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Evaluation of the persistence of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* in cattle manure under laboratory conditions

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Flies on livestock farms pose a significant problem by spreading diseases and causing stress to animals, which leads to reduced milk production and meat quality. Flies mainly breed in calf barns with wet bedding, as well as in manure, piles, and slurry under slatted floors. Non-chemical solutions, such as biopesticides containing entomopathogenic nematodes, are increasingly favored due to their high efficacy in controlling insects without harming the environment or animals. The aim of the study was to evaluate which of the commercially available nematode preparations, containing different nematode species, exhibits the highest survival in cattle manure and the highest efficacy against *Galleria mellonella* larvae under laboratory conditions. The study was conducted in three experimental variants: the Entonem preparation containing larvae of *Steinernema feltiae* (Sf), the Capsanem preparation containing larvae of *Steinernema carpocapsae* (Sc), and the Larvanem preparation containing larvae of *Heterorhabditis bacteriophora* (Hb). Each variant included 15 containers of cattle manure to which 2000 infective juveniles were applied on the first day of the experiment. On the day of application, and subsequently on days 2, 4, 6, and 8 after nematode application, ten *G. mellonella* larvae were placed in three containers from each variant. Forty-eight hours after adding the *G. mellonella* larvae to the containers, the dead larvae were dissected, and the presence of nematodes was determined using a stereomicroscope. The results indicated that the

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preparations differed significantly in their persistence in cattle manure and insecticidal efficacy. For Capsanem, the highest mortality of *G. mellonella* larvae (100%) was achieved 4 days after nematode application. Entonem showed variable efficacy, with the highest mortality of *G. mellonella* larvae (76.7%) recorded on day 6 after nematode application. In contrast, Larvanem maintained high efficacy from day 2 to the end of the study (83.3-96.4%). These results indicate that Larvanem can be an effective tool for long-term control of insects that develop in cattle manure.

KEY WORDS: cattle manure / biological control / animal welfare / entomopathogenic nematodes

Flies are common in areas with livestock due to easy access to food. These insects pose a significant problem on farms, particularly those with cattle and pigs. Warm and humid conditions in livestock buildings create an ideal environment for insect larvae development. Flies primarily breed in calf barns with wet bedding and spilled milk residues. Manure, manure piles, and slurry under slatted floors also serve as breeding grounds for various flying insects [Hansen *et al.* 2023]. Affected animals experience both physical and psychological discomfort, leading to a notable decline in livestock production efficiency. Dairy cows produce less milk with deteriorated quality. Flies can contaminate milk through milking equipment and teat cups, introducing bacteria and viruses. Milk yield can decrease by up to 20%, and weight losses in fattened cattle can reach up to 6 kg per head. Flies also significantly impair cattle reproduction. Besides being a nuisance and negatively affecting reproduction, flies pose a serious epidemic threat. Research indicates that a fly can carry up to 6 million microorganisms on its body surface and up to 30 million in its intestines. These microorganisms can cause severe diseases such as typhoid fever, cholera, tuberculosis, polio, salmonellosis, and anthrax [Geden *et al.* 2021]. For livestock, the most dangerous diseases include dysentery, salmonellosis, colibacillosis, brucellosis, and tuberculosis. In dairy herds, flies can contribute to mastitis and carry parasitic spores. Current chemical control methods for flies in cattle farms often involve the use of synthetic pyrethroids. For example, permethrin, applied as a backline treatment to cattle, has been shown to control stable flies for up to two weeks and improve milk yield in dairy cows. Moreover, insect growth regulators (IGRs) like cyromazine and diflubenzuron are used to interrupt the development of fly larvae, significantly reducing adult fly populations when added to animal bedding and other larval developmental sites [Cook 2020].

Increased awareness of the consequences of the overuse of chemical insecticides, together with the implementation of new legislation regulating their trade and use, has led to a growing interest in biological methods of pest control [Matyjaszczyk 2012, Pruszyński and Pruszyński 2013]. Non-chemical alternatives, such as biopesticides containing entomopathogenic nematodes, are gaining importance due to their high insecticidal efficacy and lack of negative impact on the natural environment and vertebrates.

Entomopathogenic nematodes (EPNs) have been used for many years to control insect pests in various agricultural crops, forests, mushroom farms and golf courses. However, there has been limited research on the use of nematodes for the control of

troublesome insects in livestock facilities. Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are obligate insect parasites. These nematodes have a symbiotic relationship with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively. Infective juveniles (IJs) enter the host through natural openings such as the mouth, anus, or spiracles, but the IJs of some species can also enter through the cuticle. After penetrating the host's hemocoel, nematodes release their symbiotic bacteria, which begin to produce toxins that kill the insect within 24-48 hours. [Poinar 1979, Laznik *et al*. 2010, Kucharska *et al*. 2023].

We verified two research hypotheses:

– different preparations would vary in their persistence in cattle manure and insecticidal efficacy.

– the species *Heterorhabditis bacteriophora* (Larvanem preparation) would show the longest persistence and the highest insecticidal efficacy because it is a thermophilic species.

The aim of the study was to evaluate which of the commercially available nematode preparations containing different nematode species has the highest survival rate in cattle manure and efficacy against the larvae of the greater wax moth, *Galleria mellonella* L., under laboratory conditions.

Material and methods

Commercial preparations

Three commercial preparations were used in this study:

Larvanem produced by Koppert, The Netherlands, with infective juveniles (IJs) of *Heterorhabditis bacteriophora* (Poinar, 1976) (1 pack contains 50 million IJs).

Capsanem produced by Koppert, The Netherlands, with infective juveniles (IJs) of *Steinernema carpocapsae* (Weiser, 1955) (1 pack contains 50 million IJs).

Entonem produced by Koppert, The Netherlands, with infective juveniles (IJs) of *Steinernema feltiae* (Filipjev, 1934) (1 pack contains 50 million IJs).

Larvae of G. mellonella. The *G. mellonella* larvae used in this study were obtained from the Department of Animal Environmental Biology at the Warsaw University of Life Sciences (SGGW), where this species has been reared for many years.

Cattle manure. The cattle manure was sourced from the Wilanów-Obory Agricultural Experimental Station (Obory, Konstancin-Jeziorna), located at the geographical coordinates N 52°04'52.99" E 21°08'50.06". Currently, the station maintains approximately 360 high-yielding dairy cows. The cows are fed year-round using the Total Mixed Ration (TMR) system. The mix consists of maize silage, ensiled maize grain, grass and alfalfa silage, soya and rapeseed meal, cereal meal, yeast and mineral-vitamin supplements.

The cattle manure used in the experiment was sourced from healthy, disease-free cows. Samples were collected from various locations within the manure pile to ensure the representativeness of the material. The manure was transported in plastic

containers to the laboratory of the Department of Animal Environment Biology at the Warsaw University of Life Sciences and stored in a cool, shaded place to minimise the decomposition of organic matter and microbial activity prior to the start of the experiment (the pH of the manure was 8.84).

Bioassay. Forty-five 250 ml plastic containers were used for the study. Each container was filled with 20 g of fresh manure. Before the experiment, the manure was thoroughly mixed using a laboratory stirrer to achieve a uniform consistency and ensure even distribution of organic and microbial components.

The containers were divided into three groups, each treated with different nematodes: *S. feltiae* (Sf), *S. carpocapsae* (Sc), and *H. bacteriophora* (Hb). Each group consisted of 15 containers. On the first day of the experiment, 1 ml of nematode larval suspension (IJs) was applied to all containers at a dose of 200 infective juveniles per *G. mellonella* larva, which equated to 2000 IJs per container. The nematode preparations were made following manufacturer's recommendations to ensure their viability and biological activity. The nematodes were applied to the containers using an Eppendorf pipette to ensure precise dosing. The containers were covered with lids containing ventilation holes to ensure proper airflow.

On the day of application and subsequently on days 2, 4, 6, and 8 after nematode application, 10 greater wax moth larvae (approximately 2 cm long and weighing 200-300 mg) were placed in 3 containers from each group (Sf, Sc, Hb), giving a total of 30 larvae per nematode species for each day of the experiment.

Each group used 150 *G. mellonella* larvae, with 30 larvae for each day: day of application, 2, 4, 6, and 8 (30 larvae x 5 days) x 3 groups, for a total of 450 insects.

Forty-eight hours after adding the greater wax moth larvae to the containers,

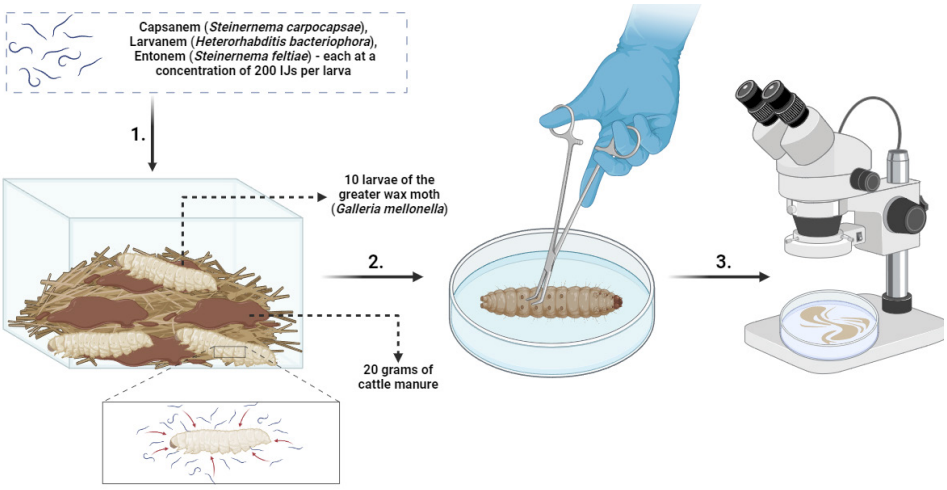

Fig.1 Scheme of the experiment.

the dead larvae were removed from the manure using sterile tweezers and placed on Petri dishes lined with filter paper. The dishes were then left in a Sanyo incubator (20°C and 60% humidity) for 24 hours. Subsequently, the dead larvae were dissected, and the presence of nematodes was determined using an Olympus SZX9 stereomicroscope (magnification × 3-5) (Fig. 1). In the control group, 30 larvae were used (in 3 containers with manure), following the same scheme as in the experimental treatments, except that instead of the nematode suspension, 1 ml of sterile water was added to the containers.

All procedures were carried out according to established laboratory protocols, and the containers were maintained in a Sanyo incubator (20°C and 60% humidity).

Statistical analysis

The normality of the data was checked using the Shapiro-Wilk test, and the homogeneity of group variances was examined using Levene's test. These preliminary tests showed that the assumptions of normality and homogeneity of variances were not met. Therefore, the Kruskal-Wallis test, a non-parametric alternative to ANOVA, was used to determine significant differences in the survival and insecticidal efficacy of the nematodes in cattle manure and their ability to infect *G. mellonella* larvae, depending on the day after nematode application.

When significant differences were found, post hoc Dunn's test with Bonferroni correction was used to identify specific groups that differed from each other. To obtain more comprehensive insights into the relationship between the efficacy of individual preparations and the time of their application, Principal Component Analysis (PCA) was conducted using the R function "prcomp". The PCA considered variables such as nematode species, time after application, and the mortality of *G. mellonella* larvae, enabling dimensionality reduction and providing a clearer understanding of the interrelationships between these factors.

All statistical analyses were performed using RStudio software, version 2024.04.2.

Results and discussion

Since the infective juveniles of nematodes are associated with the soil environment, it is important to verify whether they can survive in manure and retain their insecticidal properties. The conducted studies revealed differences in the persistence and efficacy of the analysed commercial preparations in cattle manure. Non-parametric tests and Principal Component Analysis confirmed these differences, highlighting variations in efficacy patterns among preparations.

The Capsanem preparation demonstrated high efficacy of 76.7% on the day of application, which increased to 92.9% after 2 days and reached 100% after 4 days. After this period, efficacy decreased significantly to 16.7% by day 6 and persisted at this level until the end of the experiment (Fig. 2). Statistical analysis revealed significant differences in efficacy between days 4 and 6 ($p<0.001$) and between days

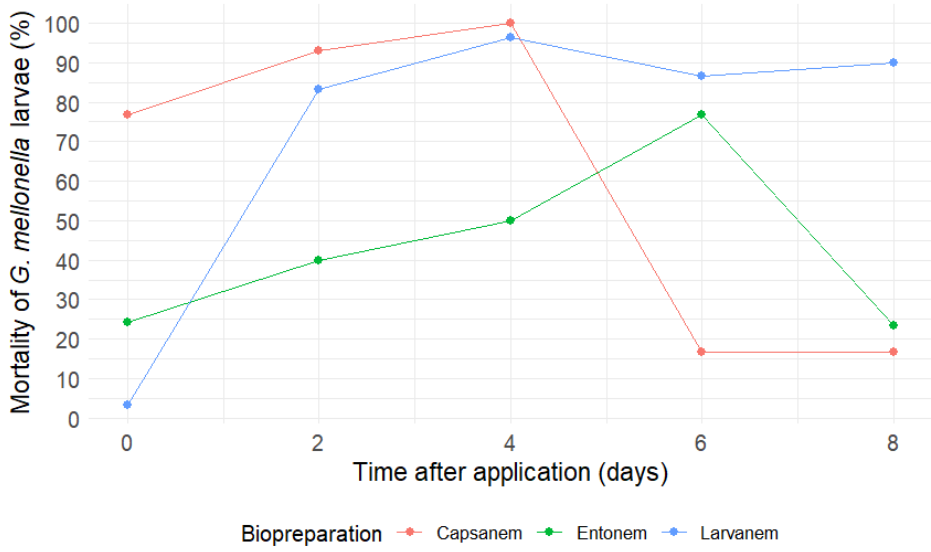

Fig. 2. Efficacy of three biopreparations in cattle manure over time.

Table 1. Comparison of mortality of *G. mellonella* larvae on different days after application of Capsanem

Comparison of experimental days	Mean mortality rate (first day in comparison)	Mean mortality rate (second day in comparison)	Adjusted p-value
Day of application – Day 2	0.77	0.93	1.00
Day of application – Day 4	0.77	1.00	0.33
Day of application – Day 6	0.77	0.17	<0.001
Day of application – Day 8	0.77	0.17	<0.001
Day 2 – Day 4	0.93	1.00	1.00
Day 2 – Day 6	0.93	0.17	<0.00001
Day 2 – Day 8	0.93	0.17	<0.00001
Day 4 – Day 6	1.00	0.17	<0.00001
Day 4 – Day 8	1.00	0.17	<0.00001
Day 6 – Day 8	0.17	0.17	1.00

4 and 8 ($p<0.001$), indicating that the efficacy of the nematodes decreases after 4 days (Tab. 1).

For the Entonem preparation, variable efficacy over time was observed. The mortality of *G. mellonella* larvae on the day of application was 24.1%, gradually increasing to 40% after 2 days and 50% after 4 days. The highest efficacy, 76.7%, was recorded 6 days after nematode application; however, efficacy decreased to 23.3% after 8 days (Fig. 2). Statistical analysis showed significant differences between the day of application and day 6 ($p<0.001$) and between days 4 and 6 ($p<0.001$), indicating a gradual increase in the efficacy of the preparation. Nevertheless, a statistically

Table 2. Comparison of mortality of *G. mellonella* larvae on different days after application of Entonem

Comparison of experimental days	Mean mortality rate (first day in comparison)	Mean mortality rate (second day in comparison)	Adjusted p-value
Day of application – Day 2	0.24	0.40	1.00
Day of application – Day 4	0.24	0.50	0.23
Day of application – Day 6	0.24	0.77	<0.001
Day of application – Day 8	0.24	0.23	1.00
Day 2 – Day 4	0.40	0.50	1.00
Day 2 – Day 6	0.40	0.77	<0.05
Day 2 – Day 8	0.40	0.23	0.97
Day 4 – Day 6	0.50	0.77	0.19
Day 4 – Day 8	0.50	0.23	0.19
Day 6 – Day 8	0.77	0.23	<0.001

Table 3. Comparison of mortality of *G. mellonella* larvae on different days after application of Larvanem

Comparison of experimental days	Mean mortality rate (first day in comparison)	Mean mortality rate (second day in comparison)	Adjusted p-value
Day of application – Day 2	0.03	0.83	<0.00001
Day of application – Day 4	0.03	0.96	<0.00001
Day of application – Day 6	0.03	0.87	<0.00001
Day of application – Day 8	0.03	0.90	<0.00001
Day 2 – Day 4	0.83	0.96	1.00
Day 2 – Day 6	0.83	0.87	1.00
Day 2 – Day 8	0.83	0.90	1.00
Day 4 – Day 6	0.96	0.87	1.00
Day 4 – Day 8	0.96	0.90	1.00
Day 6 – Day 8	0.87	0.90	1.00

significant decrease in efficacy was observed on the last day ($p<0.001$) (Tab. 2).

In contrast, Larvanem showed a unique pattern of efficacy compared to the other preparations. On the day of application, the mortality of *G. mellonella* larvae was only 3.3%, but increased significantly to 83.3% after 2 days, reaching a maximum of 96.4% on day 4. The high efficacy was maintained until the end of the study, with 86.7% on day six and 90% on day eight (Fig. 2). Statistical analysis revealed significant differences between the day of application and the other days of the experiment ($p<0.001$). These results indicate that the Larvanem preparation has the greatest persistence in cattle manure, maintaining high efficacy throughout the study period (Tab. 3).

A comparative analysis of the efficacy of the preparations based on the time elapsed since the application of IJs to the cattle manure revealed significant differences (Tab. 4). On the day of IJ application, Capsanem showed higher efficacy than both Entonem and Larvanem, with a statistically significant difference ($p<0.001$). On the second day after application, Capsanem and Larvanem were more effective than Entonem ($p<0.001$). This trend continued on the fourth day, with Capsanem and Larvanem

Table 4. Comparison of mortality of *G. mellonella* larvae depending on the preparation used on different days after application

Comparison of preparations	Mean mortality rate (first preparation in comparison)	Mean mortality rate (second preparation in comparison)	Adjusted p-value
		day of application	
Capsanem – Entonem	0.76	0.24	<0.001
Capsanem – Larvanem	0.76	0.03	<0.00001
Entonem – Larvanem	0.24	0.03	0.14
		two days after application	
Capsanem – Entonem	0.93	0.40	<0.001
Capsanem – Larvanem	0.93	0.83	0.64
Entonem – Larvanem	0.40	0.83	<0.001
		four days after application	
Capsanem – Entonem	1.00	0.50	<0.00001
Capsanem – Larvanem	1.00	0.96	1.00
Entonem – Larvanem	0.50	0.96	<0.00001
		six days after application	
Capsanem – Entonem	0.17	0.77	<0.00001
Capsanem – Larvanem	0.17	0.87	<0.00001
Entonem – Larvanem	0.77	0.87	0.65
		eight days after application	
Capsanem – Entonem	0.17	0.23	0.91
Capsanem – Larvanem	0.17	0.90	<0.00001
Entonem – Larvanem	0.23	0.90	<0.00001

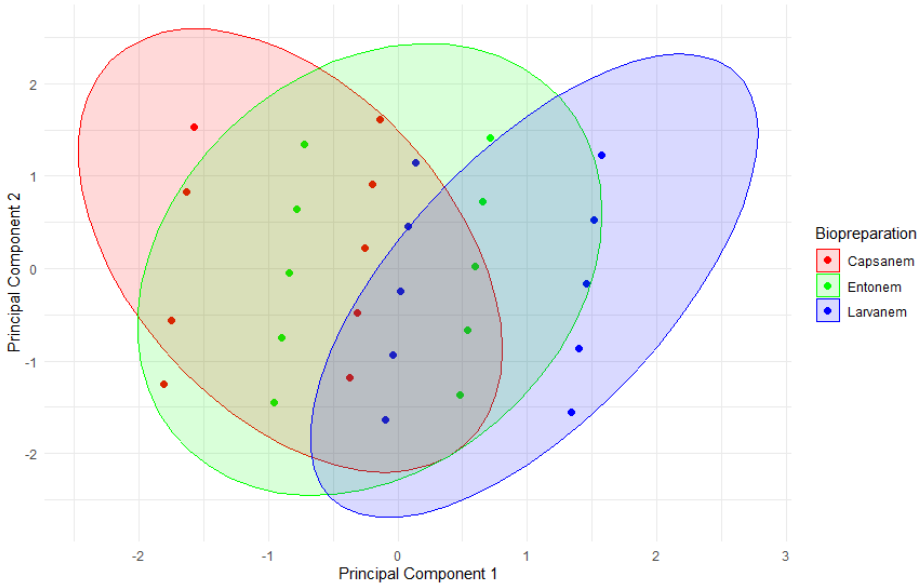

Fig. 3. Principal Component Analysis (PCA) score plot of three biopreparations: Capsanem, Entonem, and Larvanem (PC1 - 36.52%, PC2 - 33.37%).

showing superior efficacy compared to Entonem ($p<0.001$). However, on day six, Entonem and Larvanem outperformed Capsanem in terms of efficacy ($p<0.001$). Finally, on the eighth day after IJ application, Larvanem proved to be the most effective, significantly outperforming both Capsanem and Entonem ($p<0.001$). The PCA enabled the visualisation of complex relationships between the variables, while simultaneously reducing the dimensionality of the data and preserving the key information on variability. The results of the PCA indicate that the first two principal components together explain 69.89% of the total variance in the dataset (with PC1 accounting for 36.52% and PC2 for 33.37%), suggesting that they are sufficiently representative to describe the differences between the nematode preparations analysed. The PCA score plot (Fig. 3) clearly illustrates distinct groupings for the Larvanem and Capsanem preparations within the principal component space, indicating significant differences in their efficacy and persistence. In contrast, Entonem exhibited an intermediate pattern, characterised by an initial increase in efficacy followed by a subsequent decline over time. The PCA analysis confirmed that both the type of biopreparation and the time since application are key factors influencing the effectiveness of pest control.

Based on the results obtained, it was concluded that the most persistent and effective preparation for long-term use in cattle manure is Larvanem, which contains infective juveniles of *H. bacteriophora*. The variable efficacy of preparations containing different nematode species is supported by studies such as Smits [1996], who found that the persistence of nematodes after application varied by species, strain, and multiple parameters, with each species being characterised by a unique set of such parameters. Furthermore, the quality of the product itself and the conditions of application can significantly influence the persistence of nematodes in each environment. In studies conducted by Solano *et al.* [2004], it was demonstrated that among the nine species from the genera *Heterorhabditis* and *Steinernema*, only *S. carpocapsae* was able to survive in manure without losing its insecticidal properties. This is not confirmed by the results of this study, where *H. bacteriophora* had the longest survival time in cattle manure while maintaining the highest infection efficacy. Therefore, this preparation can be considered for a practical use against flies developing in animal manure.

This is particularly important, as studies by Archana *et al.* [2017] have shown that Heterorhabditidae are more effective than Steinernematidae in controlling *M. domestica* at both the adult and larval stages. The infective juveniles of *Heterorhabditis* species exhibit greater motility and probing potential [Pinnock and Mullens 2007], which enhances the efficacy of infection against houseflies and contributes to greater population reduction. In addition, the smaller size of *Heterorhabditis* IJs facilitates penetration and entry into the hemocoel of both larval and adult *M. domestica*.

Studies conducted by Shapiro *et al.* [1996] showed that animal manure reduces the pathogenicity of nematodes. Similar results were obtained by Belton *et al.* [1987], who found that both *Heterorhabditis* and *Steinernema* could survive in manure for only a few days and had limited potential for controlling *M. domestica*. However, the results of the authors' study demonstrated that all three species, from both the

Heterorhabditis and *Steinernema* genera, are capable of surviving in manure without losing their insecticidal properties. It is consistent with the results of Taylor *et al.* [1998], which showed that the species that survived the longest in manure (10 weeks) were *H. bacteriophora* and *S. feltiae*.

Comparing this study's results with the research conducted by Khwanket [2021] and Khwanket *et al.* [2024], who evaluated the persistence and efficacy of a native isolate of *H. bacteriophora* in 7-day-old cattle manure under laboratory conditions, revealed both similarities and differences. In this study, the mortality of greater wax moth larvae on the day of application was only 3.3%, whereas in Khwanket's [2021] study, the mortality on the day of application was 100%. Such discrepancy may be due to nematodes from commercial preparations needing more time to adapt and penetrate *G. mellonella* larvae. The lower mortality on the day of application could also be due to differences in application methods and manure characteristics. The 83.3% mortality of *G. mellonella* larvae observed in our study two days after application is comparable to the results obtained by Khwanket [2021] on the third day, where the mortality was 86.6%. Four days after application, larval mortality in our study wa higher (96.4%) compared to Khwanket's [2021] results on day five (90%). Both values indicate a consistently high efficacy of *H. bacteriophora* against *G. mellonella* larvae in cattle manure. Six days after application, the mortality in this study was 86.7%, while it reached 100% on the seventh day in Khwanket's [2021] . Eight days after application of the Larvanem preparation, this study reported 90% mortality of *G. mellonella* larvae, whereas on day 9 of Khwanket's [2021] study, mortality was only 40% indicating that *H. bacteriophora* nematodes from the commercial preparation exhibited greater persistence in cattle manure. The differences could result from the chemical composition of the manure used in in both pieces of research. Moreover, differences in humidity, temperature, and the method of storage of the manure prior to use in the trials could have affected the survival and efficacy of the nematodes.

This research has shown promising results that could contribute to the development of sustainable methods for controlling important livestock pests. The high efficacy of nematodes of the genera *Steinernema* and *Heterorhabditis* suggests the potential for their combined or alternating use with other insecticidal agents, which is particularly important for reducing the selective pressure that can lead to the development of resistant insect strains. The use of biopreparations containing EPNs in livestock production can help improve sanitary conditions, enhance animal welfare, and reduce the use of chemical insecticides.

Conclusions

The results of the studies indicate statistically significant differences in the insecticidal efficacy of the tested nematode preparations in fresh cattle manure. For the Capsanem preparation, the highest mortality of *G. mellonella* larvae (100%) was achieved 4 days after nematodes application, followed by a statistically significant

decrease. The Entonem preparation showed variable efficacy, with the highest *G. mellonella* larval mortality (76.7%) recorded on day 6 after nematode application. In contrast, the Larvanem preparation maintained a high level of efficacy from the second day after application until the end of the study (83.3-96.4%) and can therefore be recommended as a tool for long-term control of insects developing in cattle manure. The combination of Larvanem with Capsanem, characterised by high insecticidal efficacy immediately after application, could further improve the effectiveness of biological control in livestock buildings. Furthermore, the use of nematode preparations in manure will serve the dual purpose of controlling insects, particularly on organic farms where manure is used as a fertiliser during the initial growth phase of plants. This is when insect pests pose the greatest threat to crops.

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