($2,309.80 \pm 858.64$ IJs/mL) and dog food ($1,165.40 \pm 156.49$ IJs/mL) exhibited increases, with a near-significant treatment effect ($F = 3.805$, $p = 0.053$). Tukey indicated a significant difference between fish meal and dog food, while mung bean showed intermediate values and did not differ significantly from either treatment. In Week 3, all treatments experienced declines, with fish meal ($-7,506.60 \pm 461.74$ IJs/mL) showing a significantly greater reduction compared to mung bean ($-3,431.20 \pm 1,365.59$ IJs/mL) and dog food ($-4,029.40 \pm 763.57$ IJs/mL) ($F = 27.299$, $p < 0.001$), indicating a marked decrease in fish meal relative to the other substrates. Mung bean and dog food were statistically grouped in the Tukey analysis, suggesting similar mid-phase population retention despite differences in mean magnitudes. In Week 4, declines continued across treatments, with dog food ($-4,395.20 \pm 906.05$ IJs/mL) exhibiting a significantly greater decrease than fish meal ($-1,654.20 \pm 775.62$ IJs/mL) and mung bean ($-1,341.20 \pm 1,240.29$ IJs/mL) ($F = 14.399$, $p = 0.001$). Notably, this final week showed relatively consistent variability across treatments, with no zero-variance conditions, ensuring that statistical comparisons remained valid even under uniformly negative population changes. Overall, these results indicate that population dynamics varied markedly across weeks and treatments, suggesting that nutrient composition and media suitability exert a stronger influence on EPN population changes than initial inoculum density alone. It was also observed that although the mung bean generally had the lowest populations among all media, its population decline occurred progressively, indicating limited but gradually diminishing nutrient support.

Table 8. Weekly mean population change (+/-) of EPNs in various artificial media in four weeks.

Week	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 1	+ 4,813.60 ± 1,320.81a	+ 5,533.80 ± 2,679.36a	+ 6,002.20 ± 1,019.55a	1.531 (2,12)	0.256	Same subset
Week 2	- 1,840.60 ± 736.94ab	+ 2,309.80 ± 858.64b	+ 1,165.40 ± 156.49a	3.805 (2,12)	0.053	Fish meal ≠ Dog food
Week 3	- 3,431.20 ± 1,365.59a	- 7,506.60 ± 461.74b	- 4,029.40 ± 763.57a	27.299 (2,12)	<0.001	Mung bean = Dog food > Fish meal
Week 4	- 1,341.20 ± 1,240.29a	- 1,654.20 ± 775.62a	- 4,395.20 ± 906.05b	14.399 (2,12)	0.001	Dog food < Fish meal = mung bean

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

Parallel physicochemical assessment (Table 9) showed identical baseline properties at Week 0 (color = 1.00 ± 0.00 , aroma = 5.00 ± 0.00 , shape = 5.00 ± 0.00 ; pH = 6.38–6.60), with zero variance across treatments; thus, results were interpreted descriptively. By Week 1, divergence began to emerge. Color differed among treatments, with fish meal (3.20 ± 1.10) showing higher values than mung bean and dog food (both 1.00 ± 0.00). However, because mung bean and dog food exhibited zero variance, these results were interpreted descriptively. Shape also differed, with fish meal and dog food (both 5.00 ± 0.00) showing higher structural integrity than mung bean (3.80 ± 0.45), although zero variance limited inferential robustness, thus, descriptively presented. Aroma remained statistically similar across treatments ($F(2,12) = 0.167$, $p = 0.848$), indicating minimal differentiation in odor-related changes during early culture stages. By Week 2, divergence intensified. Color values exhibited zero variance across treatments and were therefore interpreted descriptively, with fish meal (4.00 ± 0.00) showing the most advanced discoloration, followed by mung bean (1.00 ± 0.00) and dog food (1.00 ± 0.00). Shape differed descriptively, with fish meal and dog food (both 5.00 ± 0.00) maintaining structural integrity compared to mung bean (2.60 ± 0.55); however, zero variance in the former treatments again limited inferential strength. Aroma remained non-significant ($F(2,12) = 0.222$, $p = 0.804$), suggesting that odor-related changes were not yet strongly differentiated despite increasing structural divergence. By Week 3, a clear separation among treatments was observed. Color differed, with fish meal (5.00 ± 0.00) showing the highest values, followed by mung bean (1.40 ± 0.55) and dog food (1.00 ± 0.00). However, zero variance in fish meal limited strict inferential interpretation and was therefore presented descriptively. Aroma also differed significantly ($F(2,12) = 10.750$, $p = 0.002$), with mung bean (1.40 ± 0.55) showing greater deterioration than fish meal (2.60 ± 0.55) and dog food (2.80 ± 0.45). Shape values exhibited zero variance across all treatments (mung bean = 1.00 ± 0.00 ; fish meal and dog food = 3.00 ± 0.00), and were therefore interpreted descriptively, indicating structural collapse in mung bean and partial integrity in the animal-based fish meal and dog food media. In Week 4, color and shape again exhibited zero variance in one or more treatments and were interpreted descriptively. Fish meal maintained the highest color score (5.00 ± 0.00), while mung bean (1.00 ± 0.00) showed complete structural collapse compared to fish meal and dog food (both 2.00 ± 0.00). Aroma showed significant differentiation ($F(2,12) = 16.333$, $p < 0.001$), with dog food (2.60 ± 0.55) highest, followed by fish meal (1.60 ± 0.55) and mung bean (1.00 ± 0.00), indicating slower degradation in the dog food medium. pH also differed significantly at Week 4 ($F(2,12) = 4.421$, $p = 0.036$), with dog food exhibiting a lower pH (5.91) than mung bean (6.85) and fish meal (6.94), suggesting greater accumulation of acidic metabolic by-products. However, since pH was measured only at the beginning and end of the experiment, these results reflect overall shifts rather than continuous trends. Overall, physicochemical parameters showed increasing divergence over time, with fish meal and dog food developing more distinct biochemical and structural profiles compared to mung bean. These changes paralleled differences in nematode population dynamics, suggesting that substrate-dependent microbial and degradation processes may contribute to cultivation outcomes, although direct causality was not experimentally established.

Table 9. Changes in Color, Aroma, Shape, and pH in four weeks.

Week	Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 0	Color	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
	Aroma	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	Shape	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	pH	6.60	6.49	6.38	—	—	Descriptive only
Week 1	Color	1.00 ± 0.00	3.20 ± 1.10	1.00 ± 0.00	—	—	Descriptive only
	Aroma	4.20 ± 0.84	4.20 ± 0.45	4.40 ± 0.55	0.167 (2,12)	0.848	ns
	Shape	3.80 ± 0.45	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
Week 2	Color	1.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
	Aroma	3.40 ± 0.55	3.60 ± 0.55	3.60 ± 0.55	0.222 (2,12)	0.804	ns
	Shape	2.60 ± 0.55	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	Color	1.40 ± 0.55	5.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Week 3	Aroma	1.40 ± 0.55	2.60 ± 0.55	2.80 ± 0.45	10.750 (2,12)	0.002	Mung bean < Fish meal = Dog food
	Shape	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	—	—	Descriptive only
	Color	3.00 ± 0.00	5.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
Week 4	Aroma	1.00 ± 0.00	1.60 ± 0.55	2.60 ± 0.55	16.333 (2,12)	<0.001	Dog food > Fish meal > Mung bean
	Shape	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
	pH	6.85	6.94	5.91	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively. pH values represent single measurements per treatment.

In terms of cost-effectiveness, the media used in this study (mung bean, fish meal, and dog food) were far more economical than other media, such as chicken liver and soybean powder, used in other studies. Each media required a fixed inoculum cost — wax moth larvae at ₱2–₱3 each, plus ₱50–₱100 for maintaining starter cultures, which was uniform across all substrates. This means the real differentiator lies in ingredient prices and biological sustainability. Mung bean (₱95–₱160/kg), fish meal (₱116–₱234/kg), and dog food (₱80–₱220/kg) were all relatively inexpensive and locally available. In contrast, chicken liver (₱184–₱406/kg) and soybean powder (₱311–₱824/kg) add significantly higher ingredient costs on top of the same inoculum acquisition, while being limited by perishability or import dependence. When comparing dog food and soybean powder, the top-performing media in this study and in Indriyanti and Muharromah's [8] study, respectively, an estimated cost reduction of approximately 60–75% is observed, depending on price variation.

4. Conclusions

Mass cultivation of *Heterorhabditis* spp. in locally available semi-solid media produced varying population densities across weeks, associated with changes in physicochemical and sensory properties (color, aroma, shape, and pH), likely reflecting underlying microbial and biochemical activity. However, these parameters were not directly tested as causal factors. All treatments showed an initial population increase in Week 1. Media A (egg yolk + mung bean) declined from Week 2 onward, while Media B (egg yolk + fish meal) and Media C (egg yolk + dog food) continued increasing initially. By Weeks 3–4, all media exhibited reduced populations. Media C demonstrated the most stable performance, reaching a peak of $8,711.60 \pm 1,303.25$ IJs/mL in Week 2 and maintaining relatively higher densities through Week 4. Media B achieved the highest peak population ($9,221.60 \pm 1,170.95$ IJs/mL in Week 2) but showed a sharp decline in Week 3, indicating lower sustainability despite superior short-term yield. Media consistently performed the least favorably. Based on

these trends, the optimal harvest for Media B and C is Week 2, when populations are at their maximum. Overall, Media C (egg yolk + dog food) provides the best balance of yield stability and economic feasibility. This aligns with Indriyanti and Muharromah [8], who identified egg yolk + dog food as a strong EPN substrate alongside soybean powder and chicken liver. Considering cost and availability, dog food is more economical and scalable in the Philippine context, consistent with Mathew et al. [18].

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