Ticks and Tick-borne Diseases 10 (2019) 781–786

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Entomopathogenic nematodes in pharmaceutical formulations for *Rhipicephalus microplus* (Acari: Ixodidae) control: In vitro evaluation of compatibility, thermotolerance, and efficiency

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ARTICLE INFO

Keywords:
Cattle tick
Biological control
Pharmacotechnical development
Heterorhabditis baujardi

ABSTRACT

This work aimed to investigate candidate biological control agents against the cattle tick *Rhipicephalus microplus* by studying three entomopathogenic nematodes (EPN) isolates from two species, *Heterorhabditis bacteriophora* (isolates LPP30 and HP88) and *Heterorhabditis baujardi* (isolate LPP7). The study comprised the pharmacotechnical development of four test formulations, and their biological evaluation, in three stages: 1) selection of the fittest EPN isolate at 16 ± 1 °C after 120 h of incorporation in the formulations; 2) determination of the effects of temperature and incubation time on the survival of the selected isolate in formulations; 3) evaluation of the *in vitro* efficacy (infectivity) of the selected EPN formulations and their components against *R. microplus* females after 72 h exposure. *H. baujardi* LPP7 presented the highest survival rates in 0.1% carboxymethyl cellulose sodium (CMC) formulations (100% at 25 °C, up to 10 days after incorporation). At the highest tested temperature (34.2 °C), 54% of LPP7 survived for up to 72 h in CMC formulations. The infectivity of LPP7-containing formulation against *R. microplus* was 99%, reducing oviposition, egg production index and larval hatching. Considering the pressing need for safer acaricide products, incorporation of EPNs in pharmaceutical formulations is presented as a promising approach for the biological control of cattle tick infestations, potentially reducing environmental impact and resistance development associated with chemical acaricides.

1. Introduction

Ectoparasites cause significant economic losses to dairy and meat production in Brazil, where the cattle tick *Rhipicephalus microplus* (Canestrini, 1888) alone is responsible for losses of 3.24 billion dollars every year (Grisi et al., 2014). This tick can cause direct damage to animals due to blood feeding and its consequences, and indirect damage through transmission of pathogenic agents, adding costs with medication and specialized care (Furlong et al., 2007).

Currently, the use of chemical acaricides is still the most common and efficient method to control tick infestations (Georgis et al., 2006; Lacey et al., 2015). However, results are frequently suboptimal due to

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https://doi.org/10.1016/j.ttbdis.2019.03.012
Received 18 October 2018; Received in revised form 28 February 2019; Accepted 15 March 2019
Available online 18 March 2019
1877-959X/

resistance acquired by ticks against the active compounds, induced mainly by widespread and inadequate application (Furlong et al., 2004, 2007). Another critical concern about these methods is the toxicity of acaricides to animals, humans, and the environment (Amaral et al., 2011; Gomes et al., 2011), including histopathological changes in specific organs and genotoxic and mutagenic effects in vertebrares (Oliveira et al., 2012; Cunha et al., 2017). In this context, there is a pressing and growing demand for the development of alternative approaches to control ectoparasites such as *R. microplus*, in particular for methods that have reduced negative impact on ecosystems, at the same time promoting animal health and well-being, and preventing economic losses (Monteiro et al., 2012a; Andreotti and Koller, 2013). Biological control using entomopathogenic nematodes (EPNs) is reported as a promising option (Monteiro et al., 2010a; Monteiro and Prata, 2015; Monteiro et al., 2014a).

EPNs are most commonly used in agricultural crop systems, whereas their application in cattle production represents a yet unexplored resource (San-Blas, 2013; Lacey et al., 2015). Several studies *in vitro* have reported the successful use of EPNs against the cattle tick *R. microplus* (Vasconcelos et al., 2004; Freitas-Ribeiro et al., 2005; Monteiro et al., 2010a, b; Carvalho et al., 2010; Silva et al., 2012). However, two EPN species tested *in vivo* (*Steinernema glaseri* isolate CCA, and *Heterorhabditis baujardi* isolate LPP7) were unable to protect cattle from tick infestation when administered topically in aqueous solution, which can be in part attributed to the action of abiotic stressors, including high temperature on cattle skin (34 °C–38 °C) (Carvalho et al., 2010). The survival and virulence of EPNs can be affected by different abiotic factors, particularly temperature, with optimal conditions varying at the species level, and even among isolates of the same species (Georgis, 1990; Rohde et al., 2010; Glazer, 2002). The temperature threshold at which a nematode species can survive, infect, and reproduce is generally close to the climatic conditions of its geographic origin (Molyneux and Bedding, 1984).

The addition of certain protective compounds to formulations has been shown to be a viable strategy to reduce the deleterious effect of high temperatures on entomopathogens in the field (Alves, 1998). Other important, desirable characteristics of EPN formulations are: being hygroscopic, having sufficient viscosity to adhere to the application surface without restricting motility of infective juveniles, being nontoxic to EPNs, and being stable over a wide range of temperature and pH conditions (Grewal, 2002). Proper formulation development is required for the implementation of pest control strategies. However, there are only a few studies on EPNs, and scarce information available on the compatibility of EPNs with inert components of potential use in formulations. This aspect represents a challenge for research in the area, and investigations in this direction deserve to be prioritized (Alves, 1998).

The incorporation of nematodes into a hydrophilic formulation could contribute to enhanced thermotolerance, required for viability in the environment. Therefore, the present work aimed to develop formulations containing EPNs and to evaluate their survival, thermotolerance, and infectivity rates (efficacy) *in vitro* against *R. microplus*, to guide the selection of promising approaches for future field studies.

2. Material and methods

2.1. Nematodes

The EPN isolates, *Heterorhabditis bacteriophora* HP88, *Heterorhabditis bacteriophora* LPP30, and *H. baujardi* LPP7 were provided by Universidade Estadual do Norte Fluminense Darcy Ribeiro - UENF (Campos dos Goytacazes, Rio de Janeiro, Brazil), and maintained at the Parasitology Laboratory of Embrapa Gado de Leite – Juiz de Fora/MG, Brazil by *in vivo* multiplication in *Galleria mellonella* (Lindegren et al., 1993; Kaya and Stock, 1997). The “White Traps” method (Kaya, 1990) was used to collect nematodes emerged from larvae. Nematodes were conditioned in distilled water (20 mL aliquots) in cell culture bottles (40 mL) and stored in a climate-controlled chamber (16 ± 1 °C). Infective juveniles (IJ) up to 15 days post-emerged were used in the assays.

Table 1
Gelling agents and humectants used in gel formulations developed for incorporation of entomopathogenic nematodes of the genus *Heterorhabditis*.

Gel	Gelling agent (0.1%)	Humectant (1.0%)
1	Carboxymethyl celulose sodium (CMC)	Glycerin
2	Carboxymethyl celulose sodium (CMC)	Propyleneglycol (PG)
3	Hydroxyethyl cellulose	Glycerin
4	Hydroxyethyl cellulose	Propyleneglycol (PG)

2.2. Ticks

R. microplus engorged females were collected from natural field infestations in a farm of Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Leite, located in the city of Coronel Pacheco (21° 35′16″S 43° 15′ 57″ W), Minas Gerais, Brazil.

2.3. Pharmacotechnical study

Four different gel formulations were developed for incorporation of EPNs and subsequent evaluation of survival, thermotolerance, and infectivity. The formulations were composed of various combinations of gelling agents carboxymethyl cellulose sodium (CMC) or hydroxyethyl cellulose, with humectants glycerin or propylene glycol (PG), as specified in Table 1. All ingredients are commonly used in cosmetic and pharmaceutical products, and were selected considering the low toxicity and cost, easy acquisition, and low formula complexity (Swamy and Yun, 2015; Farhadnejad et al., 2018; Mahinroosta et al., 2018). The formulations were prepared according to the guidelines for good laboratory practice and techniques adapted from Amaral et al. (2009). The concentration of gelling agents was set to obtain a low viscosity, to prevent clogging the spray equipment during field applications. The humectants were added at amounts sufficient to solubilize and incorporate the gelling agent into the formulations.

2.4. Assessment of EPN compatibility with different formulations

Survival rates of three EPN isolates (*H. bacteriophora* HP88 and LPP30, and *H. baujardi* LPP7) were assessed in the four gel formulations at 16 ± 1 °C. Five conditions were tested: control (IJs in distilled water), formulation 1, formulation 2, formulation 3, and formulation 4. Samples were prepared 30 min before the beginning of the survival assay by incorporation of EPNs in the corresponding formulations or distilled water at 1:1 ratio. Nematodes were counted by collecting a 10 µL sample from each condition in ten replicates. Live and dead nematodes were counted under microscope 120 h after incorporation, and percent survival was calculated as follows:

$$\% \text{ Survival} = (\text{total number of live IJs} / \text{total number of IJs}) \times 100$$

Live EPNs were those that showed motility during microscopy observation. Nematodes were considered dead when lying straight and motionless (Grewal, 2000).

2.5. EPN survival in the formulations at different temperature conditions

The selected EPN isolate was tested for survival 240 h after incorporation in the four formulations at the following temperature conditions: refrigerated temperature (approximately 4 °C), storage temperature (16 ± 1 °C), or ambient temperature (approximately 25 °C). Evaluation was performed following the same methodology

described above.

2.6. Thermotolerance of EPNs incorporated in different formulations

The survival of selected EPN isolate was investigated at 34.2 ± 1 °C temperature at time-points of 24 h, 72 h, and 120 h after being incorporated in the formulations. Evaluation was performed following the same methodology described above.

2.7. In vitro infectivity assay against R. microplus infectivity assay against R. microplus

Biological parameters of *R. microplus* engorged females were determined after exposure to EPN-containing formulations or isolated components, to investigate the infectivity of *H. baujardi* isolate LPP7. EPN suspensions in distilled water (2000 IJs/mL) were incorporated into each formulation at 1:1 ratio to achieve an estimated concentration of 1000 IJs/tick for infectivity assay.

Fourteen experimental groups with 10 engorged females each were treated as follows: 1) distilled water control; 2–5) each isolated formulation component (sodium carboxymethylcellulose, hydroxyethylcellulose, glycerin, propyleneglycol) without EPN; 6) aqueous solution of EPN isolate LPP7 in the absence of formulation components; 7–10) LPP7 isolate combined with each of the formulation components separately; and 11–14) LPP7 isolate incorporated into each of the four studied formulations.

For each group, treatment was applied by spreading a 1-mL sample onto a double layer of sterilized filter paper in 10 separate Petri dishes. One engorged *R. microplus* female was individually weighed and placed in each Petri dish, which was wrapped in plastic foil (PVC) and kept in a controlled climate chamber (27 ± 1 °C and 80 ± 10% RH) for 72 h. After incubation, ticks in each group were transferred to a new Petri dish, fixed with adhesive tape and individually identified. These were incubated again under controlled conditions (27 ± 1 °C and 80 ± 10% RH) suitable for ticks and nematodes. Tick mortality was recorded daily. After 15 days of incubation, the egg mass laid by each female was separately weighed, collected in clean disposable plastic syringes with cotton plugs, and incubated at 27 ± 1 °C and 80 ± 10% RH. Larval hatching was assessed after 20 days. Biological parameters evaluated were: weight of engorged female, weight of egg mass, egg production index and percent of larval hatching. These measurements were used to calculate formulation efficacy (Drummond et al., 1973).

2.8. Statistical analysis

Statistical tests were performed by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) using the software Instat3. In cases where the data did not present a normal distribution, even after using logarithmic or reciprocal function transformation, nonparametric Kruskal Wallis and Student Newman Keulls tests were used ($p < 0.05$).

3. Results and discussion

3.1. Viability of different EPN isolates incorporated in the proposed formulations

Incorporation of *H. bacteriophora* HP88, *H. bacteriophora* LPP30, or *H. baujardi* LPP7 into the four tested formulations did not significantly affect nematode viability after 120 h at 16 °C. Survival was greater than 80% in all cases, and no statistical difference was observed when compared to the control ($p < 0.05$), with survival rates of 86% to 100% (Table 2). The use of any of the four different combinations of the proposed gelling and humectant agents in formulations appeared to be applicable for EPN incorporation.

H. baujardi LPP7 was selected for further experiments due to higher survival rates in the formulations. Moreover, this isolate, identified by molecular biology techniques, is native from the Brazilian Amazon Forest (locality of Monte Negro, RO - Latitude: 10° 15′ 6″ South, Longitude: 63° 17′ 14″ West.), and therefore likely adapted to tropical environments (Dolinski et al., 2007; Minas et al., 2016). Previous studies had shown that EPNs isolated from regions of hot climate perform better under high-temperature conditions (Minas et al., 2016).

Table 2
Survival rate (%) of *Heterorhabditis bacteriophora* HP88, *Heterorhabditis bacteriophora* LPP30, and *Heterorhabditis baujardi* LPP7, after 120 h of incorporation into the four proposed formulations at 16 ± 1 °C, 80 ± 10% RH. Mean ± standard deviation values.

Treatments	HP88	LPP30	LPP7
Control (H_2O)	$91.9^{Aab} \pm 16.2$	$86.6^{Aa} \pm 13.1$	$100.0^{Ab} \pm 0.0$
Formulation 1 – CMC + Glycerin	$95.0^{Aa} \pm 15.8$	$90.1^{Aa} \pm 11.7$	$100.0^{Aa} \pm 0.0$
Formulation 2 – CMC + PG	$98.3^{Aa} \pm 5.3$	$91.7^{Aa} \pm 16.2$	$100.0^{Aa} \pm 0.0$
Formulation 3 – Hydroxyethyl cellulose + Glycerin	$92.5^{Aa} \pm 16.9$	$83.9^{Aa} \pm 15.8$	$95.8^{Aa} \pm 9.0$
Formulation 4 – Hydroxyethyl cellulose + PG	$88.6^{Aa} \pm 20.8$	$83.4^{Aa} \pm 31.7$	$96.7^{Aa} \pm 10.5$

Different uppercase letters indicate statistical difference ($p < 0.05$) between means in a given column, whereas different lowercase letters indicate statistical difference ($p < 0.05$) between means across each line. CMC: carboxymethyl cellulose Sodium; PG: Propylene glycol.

3.2. Effect of temperature and exposure time on EPN viability in different formulations

Table 3 shows the survival rates of *H. baujardi* LPP7 after 240 h of incorporation in the proposed formulations at different temperature conditions. Infective juveniles were able to survive at 16 °C and 25 °C when incorporated into formulations containing CMC and glycerin, or CMC and PG (1 and 2), presenting mean survival rates higher than 90%, which was statistically equivalent to the control ($p < 0.05$). Formulations containing hydroxyethyl cellulose (3 and 4) yielded lower survival rates ($p < 0.05$) at all temperatures tested at this time-point, suggesting that these formulations were disadvantageous to the nematodes for longer periods. Nematode survival was low after 240 h at 4 °C regardless of the formulation, with significant differences compared to the control.

The optimal temperature reported for *H. baujardi* LPP7 replication and infection was 28 °C (Dolinski et al., 2007; Minas et al., 2016). According to our data, this isolate showed high survival rates for up to 240 h at room temperature (15 °C–25 °C), particularly when incorporated in formulations 1 and 2 (97.3% and 100% survival, respectively; Table 3). Temperature can influence many physiological and

Table 3
Survival rate (%) of *Heterorhabditis baujardi* isolate LPP7, after 240 h of incorporation into the 4 proposed formulations at different temperature conditions: refrigerated (4 °C), storage temperature (16 ± 1 °C) or room temperature (25 °C). Mean ± standard deviation values.

Treatments	4 °C	16 °C	25 °C
Control (H_2O)	$45.6^{Ab} \pm 22.3$	$96.1^{Aa} \pm 4.2$	$98.4^{Aa} \pm 3.4$
Formulation 1 – CMC + Glycerin	$10.0^{Bb} \pm 11.7$	$96.7^{Aa} \pm 7.0$	$97.3^{Aa} \pm 5.8$
Formulation 2 – CMC + PG	$12.8^{Bc} \pm 7.3$	$92.0^{Ab} \pm 8.4$	$100.0^{Aa} \pm 0.0$
Formulation 3 – Hydroxyethyl cellulose + Glycerin	$6.3^{Bb} \pm 6.5$	$55.6^{Ba} \pm 21.6$	0.0 ± 0
Formulation 4 – Hydroxyethyl cellulose + PG	$16.5^{Bb} \pm 10.2$	$59.0^{Ba} \pm 19.2$	$63.3^{Ba} \pm 23.9$

Different uppercase letters indicate statistical difference ($p < 0.05$) between means in a given column, whereas different lowercase letters indicate statistical difference ($p < 0.05$) between means across each line. CMC: Sodium carboxymethyl cellulose; PG: Propylene glycol.

metabolic processes in infective juveniles, including mobilization of energy reserves (lipids, proteins, and carbohydrates), motility, survival, infectivity, development, and reproduction (Dunphy and Webster, 1986; Andaló et al., 2011). The temperature range compatible with EPN efficiency as biological control agents is narrow. Infectivity and persistence are reduced at temperatures lower than 15 °C and higher than 30 °C (Kaya, 1990). However, González (2006) reported that *H. baujardi* isolate LPP7 endured temperatures of up to 35 °C for 2 h with no losses in viability, mobility, and pathogenicity towards *G. mellonella* larvae. These parameters were negatively affected at temperatures between 40 °C and 45 °C. Therefore, a temperature range of 25 °C–35 °C was defined as optimal for this EPN, considering the period of 2 h as relevant in the context of irrigation systems approached by the study (González, 2006).

Incubation time is selected based on the relationship between EPN virulence and the period of contact with the target organism, as has been previously described by Monteiro et al. (2012b). The authors observed 82.45% and 100% efficacy of the treatment with *H. bacteriophora* isolate HP88 after 24 h and 48 h respectively, suggesting that a critical period is required for the nematode to exert an adverse impact on the physiology of *R. microplus* females. Carvalho et al. (2010) evaluated the exposure time required for infection of *R. microplus* females by *S. glaseri* CCA, and found that at least 24 h were necessary to achieve efficacy higher than 90%.

In the present work, survival of LP77 IJ in the different formulations was evaluated at a temperature representative of the cattle haircoat surface (34 °C), for periods of up to 120 h (Table 4). The results after 24 h showed highest survival rates of nematodes in formulation 4 (72.4%). Survival of EPNs incorporated into formulation 1 was significantly lower compared to formulation 4 at 24 h and 120 h, but statistically equivalent to the control group at all time points ($p < 0.05$), suggesting the components CMC and glycerin were not unfavorable to the nematodes compared to distilled water, at this temperature. In order to analyze the performance of humectant components, a direct comparison must be drawn between formulations that differ only by this variable, *i.e.* formulations that contain the same gelling agent, but different humectants. Thus, the formulations fall into two groups: one comprised of formulations 1 and 2, the other comprised of formulations 3 and 4 (Table 4). No significant difference was observed in nematode survival in the presence of glycerin or propilenglycol up to 72 h after formulations were produced. Considering potential application in the field, this period would be enough for an effective nematode treatment. The results of the thermotolerance tests should not be considered as an isolated parameter, but in combination with costs and innocuity of the components for ticks and the host. Therefore, we conclude that glycerin may represent the best option.

In vitro infectivity of R. microplus females by H. baujardi isolate LPP7 incorporated in formulations

Fig. 1 shows representative images of female ticks in the control group after oviposition (Fig. 1a), and of a female treated with *H. baujardi* LPP7 (Fig. 1b), which was killed before laying eggs and presented signs of bleeding beneath the cuticle. Biological parameters of ticks in the control group were consistent with previous reports (Benett, 1974; Gonzales, 1974). Our results demonstrate that *H. baujardi* isolate LPP7 was able to infect *R. microplus* when incorporated into the proposed formulations at 1000 EPN/female (concentration after incorporation at 1:1 ratio). *H. baujardi* isolate LPP7 has been previously evaluated as a biological control agent against the guava weevil (*Conotrachelus psidii*) larvae, with virulence and control efficacy higher than 80% in laboratory and field studies (Minas et al., 2016; Del Valle et al., 2005). Also, *H. bacteriophora* isolate HP88, and *H. baujardi* isolate LPP7, under laboratory and semi-natural conditions, caused higher than 90% egg-laying inhibition and mortality in *R. microplus* females. Infectivity tests performed in the current study confirmed that LPP7 presents high virulence against *R. microplus*, and demonstrated for the first time that these nematodes are able to kill ticks before the onset of oviposition when applied alone or incorporated in the formulation containing hydroxyethyl cellulose and propylene glycol (formulation 4), as shown in Table 5.

To address the efficacy of EPNs in formulations for *in vivo* applications, the first step is to select nematode isolates with the best performance *in vitro*, looking for features such as compatibility with the proposed formulation, tolerance to high temperatures, and preserved infectivity after incorpotation. In this context, our results show formulations 1 and 4 as having the best performance in infectivity test (99% and 100%, respectively). Despite the results showing susceptibility of nematodes after long periods at 34 °C using formulation 1, it is considered the most promising formulation, based on satisfactory survival rates at this temperature during up to 72 h, (Table 4), as well as a wider compatible temperature range (Table 3), and good performance on infectivity tests (Table 5). Also, the components meet important aspects required in a formulation, including low toxicity, low cost, and easy obtainability.

Exposing *R. microplus* engorged females to *H. baujardi* LPP7 alone in distilled water caused 100% mortality. Nevertheless, the use of formulations is beneficial for preservation of the infective juveniles against desiccation during field application. Additionally, incorporating EPNs into pharmaceutical formulations will support their possible commercial use. In the absence of LPP7, formulation components (except hydroxyethyl cellulose) seem to be toxic to ticks to some extent, mainly inhibiting larval hatching (Table 5); however, in light of the strikingly high efficacy of the treatment in the presence of nematodes, the results still support their potential use as biological control agents. It is possible to observe that formulation components individually did not affect egg mass weight or egg production index, but interfered with larvae hatching in all cases except hydroxyethyl cellulose (Table 5). When associated to EPNs, this component significantly reduced all evaluated parameters. It is noteworthy that the combination of LPP7 with CMC or CMC-containing formulation 1, although still allowing oviposition, did not differ statistically from the treatment in the presence of hydroxyethyl cellulose and LPP7, demonstrating that EPN treatment using CMC as a gelling agent is also effectively toxic to ticks. In fact, all formulations proposed in this study were compatible with the effect of *H. baujardi* LPP7 on *R. microplus* regarding the analyzed biological

Table 4
Survival rate (%) of *Heterorhabditis baujardi* LPP7, after 24 h, 72 h and 120 h of incorporation into the 4 proposed formulations at 34 °C. Mean ± standard deviation values.

Treatments	24 h	72 h	120 h
Control (H_2O)	$50.2^{Ba} \pm 21.0$	$54.9^{Aa} \pm 23.6$	$19.9^{BCb} \pm 14.9$
Formulation 1 – CMC + Glycerin	$47.1^{Ba} \pm 16.2$	$54.0^{Aa} \pm 18.0$	$18.5^{Bb} \pm 7.5$
Formulation 2 – CMC – PG	$62.7^{ABa} \pm 19.8$	$62.1^{Aa} \pm 10.7$	$35.6^{Bb} \pm 16.9$
Formulation 3 – Hydroxyethyl cellulose + Glycerin	$50.8^{ABa} \pm 6.8$	$21.6^{Bab} \pm 12.1$	$7.8^{Cb} \pm 7.3$
Formulation 4 – Hydroxyethyl cellulose + PG	$72.4^{Aa} \pm 14.5$	$48.6^{ABb} \pm 13.0$	$48.6^{Ab} \pm 21.4$

Different uppercase letters indicate statistical difference ($p < 0.05$) between means in a given column, whereas different lowercase letters indicate statistical difference ($p < 0.05$) between means across each line. CMC: Sodium carboxymethyl cellulose; PG: Propylene glycol.

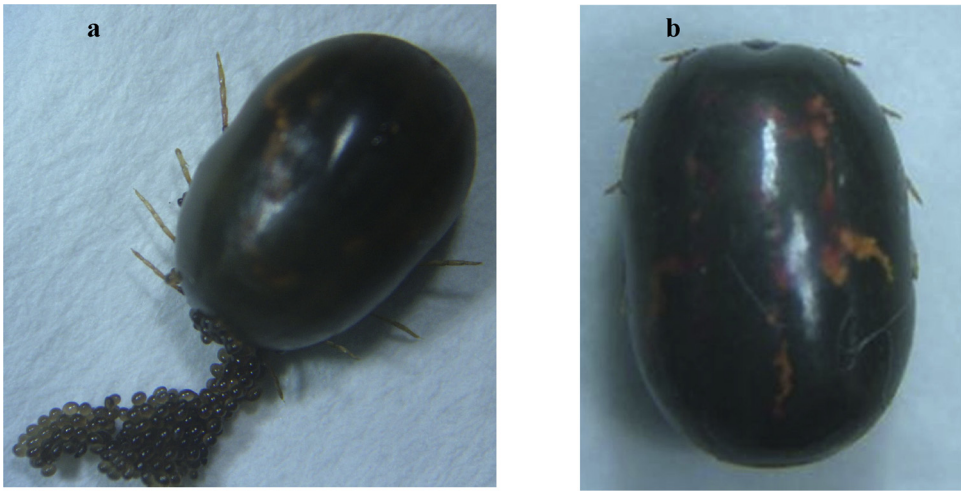

Fig. 1. *Rhipicephalus microplus* engorged females. a: Specimen of the control group after oviposition; b: Dead specimen from the group infected with *Heterorhabditis baujardi* LPP7.

Table 5
Reproduction parameters (engorged female weight, egg mass weight, egg production index, and larvae hatching) and percent efficacy of *in vitro* treatment of *Rhipicephalus microplus* ticks with EPN *Heterorhabditis baujardi* isolateLPP7 incorporated in four different proposed formulations or their components individually, at 27 ± 1 °C, 80 ± 10% RH. Mean ± standard deviation values (n = 10).

Treatments	Engorged female weight (mg)	Egg mass weight (mg)	Egg Production Index (%)	Larvae hatching (%)	Percent Efficacy (%)
Control – H_2O	$246.5^{A} \pm 21.1$	$128.5^{A} \pm 26.6$	$52.5^{A} \pm 11.0$	$100.0^{A} \pm 0.0$	
CMC	$239.0^{A} \pm 18.6$	$86.3^{AB} \pm 46.5$	$36.4^{AB} \pm 20.0$	$23.5^{BC} \pm 36.5$	84.0
Hydroxyethyl cellulose	$244.4^{A} \pm 24.4$	$139.7^{A} \pm 14.7$	$57.3^{A} \pm 4.0$	$96.5^{A} \pm 6.3$	−6.0
Glycerin	$243.5^{A} \pm 29.9$	$122.1^{A} \pm 13.5$	$50.3^{A} \pm 3.0$	$36.5^{B} \pm 35.7$	65.0
PG	$241.4^{A} \pm 22.8$	$110.9^{A} \pm 28.7$	$46.0^{A} \pm 11.0$	$29.0^{B} \pm 36.9$	74.0
LPP7 + H_2O	$246.7^{A} \pm 19.8$	0.0 ± 0.0	00.0 ± 0.0	0.0 ± 0.0	100.0
LPP7 + CMC	$243.2^{A} \pm 18.0$	$75.2^{AB} \pm 51.9$	$31.0^{AB} \pm 21.0$	$32.0^{BC} \pm 41.3$	81.0
LPP7 + Hydroxyethyl cellulose	$245.3^{A} \pm 16.0$	$0.01^{B} \pm 0.0$	00.0 ± 0.0	$9.0^{BC} \pm 28.5$	100.0
LPP7 + Glycerin	$246.5^{A} \pm 19.7$	$102.6^{A} \pm 41.6$	$41.6^{A} \pm 17.0$	$30.5^{B} \pm 33.2$	76.0
LPP7 + PG	$246.4^{A} \pm 26.3$	$100.5^{A} \pm 45.7$	$40.1^{A} \pm 17.0$	$17.0^{B} \pm 23.4$	87.0
LPP7 + Formulation 1	$241.8^{A} \pm 19.5$	$7.3^{B} \pm 24.7$	$3.6^{B} \pm 11.0$	$19.0^{BC} \pm 40.1$	99.0
LPP7 + Formulation 2	$247.1^{A} \pm 21.7$	$29.5^{B} \pm 50.9$	$12.0^{B} \pm 20.0$	$38.0^{BC} \pm 49.1$	91.0
LPP7 + Formulation 3	$241.7^{A} \pm 18.3$	$19.1^{B} \pm 34.0$	$8.4^{B} \pm 15.0$	$35.0^{BC} \pm 27.1$	94.0
LPP7 + Formulation 4	$247.2^{A} \pm 21.2$	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0

Different uppercase letters indicate statistical difference ($p < 0.05$) between means in a given column.
CMC: Sodium carboxymethyl cellulose. PG: Propylene glycol. Formulations 1 - CMC + Glycerin; Formulation 2 - CMC + PG; Formulation 3 - Hydroxyethyl cellulose + Glycerin; Formulation 4 - Hydroxyethyl cellulose + PG.

parameters. The CMC-formulations (1 and 2) appeared to have advantages over formulations with hydroxyethyl cellulose, such as mantaining nematode pathogenicity against *R. microplus in vitro,* and superior performance in survival assays.

Previous research using EPN-infected remains of *G. mellonella* and *Tenebrio molitor* larvae as a delivery system indicated positive outcomes with the use of EPNs to control cattle tick during the free stages of life cycle, under laboratory and semi-natural conditions (Monteiro et al., 2014b). However, the approach has so far been unsatisfactory against the parasitic stages (Carvalho et al., 2010), which might be attributed to the use of water as the sole vehicle for administration on the bovine surface, highlighting the need for a formulation to protect the nematodes from environmental stressors.

Formulation development and administration techniques aim to reduce the negative impact of climate and environmental factors on the nematodes, supporting their successful use as biological pest control. In addition, integrated pest control practices can play an important role in the effectiveness of such a method (Georgis, 1990). The production of formulations for commercial use, however, is still largely impaired by issues related to costs, shelf-life, and legal aspects (Lacey et al., 2015). Further research is required, including *in vivo* studies, to validate the use of the proposed formulations.

The evaluated entomopathogen met the main quality requirements for a possible application in formulations that have the purpose of animal use in the field: it was virulent, compatible with inerts, and tolerant to high temperature.

4. Conclusions

The gel formulation containing CMC 0.1%/glycerin 1% (formulation 1) is brought forward as a potentially successful vehicle for *H. baujardi* LPP7, promoting the delivery of live nematodes able to infect *R. microplus*. The present study shows an innovative approach using pharmacotechnical development, a so far unexplored resource to advance the use of EPNs in the biological control of cattle tick infestations.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo à

Pesquisa de Minas Gerais (FAPEMIG, Brazil), Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM, Brazil), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil) for the financial support to this research, and to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support provided by the scholarship awarded to the researcher.

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