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Research Article

Different Formulations of Entomopathogenic Nematodes for the Control of False Codling Moth

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ABSTRACT

Most previous research on entomopathogenic nematodes (EPNs) in South Africa has focused on the use of EPNs against false codling moth (*Thaumatotibia leucotreta*, FCM) in citrus orchards. No control measures in viticulture are currently directed at the soil-dwelling stages of FCM, presenting a niche that EPNs have the potential to fill. Despite significant progress in formulation techniques for EPNs, challenges such as limited shelf-life and microbial contamination remain problematic. In this study, locally sourced isolates of *Steinernema yirgalemense* and *Heterorhabditis bacteriophora* were produced through *in vitro* liquid culture and the shelf-life and pathogenicity of different formulations were assessed, along with the use of *Xenorhabdus indica* bacterial metabolites as a possible strategy to reduce microbial contamination. The results demonstrated that carboxymethylcellulose (CMC) and cellulose nanofibril (CNF) gel formulations enhanced the longevity of infective juveniles (IJs), while maintaining virulence over time. Soil bioassays showed no differences in FCM larval pathogenicity between the formulated and unformulated nematodes, with an infection rate of > 90% after 48 h.

Keywords: Bacterial metabolites, false codling moth, formulation, *Heterorhabditis* bacteriophora, pathogenicity, shelf-life, *Steinernema yirgalemense*

INTRODUCTION

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), is an important pest of citrus (Malan *et al*., 2018, Malan & Knoetze, 2024). FCM has developed resistance to certain pesticides (Malan *et al*., 2018). The soil-dwelling life stages of FCM in grapevine are not yet targeted for control. The use of entomopathogenic nematodes (EPN) as biocontrol agents could therefore act as a supplementary approach to existing insect management practices in vineyards.

To naturally suppress FCM, citrus growers can use a combination of biological control techniques and products within a system approach that has been successfully applied in South Africa (Moore, 2021). Multiple biologically based products are available on the market, using such organisms as bacteria, fungi and viruses. According to Gaugler (2002), entomopathogenic nematodes (EPNs), which are pathogenic parasites of insects, have emerged as one of the most effective nonchemical alternatives for the management of insect pests since the late 1970s. EPNs are especially important because of their safety in use, the relative ease of their mass production, their high reproductive capacity, and their specificity to the intended target pest. EPNs would ideally fit an integrated pest management system that is currently applied in grape production.

EPNs of the families Steinernematidae and Heterorhabditidae were first discovered in 1923, although they were not commercially available until 1981 (Poinar & Grewal, 2012). Since their discovery, EPNs have become increasingly used as biocontrol agents for insect pests. However, their high market value is heavily influenced by the costs that are associated with their manufacturing, formulation and storage.

The first study on the possible use of EPNs to regulate the soilborne life stages of FCM was conducted by Malan *et al*. (2011). Promising results were achieved during laboratory bioassays, when *Steinernema yirgalemense* Nguyen *et al*. applied at a concentration of 50 IJs/FCM larva were found to cause 100% mortality, marking the first successful report of EPN use against the soilborne stages of FCM in South Africa. Furthermore, in a semi-field trial, Steyn *et al*. (2019) applied four different EPN species (30 IJs/cm^2) in avocado, macadamia and lichi orchards to control FCM. The results from the study indicated that the highest FCM mortality (86%) was obtained with the use of *S. yirgalemense*.

The only stage of the EPN life cycle that can be formulated is that of the free-living, third larval stage, called the infective juveniles (IJs). The IJs penetrate their target host through natural openings, whereupon the symbiotic bacteria associated with the nematode are released, causing septicaemia and host death within 48 h (Poinar & Grewal., 2012). However, EPNs can only be delivered effectively and efficiently if the formulation and application techniques are carefully considered, along with knowledge of the characteristics and constraints of the biocontrol agent and target pest.

Although multiple companies have developed and commercially sold formulated EPN products, the literature on successful commercial production and formulation is scarce due to the commercial sensitivity of the technology involved. Thus, with the formulation and commercialising of local EPN isolates, there is minimal guidance and basic information available on aspects such as shelf-life and desiccation stress potential. According to Moore (2021), two exotic species, *Heterorhabditis bacteriophora* Poinar and *Steinernema feltiae* (Filipjev, 1934), have been registered for commercial use in South Africa, although no local EPN species are currently commercially available (Hatting *et al*., 2019; Moore, 2021). The predominant method of EPN application is through spraying equipment or irrigation systems as an aqueous suspension (Shapiro-Ilan & Dolinski, 2015). However, recent research has focused on employing polymer gels to improve liquid formulations.

Predictably, improved nematode formulations could be achieved through the addition of water-soluble polymers to form gels. One such polymer is carboxymethylcellulose (CMC), which is a derivate of cellulose. CMC is widely used in the food industry as a thickening or binding agent to increase shelf life (BeMiller *et al*., 2018; Schlesinger *et al*., 2026). The potential of CMC as a carrier for the formulation of EPNs is due to its biodegradable nature, solubility in water and low cost. Furthermore, cellulose nanofibrils (CNFs), also known as micro- or nano-fibrillated cellulose, are often used in pharmaceutical applications for formulations in the field of drug delivery, but also for tissue engineering and cosmetics, among others (Pradeep *et al*., 2022). However, the suitability of the nanofibrils to improve EPN formulations has not yet been explored and, due to their nontoxic and biocompatible nature, it is believed that the substance would be an ideal material for EPN applications.

The objective of this study was to evaluate different formulations of EPNs for improvement in terms of storage, whilst maintaining pathogenicity over time. Soil bioassays were conducted in the laboratory to test EPN efficacy using different formulations.

MATERIALS AND METHODS

Nematode culture

Infective juveniles (IJs) of *S. yirgalemense* were retrieved from the nematode collection at the Department of Conservation Ecology and Entomology at

Stellenbosch University, South Africa. *Heterorhabditis bacteriophora* SGI-170 was originally obtained from the Agricultural Research Council, Small Grain Institute, Bethlehem, South Africa (Claasen *et al*., 2024). The IJs of the two nematode species were mass-produced, using the technique of Dunn *et al*. (2022). A 200 µL aliquot of cryopreserved *Xenorhabdus indica* Somvanshi *et al*. (-80°C) and *Xenorhabdus* sp. SGI-170 (-8°C) (Claasen *et al*., 2025a, b) was inoculated into 30 mL sterilised tryptic soy broth (TSB) and transferred to a junior orbital shaker (Benchmark's ORBI-SHAKER™JR) (140 rpm) in a 28°C growth chamber for 48 h. A total of 600 µL (2% v/v) of the TSB bacterial culture was added to 30 mL of nutrient complex media containing the following ingredients: 4 g NaCl, 0.15 g $CaCl_2$, 0.35 g KCl, 0.1 g $MgSO_4$, 15 g yeast extract, 20 g dried egg yolk powder and 24 mL canola oil per litre of water in 250 ml Erlenmeyer flasks and placed on shakers (Labotec) for 48 h at 25°C. Erlenmeyer flasks, containing complex media and bacteria, were inoculated from stock cultures of *S. yirgalemense* and *H. bacteriophora*, and then incubated on shakers at 25°C for 14 days. Subsequently, the flasks were transferred to a temperature of 14°C and agitated at 140 rpm for three days to allow the IJs to stabilise prior to use. The experiment was repeated on a different test date with a freshly prepared batch of IJ of the two EPN species.

Concentration of IJs

One 30 mL flask each of the *in vitro*-cultured IJs of *S. yirgalemense* and *H. bacteriophora* was separated from their bacteria, secondary metabolites and waste by means of a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). The nematode paste was collected from the sieve and rinsed into a 100 mL glass beaker with sterilised Ringer's solution. One mL of the suspension was then pipetted into a flask, which was filled with 100 mL distilled water. The five-drop method was used to determine the average number of nematodes in 1 mL of the original suspension (Glazer & Lewis, 2000).

Formulations

Carboxymethylcellulose

The IJs of *S. yirgalemense* and *H. bacteriophora* were pretreated with 15% glycerol in water by leaving them in the glycerol solution for 30 min. CMC (5%) was then added to 500 mL glass beakers. An antimicrobial agent, trans-cinnamaldehyde (TCA) (0.002%), was added to the CMC (Kary *et al*., 2021; Schlesinger *et al*., 2026). The nematode-glycerol suspension was added and mixed thoroughly using an electric hand mixer (Sunbeam) to form a homogeneous gel with an IJ concentration of 7.5×10^6 per mL for *S. yirgalemense* and of 4×10^6 per mL for *H. bacteriophora*. The formulated products were divided into 35 mL plastic containers and stored at 14°C for a period of 21 days. Nematodes in distilled water served as a control. There were six containers in total, with all experiments being run in duplicate and repeated on different test dates with a fresh batch of nematodes.

Diatomaceous earth

IJs of *S. yirgalemense* were formulated in diatomaceous earth (DE) (Celite 209 Imerys Refractory Minerals SA (Pty) Ltd), following the combined method of Kagimu and Malan (2019) and Nxitywa and Malan (2022). The following ingredients were used in the preparation of the DE formulation: PEG 600, Tixosol 38 A (anticaking agent), TCA (0.002%) as an antimicrobial agent to prevent microbial contamination and, lastly, the IJs. A formulation without the addition of the antimicrobial agent TCA served as a control. The IJs were cleaned using the previously prescribed method, with the paste being collected and tapped dry with a piece of tissue paper on the back of the sieve. The ingredients were then combined manually with the IJ paste in the manner described below: for 15 g of IJ paste, 11 g of DE, 2 g of Tixosol (38 anticaking agent), 2 g of PEG 600 and 0.02% of TCA was used. A final concentration of DE formulation of 9.0×10^5 IJs/g of *S. yirgalemense* was attained. Afterwards, the formulation was added to watch-glass Petri dishes (3 cm), using six samples in total, with a control treatment without the addition of antimicrobial agent. The parafilm-sealed containers were then placed in a larger container, lined with moist tissue paper to maintain high humidity levels, and stored at 14°C. The formulation was evaluated after seven, 14 and 21 days, with the experiments being repeated on another test date, using a fresh batch of nematodes.

Alginate beads

Alginate beads were developed by using the modified technique of Kim *et al*. (2021). A nematode suspension containing 6.5×10^6 IJ per mL was used. Based on preliminary experiments, alginate-glycerol (15% glycerol, v/v; 0.5% sodium alginate, w/v; 0.5% dye, v/v; 0.002% TCA v/v) and $CaCl_2$-glycerol (2% $CaCl_2 \cdot 2H_2O$, w/v: 15% glycerol, v/v) were prepared. The nematode suspension was added to the alginate-glycerol solution and kept at room temperature for 60 min. Using a plastic syringe, 100 µL of alginate-glycerol solution with nematodes was dripped into the $CaCl_2$-glycerol. The capsules produced were taken out of the solution after 15 min and rinsed with distilled water. The beads were then tapped dry with tissue paper and transferred evenly into six plastic containers, lined with paper towels to soak up the unwanted moisture, and placed in a plastic container for storage at 14°C. Beads without the addition of antimicrobial agents served as a control. The formulation was evaluated after seven, 14 and 21 days by determining the percentage live and dead IJ. The experiments were repeated on another test date, using a fresh batch of IJ.

Survival of formulated IJs

The survival of the formulated IJs with glycerol and an antimicrobial agent was assessed weekly by means of counting the first 100 IJs with the aid of a stereomicroscope to determine the percentage mortality. Samples were evaluated after seven, 14 and 21 days. The percentage of IJs that survived at each assessment point was calculated by means of dissolving 1 mL of the formulated product (CMC and DE) in 30 mL of distilled water in vacutainer tubes. The suspension was shaken rapidly for 2 min and then left for 60 min to allow the nematodes to rehydrate and escape the formulation. For the alginate bead formulation, three beads were placed in an Eppendorf tube and crushed, whereafter the tube was filled with 30 mL distilled water and left for 60 min. One mL of each suspension was transferred to 5 cm Petri dishes and examined under a stereomicroscope.

Pathogenicity

A 24-well bioassay tray (CELLSTAR®, Cat. No. 662160) was used as the test arena to determine the pathogenicity of the *S. yirgalemense* IJs formulated with CMC and TCA after 21 days of storage. Every alternate well was lined with a circular filter paper (12.7 mm in diameter) (Ahlstrom Munksjö) to ensure that the applied formulation was evenly distributed using five trays in total and 12 wells per tray. A control treatment was prepared containing water only, as well as another control containing a fresh batch of unformulated nematodes. A concentration of 100 IJs/50 mL of formulated *S. yirgalemense* IJs in tap water was used to inoculate each of the 12 wells. *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (mealworm) larvae (n = 60) were added to each well of the bioassay plate and, to prevent the larvae from escaping, the plate was covered with a glass pane. To maintain humidity, the trays were placed in a moisture chamber, and placed in a growth chamber for 72 h at 25°C. Thereafter, mortality was assessed by observing insect movement, with nematode infection visually confirmed through the dissection of the FCM larvae using a stereomicroscope. A freshly prepared nematode inoculum that had undergone formulation and treatment was used for the bioassay on a different test date.

Bacterial strains

Xenorhabdus indica 157-C was isolated from *S. yirgalemense* (Ferreira *et al*., 2014), collected from a citrus orchard in the Mpumalanga province of South Africa (Malan et al., 2011). *Photorhabdus* sp. SGI-170 was isolated as an unknown species from *H. bacteriophora* (Claasen *et al*., 2025a) from an apple orchard in the Free State province (Hatting *et al*., 2009). Both bacterial isolates were maintained on nutrient agar and pure colonies were stored at -80°C in 40% (v/v) glycerol.

Bacterial metabolite production

Single colonies of *Photorhabdus* sp. SGI-170 and *X. indica* 157-C were streaked onto NBTA plates (Dreyer *et al*., 2017) consisting of nutrient agar, supplemented with triphenyl tetrazolium chloride (TTC) at a concentration of 0.004%, and bromothymol blue, at a concentration of 0.025% (w/v). The plates were incubated for a minimum of 48 h at 30°C. After incubation, a single blue colony was isolated and inoculated into 5 mL TSB. Inoculated test tubes were placed on an orbital shaker for 48 h at 30°C.

Amberlite™ XAD16N beads were used to extract hydrophobic compounds from *Photorhabdus* sp. and *X. indica* following the protocol of Van Staden (2015). Isopropanol (80% v/v) was used to activate the beads, which were then incubated on an orbital shaker (100 rpm) at 8°C for 30 min. Afterwards, the beads were taken out, cleaned with ddH_2O, and 5 g were aliquoted into McCartney bottles and autoclaved. The McCartney bottles containing the activated beads received the 5 mL incubated TSB cultures. The beads, inoculated with their respective bacteria on tryptone soy agar (TSA), were spread-plated and incubated at 26°C for 96 h. Afterwards, the beads were scraped from the TSA plates and rinsed with ddH_2O while being stirred for 40 and 60 min, respectively, at 8°C. Liquid was taken out of the beads using a vacuum and a filter. The beads were then resuspended in 25 mL 30% EtOH per 5 g beads and shaken at an orbital speed of 100 rpm for 45 min at 8°C. Following the removal of the ethanol using a vacuum suction, the beads were thoroughly washed with 1 L of ddH_2O. The beads were resuspended in isopropanol with 40 mL/5 g beads, which was then shaken at 100 rpm for 45 min at 8°C to elute the amphipathic compounds. The isopropanol was removed using a RotaVapor® R114, Büchi rotary evaporator, after being filtered through a 0.45 µM cellulose nitrate filter. The concentrated eluent was then stored overnight at -80°C and subsequently lyophilised for 48 h. The lyophilised eluent was referred to as solid metabolite formulation, and the eluent that did not undergo lyophilisation was referred to as liquid metabolite formulation.

Formulation with metabolites

Metabolites of *X. indica* were tested for compatibility with *S. yirgalemense* in a CMC formulation. A suspension of IJs, with a concentration of 65 000 IJs/mL, was prepared and pretreated with 15% glycerol in water in a 100 mL measuring cylinder for 30 min. The formulation was prepared by adding the IJ–glycerol suspension to 5% CMC in a 500 mL glass beaker. Metabolites, at concentrations of 700 mg solid or 2 mL liquid, were added to the glass beaker and stirred with an electronic hand mixer to form a homogenous gel. The formulated products were divided into 35 mL plastic containers, placed in a big

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plastic container with moist tissue paper, and stored at 14°C for a period of 21 days. Tap water was used that contained solely nematodes at a concentration of 2 000 IJ/mL. The experiment was repeated using a fresh batch of nematodes.

Formulation with cellulose nanofibrils formulation

CNFs were prepared at the Division of Polymer Science, Stellenbosch University according to a previously published procedure (Motloung *et al.* 2024). The CNFs were autoclaved and added to a 50 mL nematode–water suspension containing a total of 6.5 × 10^6 *S. yirgalemense* IJs. The formulated product, which was divided into six smaller 3 cm glass crucibles, was placed in a 30 cm glass Petri dish, together with moist cotton balls to increase the humidity; it was then placed at room temperature for 21 days. There were six samples in total, and the treatments were assessed weekly. The experiment was repeated with a fresh batch of *S. yirgalemense*.

Soil screening

A screening bioassay of the different formulations, using orchard soil, was performed in the laboratory. Soil was obtained from a citrus orchard at Welgevallen Experimental Farm, Stellenbosch. The orchard soil consisted of fine to medium sand with a 10% clay content. The soil was not sieved or sterilised before the trial was conducted so as to retain its natural properties. The experimental layout consisted of 24 plastic containers (25 cm × 16 cm) containing 2 kg of soil each, with six treatments and four replicates as the test arena. The most successful formulations from the survival screening were used to evaluate their effectiveness against final-instar FCM larvae contained in cages and buried in the soil.

The six treatments were: A) *S. yirgalemense* with CMC and TCA, B) *H. bacteriophora* with CMC and TCA, C) *H. bacteriophora* with CNFs, D) *S. yirgalemense* with water (control), E) *H. bacteriophora* with water (control), F) water only (control). Bioassay chambers to contain the FCM larvae were constructed using PVC pipe cut to rings of 5 cm in height and 10 cm diameter. The open ends were closed with rounds of ± 700 µm stainless steel mesh glued to the PVC pipe ring on one side using nonsolvent hot glue. Moist soil from the orchard was loaded into the bioassay chamber, together with 20 final-instar FCM larvae. The other end of the PVC circle was then also sealed with mesh. Test populations across four replicates of a treatment (A to F) consisted of a total of 480 insects. An individual bioassay chamber was buried in each plastic container, approximately 2 cm below the soil surface. The soil in each of the plastic containers was then wetted with 30 mL dH_2O distilled water, using a mist sprayer, a few hours before the addition of the bioassay chambers and during the application of nematodes in formulation.

In vitro-produced *S. yirgalemense* and *H. bacteriophora* IJs were formulated in 5% CMC, containing 15% glycerol and 0.002% TCA as an antimicrobial agent (Schlesinger *et al.*, 2026). *Heterorhabditis bacteriophora* IJs were formulated with CNFs, prepared as described, and stored overnight at 14°C. Upon application, the CMC was dissolved in water, and the CMC and CNFs were applied at a rate of 20 IJs/cm^2, with 8 000 IJs in total per container. The control treatments received IJs with water and water only. The deposition of the IJs/cm^2 was calculated on the 400 cm^2 surface area of the plastic container. Each treatment was sprayed with a hand mist sprayer to obtain 20 IJs/cm^2 of soil surface. The plastic containers were closed with a lid to retain the relative humidity and stored at 25°C. After 24 h, 20 mL of distilled water was sprayed into each container to ensure that the soil did not dry out.

The bioassay chambers were removed approximately 48 h after application of the treatments and sieved to remove the larvae. The larvae were rinsed with distilled water to remove the external IJs, then tapped dry with a paper towel, placed in Petri dishes, and further incubated for an additional 24 h at 25°C. Mortality and infection were assessed 72 h after conducting the experiment by means of visual colour observation, and infection was confirmed by means of dissecting the insects under a stereomicroscope, which enabled the presence of nematodes to be confirmed visually.

Statistical analysis

Differences in mean percentage mortality between laboratory bioassay EPNs and the control treatment were tested using one-way analysis of variance (ANOVA), with a Mann-Whitney test and descriptive statistics. Differences in the mean percentage survival of IJs of *S. yirgalemense* in different formulations over 21 days were tested, using a mixed-model ANOVA, with an LSD post-hoc test and descriptive statistics. The same statistical analysis as stated above was performed for the metabolite, as well as for the cellulose nanofiber treatments.

RESULTS

Formulations

The effects of different formulations on the survival of *S. yirgalemense* IJs during 21 days of storage at 14°C were evaluated. A significant effect of formulation and storage duration on IJ survival was detected ($F_{6,4} = 33.49$; $P = 0.001$), with survival generally declining over time (Fig. 1).

After seven days of storage, IJ survival in the control treatment (water only) was 80%. Survival in the CMC

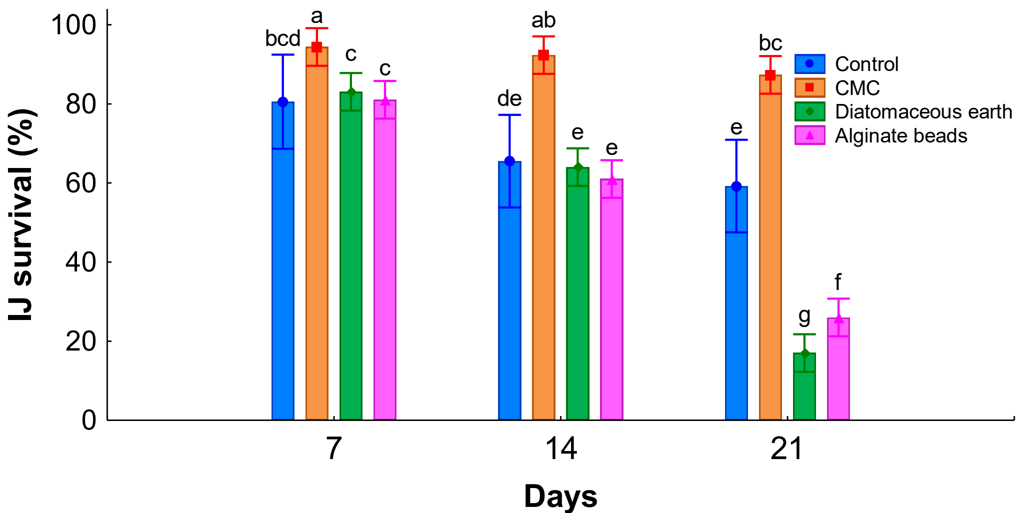

FIGURE 1 Mean survival (95% confidence interval) of the infective juveniles (IJs) of Steinernema yirgalemense formulated in in carboxymethylcellulose (CMC)-based gel, diatomaceous earth, alginate beads and water-only formulations, supplemented with trans-cinnamaldehyde, after seven, 14 and 21 days at 14°C (F6, 4 = 33.49; P = 0.001). Different letters on the bars indicate significant differences (P < 0.05) found between the formulations and the survival of the IJs.

formulation (94.33 ± 0.52%) was significantly higher than that in the control (P = 0.034). No significant differences were detected between the control and the DE (83.00 ± 3.52%; P = 0.702) or alginate bead formulations (81.00 ± 3.22%; P = 0.940). Furthermore, no significant difference was detected between the DE and alginate bead formulations (P = 0.559) (Fig. 1).

After 14 days, IJ survival in the control declined to 65%. Survival in the cellulose formulation remained high (92.33 ± 1.86%) and was significantly higher than that of the control, DE (64.00 ± 6.42%) and alginate bead formulations (61.00 ± 4.73%) (P < 0.001). No significant difference was detected between the DE and alginate bead formulations (P = 0.485) (Fig. 1).

After 21 days of storage, IJ survival in the control was 59%. Survival in the DE and alginate bead formulations declined markedly – to 17.00% ± 5.22% and 26.00% ± 15.89%, respectively. In contrast, IJ survival in the CMC remained high, at 87.33% ± 1.63%. Significant differences in IJ survival were detected among all treatments (P < 0.001) (Fig. 1).

Pathogenicity

Natural mortality in the *T. molitor* larval control group (water only) was low, ranging from 0% to 1.6% after 72 h. There was no significant difference in infectivity between the unformulated nematodes in water and the CMC-formulated IJs with added TCA ($F_{1,8}$ = 3.189; P = 0.112). The unformulated nematodes achieved an infectivity of 96.64% ± 2.64%, compared with 89.97% ± 2.64% for the CMC-formulated treatment (data not shown).

Formulation with metabolites

The IJ survival rate was significantly ($F_{4,3}$ = 1.86; P = 0.336) affected by the different metabolite treatments and storage duration (in days) (Fig. 2). When comparing the mean survival of *S. yirgalemense* over a period of 21 days, the IJ survival rate was found to decreased significantly for both metabolite treatments from day seven to day 14 (P < 0.001) and from day 14 to day 21 (P < 0.001). For the control, no significant difference (P = 0.183) was detected between seven and 14 days, although a significant difference (P < 0.001) was detected between seven, 14 and 21 days. The mean survival rate of IJs in the control was 80% after seven days, decreasing to 50% after 21 days. The survival of the IJs in the liquid treatment decreased from 93.16% ± 1.617% after seven days to 47.5% ± 6.89% at 21 days. The viability of the IJs in the solid treatment achieved a survival rate of 91.83% ± 2.14% after seven days and 54.83% ± 7.62% after 21 days.

Formulation with cellulose nanofibrils

When analysing the main effects of novel cellulose nanofibrils on the percentage IJ survival of *S. yirgalemense* and *H. bacteriophora* over 21 days, a significant difference ($F_{6,53}$ = 200.78; P < 0.001) was detected between the IJ treatments in the presence and absence of CNFs. On day 7, the survival rate of *S. yirgalemense* (SyC) in the water-only control was 75%, which differed significantly (P < 0.001) from that of the *H. bacteriophora* control (HbC), which was 70%. Significant differences (ρ < 0.001) in the survival rates were also recorded for both species when the IJs were formulated in the absence (i.e. SyC and HbC) and presence of the nanofibrils (i.e. SyF and

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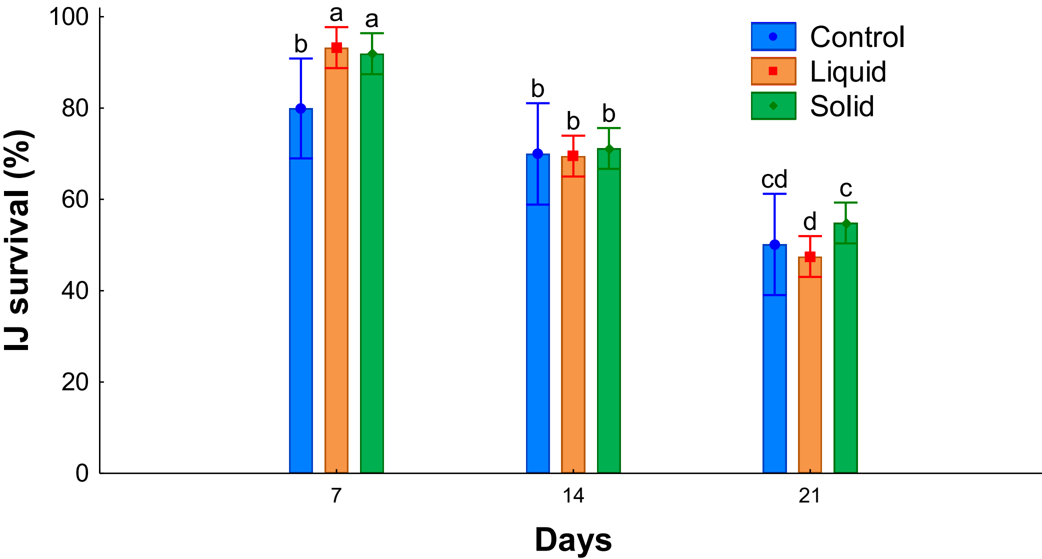

FIGURE 2 Mean survival (95% confidence interval) of the infective juveniles (IJs) of Steinernema yirgalemense formulated in carboxymethylcellulose (CMC)-based gel with the addition of liquid and solid metabolites of Xenorhabdus indica after 21 days at 14°C ($F_{4,3} = 1.86$; $P = 0.336$). Different letters on the bars indicate the significant differences ($P < 0.05$) between the treatments.

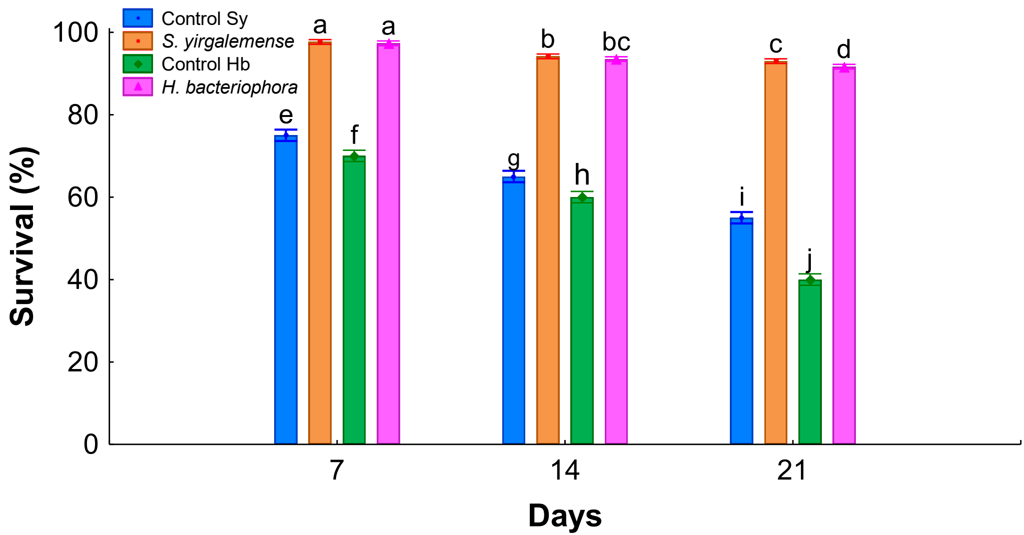

FIGURE 3 Mean survival rate (95% confidence interval) of infective juveniles (IJs) of Steinernema yirgalemense (Sy) and Heterorhabditis bacteriophora (Hb) formulated in cellulose nanofibril (CNF)-based gels, after seven, 14 and 21 days at 25°C ($F_{6,53} = 200.78$; $P < 0.001$). Different letters on the bars indicate the significant differences ($P < 0.05$) in survival between the formulated and unformulated nematodes.

HbF). For the nanofibril treatment, after seven days storage, no significant difference (P = 0.414) was found between the IJ survival of *S. yirgalemense* (97.66% ± 0.52%) and that of *H. bacteriophora* (97.33% ± 0.52%) (Fig. 3).

After 14 days, the survival rate of *S. yirgalemense* in the control was 65%, whereas the HbC survival rate was 60%. A significant difference was detected (P < 0.001) between the SyC and the HbC, as well as between the SyC and the SyF (P < 0.001) and the HbC and the HbF (P < 0.001). However, no significant difference (P = 0.102) was detected between the SyF (94.17% ± 0.98%) and the HbF (93.50% ± 1.22%) (Fig. 3).

After 21 days at 25°C, the survival rate of IJs in the SyC was 55%, whereas, for the HbC, the survival rate was found to be 40% (P < 0.001). When analysing the nanofibril treatment (P < 0.001), survival rates of 93% ± 0.89% and 91.66% ± 1.03% were obtained for *S. yirgalemense* and *H. bacteriophora*, respectively. A significant difference (P < 0.001) was detected between the two control treatments and the two nanofibril treatments (Fig. 3).

Soil screening

Between 85% and 100% of FCM larvae were recovered from the cages after 48 h exposure to the different treatments. After 72 h, the mean infectivity of FCM, when treated with *S. yirgalemense*, CMC and TCA (Sy + CMC + TCA) was 93.15% ± 2.6%. No significant difference (P = 0.484) was detected between Sy + CMC + TCA and the *H. bacteriophora*, CMC and TCA (Hb + CMC + TCA) treatment, the *S. yirgalemense*-water control (Sy control) (P = 0.501), the *H. bacteriophora*-water control (Hb control) (P = 0.338), and the *H. bacteriophora* cellulose fibres (Hb + fibres) (P = 0.156). The same results are seen for the *S. yirgalemense* control, with an infectivity of 95.67% ± 2.6%. No significant difference (P = 0.179) was detected between the Sy control, Hb + CMC + TCA, the Hb control (P = 0.112) and the Hb + fibres (P = 0.437) treatments (Fig. 4).

When analysing the Hb + fibre treatment (P = 0.001), an infectivity rate of 98.6% ± 2.6% was obtained. A significant difference (P = 0.042) was detected between the Hb + fibre treatment and the Hb + CMC + TCA treatment (90.52 ± 2.6%), as well as between the Hb + fibre treatment and the Hb control (P = 0.024), with an infectivity rate of 89.53% ± 2.6%. No significant difference (P = 0.790) was detected between the Hb + CMC + TCA treatment and the Hb control (Fig. 4).

DISCUSSION

Maximum IJ survival and pathogenicity should be achieved by the storage and formulation of EPNs. Their short shelf-life has been a restraint (Grewal, 2002) since EPNs were first used commercially as a biocontrol agent of insect pests, and microbial contamination adds to a decrease in their survival. One of the most crucial steps in the development of EPN formulations is choosing the proper carrier for IJ trapping to reduce their metabolic activity. The current study reports on the suitability of different EPN formulations of *S. yirgalemense* and *H. bacteriophora* and the success of TCA and metabolites to be used as antimicrobial agents in EPN formulations. The findings of this study demonstrate how an effective IJ formulation can lengthen the lifespan of IJs without negatively affecting pathogenicity.

Three different formulations were selected as potentially successful formulations that can be used in commercial settings. Alginate beads initially showed promising results after seven days of storage at 14°C with a viability > 80%; however, the survival of the IJs decreased over time, achieving a survival rate < 30% after 21 days. Hiltpold *et al.* (2012) demonstrated how alginate beads showed good prospects against western corn rootworm in laboratory experiments; however, EPNs were found to be poorly retained in the soft capsule (Kagimu & Malan, 2019). Due to this fact, Kim *et al.* (2015) adjusted the formulation properties to improve the hardness of the beads, with a 40% improvement in retention of EPNs. In a more recent paper, the authors adopted two new strategies and adjusted the formulation (used during this study) even further by adding glycerol and using sodium alginate together with $CaCl_2.2H_2O$ (Kim *et al.*, 2021). Although it is important to retain the EPNs inside the capsule during the storage period, the release of the EPNs from the formulation into the agricultural setting where they are applied is of more importance to achieve the successful control of insect pests. Therefore, the results in this study differ from those of Kim *et al.* (2021), as quiescence was not broken by adding the beads to water and the nematodes did not spontaneously escape from the beads until they were mechanically crushed. In addition, although

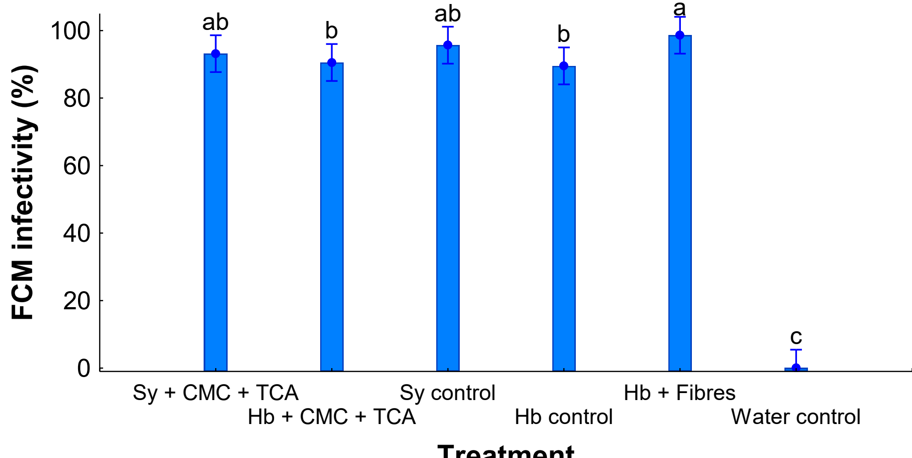

FIGURE 4 Mean percentage infectivity (95% confidence interval) by different formulations of Steinernema yirgalemense (Sy) and Heterorhabditis bacteriophora (Hb) with carboxymethylcellulose (CMC) with trans-cinnamaldehyde (TCA) for last-instar false codling moth (FCM) larvae in soil, after 72 h at 25°C ($F_{5,18}$ = 216.75; P < 0.001). Different letters on the bars indicate the significant differences (P = < 0.05) in infectivity between formulations.

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the beads were patted dry with tissue paper, they constantly release free water. As a result, the high moisture content may promote contamination of the formulation after 14 days, despite the application of TCA as an antimicrobial agent. Therefore, it is assumed that contamination caused the poor survival of the *S. yirgalemense* IJs. In contrast to the findings in this study, Nxitywa and Malan (2022) investigated the encapsulation of IJs in sodium alginate beads that were stored at 25°C and 14°C over a period of four weeks and achieved *Galleria* mortality rates of > 94% and > 86%, respectively. The pathogenicity of the IJs of *S. yirgalemense*, *Steinernema jeffreyense* Malan, Knoetze & Tiedt and *H. bacteriophora* stored in alginate beads was evaluated against the final instar of *Galleria* larvae, revealing a high percentage of mortality, of 98%, in the case of *S. yirgalemense*.

When using DE for the formulation, satisfactory results were also recorded after seven days of storage at 14°C, with a viability of > 80%; however, as time progressed, IJ survival decreased substantially, achieving a survival rate of < 20% after 21 days. In a recent paper by Kagimu *et al.* (2022), the viability of *S. yirgalemense* at room temperature when formulated with DE achieved a mean survival rate of 70% after four weeks. Adding to the authors' results, a study done by Nxitywa and Malan (2022) found that a high concentration of *S. yirgalemense* (4×10^6 IJs/g) was formulated with DE for a period of four weeks. The mean survival rate of 81% was obtained at both 25°C and 14°C, concluding that the shelf-life was maintained above a 70% mean survival rate by week 4. The results of this study are not consistent with the findings of Kagimu *et al.* (2022) or Nxitywa and Malan (2022), and the reason for the poor survival rate of IJs formulated in DE remains unclear. However, it is likely that too much moisture was lost to counteract desiccation. Due to the lack of survivability of the IJs stored in alginate beads and DE, these two formulations were not investigated further.

CMC emerged as a more effective and promising formulation for *S. yirgalemense* by maintaining survival rates of > 85% after 21 days. The results of this study are coherent with those of Kary *et al.* (2021), who reported that CMC is an efficient substrate for formulation, especially for *Steinernema carpocapsae*. After six months of storage at 15°C, they found that *H. bacteriophora* and *S. carpocapsae* had respective survival rates of 53% and 54%, whereas the equivalent values at 25°C were 32% and 43%. In addition, Kary *et al.* (2021) suggest that antimicrobial agents other than formaldehyde, which had a profoundly negative effect on IJ survival, may yield more promising results in CMC formulations. As a results, TCA was used as a proxy (Schlesinger *et al.*, 2026). The experiment in this study was terminated after 21 days. However, according to the research by Kary *et al.* (2021), it is predictable that the formulation can be stored for an extended period. In the wake of the results obtained with the *S. yirgalemense* CMC formulation, a bioassay was conducted after 21 days to determine if the IJs were still pathogenic. The formulation was evaluated against *T. molitor* larvae, and > 89% infectivity of the *T. molitor* larvae was achieved after 21 days of storage, which is in agreement with the results of Kary *et al.* (2021).

CMC was used as the standard formulation for further investigation. Even though TCA served as an effective antimicrobial agent, further research was conducted on the potential of using EPNs' own secondary metabolites as antimicrobial agents. In previous studies it was shown that secondary metabolites of EPNs exhibit strong antimicrobial activity (Fang *et al.*, 2008; Kumar *et al.*, 2014; Shan *et al.*, 2020). However, this study investigated the use of EPN secondary metabolites in a formulation as an alternative to TCA. EPN symbionts such as *Photorhabdus* spp. and *Xenorhabdus* spp. have antibacterial traits that inhibit bacterial growth by means of interfering with a variety of target systems that prevent the target bacteria from proliferating. The development of the bacteria is then compromised, as the symbionts cause membrane and metabolic pathway destruction (Tomar *et al.*, 2022). There are no reports in the literature of using EPN secondary metabolites in EPN formulations as antimicrobial agents; however, Kagimu *et al.* (2022) have suggested that the bioactive secondary metabolites associated with EPNs may be effective antimicrobial agents in EPN formulations. In this study, the results were initially promising. After seven days of storage, *S. yirgalemense* IJ survival of > 90% was achieved for both the solid and liquid metabolites in CMC formulation. However, survival decreased over time and, after 21 days, survival rates were down to > 50% for both the solid and liquid metabolites. The experiment was only conducted with *X. indica*, as the manufacturing process of *Photorhabdus* was hindered because of its viscosity, which prevents successful filter sterilisation. The viscosity of the sample might be explained by the high number of sugars, extracellular polymeric substances (EPS) and visible lipids, which resulted in a "slime" after lyophilising, rather than a powder. In addition, a set concentration of metabolites was used during this study. Additional research is recommended, and it is probable that more suitable results would be achieved if different concentrations of the metabolites are screened for their compatibility.

The most prevalent EPN found all over the world, including in South Africa, is *H. bacteriophora* (Hatting *et al.*, 2009; Malan *et al.*, 2011). Therefore, during this study, the main aim was to find a suitable and successful formulation for *H. bacteriophora*. It is known that CMC is a derivative of cellulose (Kary *et al.*, 2021) and, due to the promising results achieved, an alternative formulation consisting of cellulose

was explored. An EPN formulation consisting of cellulose nanofibril or polymer fibres has not yet been evaluated. When comparing the results of the *S. yirgalemense* and *H. bacteriophora* CNF formulations, no significant difference was detected between the two formulations after seven days, with a survival rate of > 95%. However, a significant difference was seen between the formulations and the control treatments, which had survival rates of 75% and 70%, respectively. Compared to the control treatments, the results showed that CNFs were an efficient substrate for the formulation of EPNs, as *S. yirgalemense* and *H. bacteriophora*, with the two achieving a survival rate of > 90% after 21 days of storage. In contrast, the control treatments maintained survival rates of > 40%. Even though both species maintained a survival rate of > 90% after formulation, the *S. yirgalemense* control obtained a higher survival rate than the *H. bacteriophora* control. This result may be attributed, at least in part, to the generally longer lifespan of *Steinernema* compared to *Heterorhabditis*. In support of these findings, Abate *et al*. (2019) screened the virulence and survival of native South African EPN isolates in different substrates and found that the survival of *Heterorhabditis* species (44% to 53%) was lower than that of *Steinernema* species (61% to 84%), based on direct nematode counts after 15 days. Kotliarevski *et al*. (2022) evaluated the success of individual coatings of titania (TiO_2) nanoparticles based on oil-in-water Pickering emulsion on *S. carpocapsae*, achieving equivalent results to control aqueous suspensions after 30 days, with an IJ survival of > 95%. Similarly to the current study, Van Zyl *et al*. (2013) explored the use of coconut fibres for the storage of *H. zealandica*; however, the results obtained were not as promising, as survival decreased severely and the IJ survival obtained after 21 days was 2.25%.

During the preliminary experiments, *S. yirgalemense* and *H. bacteriophora* were stored for two months in CNFs at room temperature (21°C to 24°C). IJs maintained both viability and pathogenicity, resulting in > 85% mortality of the FCM larvae. The present study indicates that CNFs show promise as a formulation for EPNs; however, further studies are required to fully assess their suitability.

Malan *et al*. (2018) concluded that EPNs have exciting potential to control FCM, and therefore the success of the CMC and CNF formulations were tested in soil from an orchard in a bioassay. The species examined in this study were local isolates that are less adapted to survive in cold climates because they are accustomed to the comparatively mild climate of South Africa (Malan & Hatting, 2015). It is known that the temperature range required for survival and infection will vary depending on the EPN species and its place of origin (Kaya, 1990; Kaya & Gaugler, 1993). In addition, Kagimu *et al*. (2017) identified that heat and cold tolerance affect IJs survival at lower temperatures as IJs' physiological activity and motility decrease (Abate *et al*., 2019). Moreover, from analysing the results of the soil laboratory bioassay, the water control indicated 0% FCM larval infectivity, which leads to the conclusion that no EPN species were present in the soil samples prior to the study. Furthermore, all treatments that were applied, including the EPN controls, had > 90% FCM larval infectivity in both species. In the case of *H. bacteriophora*, a significant difference was recorded between the CMC and CNF treatment, where the CNF treatment achieved > 95% infectivity of the FCM larvae. Similarly, Elmahdi (2020) evaluated the suitability of EPNs to be formulated in a hydrogel, using a polymer used in baby diapers. The author further explained that cotton fibres included in the gel may lessen the adherence of hydrogel particles and provide moisture to nematodes in the formulation. After one month of storage at 14°C, an IJ survival rate of > 90% was recorded for *Steinernema glaseri* Wouts et al., *Heterorhabditis indica* Poinar, Karunakar & David, *S. carpocapsae* and *H. bacteriophora*. Furthermore, the author evaluated the IJs' pathogenicity against *G. mellonella* larvae. The highest mortality was achieved by *S. glaseri* and *H. indica* (100%). In addition, Abate *et al*. (2019) screened the viability of *H. bacteriophora* when formulated in a polyacrylamide gel, STOCKOSORB®, achieving 92% mortality after 12 days when screened against white grubs and > 90% mortality of *G. mellonella* larvae after 42 days. The findings in this study confirmed that CMC gel and CNFs are effective formulation media for EPNs. However, relatively few semi-field trials and only one large-scale field trial have been reported from South Africa (Moore et al., 2024); therefore, additional research on field-scale applications is recommended.

Despite the evident potential of EPNs to control insect pests, their commercial successes have been constrained by limited shelf life and microbial contamination. An encouraging outcome of this study is the results showing that CMC gel and CNF were effective for *Steinernema* and *Heterorhabditis* species for the management of agricultural soil-borne pests. Furthermore, these formulations exhibited good persistence and may be suitable in large-scale field applications to control FCM.

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