Journal of Invertebrate Pathology 193 (2022) 107801

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Antifungal activity of "HO21-F", a formulation based on *Olea europaea* plant extract, in honey bees infected with *Nosema ceranae*

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ARTICLE INFO

Keywords:
Nosema ceranae
Honey bee
Infection level
Antifungal activity
Plant extract
Olea europaea

ABSTRACT

Nosema ceranae is a microsporidium parasite that silently affects honey bees, causing a disease called nosemosis. This parasite produces resistant spores and germinates in the midgut of honey bees, extrudes a polar tubule that injects an infective sporoplasm in the host cell epithelium, proliferates, and produces intestinal disorders that shorten honey bee lifespan. The rapid extension of this disease has been reported to be widespread among adult bees, and treatments are less effective and counterproductive weakening colonies. This work aimed to evaluate the antifungal activity of a prototype formulation based on a non-toxic plant extract (HO21-F) against *N. ceranae*. In laboratory, honey bees were infected artificially, kept in cages for 17 days and samples were taken at 7 and 14 days post infection (dpi). At the same time, in field conditions we evaluated the therapeutic effect of HO21-F for 28 days in naturally infected colonies. The effectiveness of the treatment has been demonstrated by a reduction of 83.6 % of the infection levels observed in laboratory conditions at concentrations of 0.5 and 1 g/L without affecting the survival rate. Besides, in-field conditions we reported a reduction of 88 % of the infection level at a concentration of 2.5 g/L, obtaining better antifungal effectiveness in comparison to other commercially available treatments.

As a result, we observed that the use of HO21-F led to an increase in population size and honey production, both parameters associated with colony strength. The reported antifungal activity of HO21-F against *N. ceranae*, with a significant control of spore proliferation in worker bees, suggests the promising commercial application use of this product against nosemosis, and it will encourage new research studies to understand the mechanism of action, whether related to the spore-inhibition effect and/or a stimulating effect in natural response of colonies to counteract the disease.

1. Introduction

Apis mellifera is gaining attention due to its role as a pollinator of many crops, and large-scale honey bee colonies have been growing for these services (Neumann and Carreck, 2010). Nosemosis is a highly prevalent disease that affects bees worldwide, the effects of this disease have been reported to be multifactorial and have been linked to a phenomenon called "Colony Collapse Disorder" (CCD) debilitating the whole population progressively (Manzoor et al., 2013). The increase of reports across the world of higher colony losses rate has attracted the attention of the scientific community to provide possible explanations (Higes et al., 2013; Martín-Hernández et al., 2018). Years ago, the only effective treatment available was fumagillin, but the non-controlled application has reported resistance in microsporidia and sublethal concentration can exacerbate the infection instead of suppressing it (Huang et al., 2013). Besides, new restrictions for the application of fumagillin have been established in some countries due to their effects on human health by the consumption of honey contaminated with antibiotics (European Union, 2009; SAG, 2018). Thus, the main manufacturers are discontinuing their production and distribution (Grupe and Alisha Quandt, 2020).

Today there are nutritional products commercially available that are being used as alternative therapies like Nozemat®, Apiherb, ApiX, and others in development (only tested in laboratory conditions) that are mainly based on herbal extracts that claim an effect to control nosemosis (Borges et al., 2020; Braglia et al., 2021; Chaimanee et al., 2021; Cilia et al., 2020; Go et al., 2021; Jovanovic et al., 2021; Michalczyk and Sokół, 2018; Nanetti et al., 2021; Ptaszyńska et al., 2018; Shumkova et al., 2021). During the last 5 years of studying this disease, it has become one of the most prevalent honey bee pathogens worldwide

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https://doi.org/10.1016/j.jip.2022.107801
Received 3 March 2022; Received in revised form 28 June 2022; Accepted 15 July 2022
Available online 19 July 2022

(Bekele et al., 2015; Higes et al., 2013; Martín-Hernández et al., 2018). The presence of *N. ceranae* has been detected in countries of South America, Brazil, Uruguay, Argentina (Invernizzi et al., 2011; Medici et al., 2012; Teixeira et al., 2013), in Chile was first detected in the Bío Bío Region (Martínez et al., 2012; Rodríguez et al., 2012), this represents 42 % of total country's production. Later, *N. ceranae* was detected in the V Region of Valparaíso and was correlated with more than 2.000 deaths (Bravo et al., 2014).

Nowadays, control of pathogens are being mainly controlled with apicultural practices in colonies called Good Beekeeping Practices (GBPs) and Biosecurity Measures in Beekeeping (BMBs); controlling relative humidity inside colonies, preventing spore spreading by keeping clean water resources free of faeces or drowned/dead bees, isolating spore-contaminated colonies from the apiary, replacement of queen bee's in colonies and prevent weakening of population by providing nutritional supplements (FAO, 2018). Unfortunately, these practices can only reduce the risk of the introduction and the spread of main honey bee diseases (Formato et al., 2022).

Several studies reported plant extract and propolis-derived compounds as alternatives for controlling the disease and demonstrated a reduction in infection levels (Arismendi et al., 2018; Bravo et al., 2017; Damiani et al., 2014). Most of these extracts contain compounds that reported antifungal activity against several filamentous fungi including *N. ceranae* and are well tolerated by honey bees (Arismendi et al., 2018; Bravo et al., 2017; Kim et al., 2016; Porrini et al., 2017). *Olea europaea* leaf extracts have been studied because of their high composition and variety of phenolic compounds that demonstrated antifungal activity (Báidez et al., 2006; Battinelli et al., 2006; Korukluoglu et al., 2006), but they have not been tested for specific antifungal activity against *N. ceranae*.

Most reports have analyzed plant extract at the laboratory level, which is considered an approximation of the efficacy for controlling the infection level of *N. ceranae,* but to demonstrate efficacy to control the disease several factors are not present in laboratory conditions (Manzoor et al., 2013); honey bees are not independent individuals but they live in collaboration as a colony formed by an average of 20.000 individuals (Orkin, 2022), they acquire a social behavior and their individual development is directly related with the colony requirements, where progeny would have different roles depending on bee age (Rodriguez-García, 2018). This biological equilibrium indicates that is relevant to consider colonies as a super-organism (Berenbaum and Liao, 2019).

For these reasons, several factors must be evaluated in terms to understand the effectiveness of treatment of nosemosis: i) pre-existent physiological conditions of honey bees, ii) viability and germination-inducers of *N. ceranae* spores, iv) hygienic behavior, v) immunity response of honey bees, and vi) environmental and agroecological conditions. All these factors can be controlled at the laboratory level, but in field experiments, there are complex interactions that can affect at the colony level and is not reasonably appropriate to compare both laboratory and field results. It has been demonstrated that there are differences in the level of infection at the laboratory level and field test (Cilia et al., 2020; Valizadeh et al., 2020). It is important to demonstrate the effectiveness of a new product in natural conditions.

This study aimed to determine the antifungal activity against *N. ceranae* in the laboratory and in field conditions of a product against nosemosis based on *Olea europaea* leaf extract called "HO21-F".

2. Materials and methods

2.1. Preparation of HO21-F and fumagillin laboratory working solutions

HO21-F is a prototype formulation that was kindly provided by the company ApiQuality SpA (Santiago, Chile), licensee of the formula, under Material Transfer Agreement. This formulation is based on a proprietary mixture of polyphenols from *Olea europaea* and other plant extracts (patent pending). For laboratory conditions, the powder was dissolved in syrup: water [1:1] solution at the concentrations of 0.5 and 1 g/L and then supplemented with an artificial source of organic nitrogen for bees (Promotor L, Calier® Spain). Higher concentrations in laboratory experiments have reported a decrease in survival in previous experiments. Fumagillin-B® was obtained from Medivet Pharmaceuticals ltd. (High River, AB, Canada) and was prepared at a final concentration of 600 μg/mL in syrup.

For field experiments, HO21-F in powder was dissolved in 75:25 [syrup: water solution] at a concentration of 2.5 g/L. The concentration applied for this study was calculated by estimating the consumed dosage per bee that was effective in reducing the infection level in laboratory conditions (105 μg), assuming that a colony has an average of 20.000 honey bees. Apiherb® was obtained from Agro-apicultura (Villa Alemana, Región de Valparaíso, Chile) and was applied according to the manufacturer's instructions (Chemicals Laif S.p.A., Padua, Italy).

2.2. Preparation of N. ceranae spores

To obtain fresh *N. ceranae* spores a sample of infected bees was collected in Region de la Araucanía, Chile. Samples were processed by taking the abdomen of all individuals and were manually macerated with a pestle and mortar, eluted in distilled water, and then purified by Percoll® density gradient centrifugation. Total spores were counted in a Neubauer cell chamber and adjusted to 60,000 spores / μL. Isolated *N. ceranae* spores were identified from other Nosema species by Real-time PCR as described previously (Martínez et al., 2012).

2.3. Laboratory experiments

Honey bees were obtained from incubated sealed brood frames in the laboratory at 34 °C overnight. The next day the emergent worker bees were carefully removed and confined in cages in groups of 20 individuals and fed with syrup *ad libitum* for 2 days. On day 3 a group of honey bee cages were infected individually with 120,000 *N. ceranae* spores/bee and then all cages were distributed in the following 7 experimental groups (4 cages per group): i) Control: honey bees fed with syrup, ii) Fumagillin: honey bees infected with *N. ceranae* and treated with fumagillin at the concentration of 600 μg/mL according to previous experiments (Higes et al., 2011; Nanetti et al., 2014a), iii) NC: honey bees infected with *N. ceranae*, iv) F-0.5: honey bees fed with 0.5 g/L HO21-F v) F-1: honey bees fed with 1.0 g/L HO21-F, vi) NC F-0.5: honey bees infected with *N. ceranae* and treated with 0.5 g/L HO21-F and vii) NC F-1: honey bees infected with *N. ceranae* and treated with 1.0 g/L HO21-F (Fig. 1). Survival and consumption we daily registered, and 15

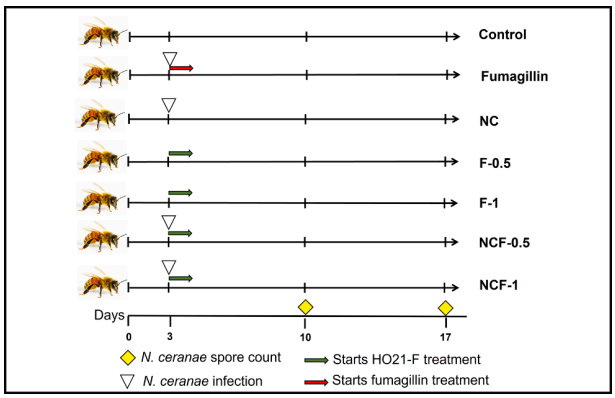

Fig. 1. Experimental design of laboratory assay in adult bees. Control: honey bees fed with syrup, Fumagillin: infected honey bees treated with syrup + fumagillin 600 μg/mL, NC: infected honey bees fed with syrup, F-0.5: honey bees fed with 0.5 g/L of HO21-F, F-1: honey bees fed with 1 g/L of HO21-F, NCF-0.5: infected honey bees treated with 0.5 g/L of HO21-F, NCF-1: infected honey bees treated with 1 g/L of HO21-F.

honey bees were taken from cages of all groups on day 10 (7th day post-infection) and on day 17 (14th day post-infection) for spore count.

2.4. Field experiment

The field experiment was performed in an apiary with naturally infected colonies located at 1.49 km from the south of Peumo (Cachapoal Province - Region de O'Higgins – Chile), location: −34.405800 S, −71.146911 W. Colonies were diagnosed 7 days before starting the experiment, classified by the level of infection (spores per bee) and equally distributed between the infected experimental groups. The experiment started labeling colonies according to the following experimental groups described in Fig. 2. Group I: infected colonies fed with syrup, group II: infected colonies treated with HO21-F, and group III: infected colonies fed with a natural alternative nutraceutical product Apiherb® (Chemicals LAIF) that has been previously suggested for controlling nosemosis (Cilia et al., 2020). Fumagillin was not available for the application of colonies in field experiments for the current Chilean regulation (SAG, 2018). Three applications of 1 L of HO21-F or syrup for each group were performed on days 0, 14, and 21 of the experiment (Fig. 2). Apiherb® was applied in colonies according to manufacturer instructions on the same days 0, 14, and 21 of the experiment. Samples of 60 forager honey bees were taken from the entrance of hives before (day 0) and after (day 28) the intervention of hives. These colonies had not been treated against nosema disease for at least 6 months before the experiments and were sampled during the spring.

2.5. Level of artificial infection in laboratory

Samples from 7 and 14 dpi were macerated and resuspended in distilled water for spore count in the Neubauer chamber (Cantwell, 1970). 10–20 bees per experimental group were individually processed for spore count.

2.6. Level of infection in field tests and parameters related to colony strength

The samples of honey bees were collected from hives on days 0 and 28 and placed in plastic disposable collection cups containing 70 % ethanol until analyzed. Samples were macerated in a pool of 60 and resuspended in distilled water, and then filtered with a mesh N°18 U.S. STD. Sieve (1 mm) to remove debris and chitin residues for spore count in Neubauer Chamber (Fries et al., 2013). Colony strength of the apiary was evaluated according to the standard methods for estimating strength with objective/subjective modes in *A. mellifera* (Delaplane et al., 2013). There were registered the number of frames containing honey from 90 % of the total area, the number of frames with covered honeycombs, and a number of frames covered with adult honey bees.

2.7. Statistical analysis

Honey bee survival rate was analyzed by a Kaplan-Meier survival method for all groups, and significant differentiation was determined by the log-rank Mantel-Cox test. To analyze the antifungal effect of HO21-F on *N. ceranae*, we performed the Shapiro-Wilk normality test data distribution. A non-parametric test was analyzed by oneway-ANOVA to identify significant differences in infection level and colony strength parameters between treated and untreated groups, with the Man-Withney test of repeated measures. Graph Prism (6.01, USA) was used to perform the statistical analyzes.

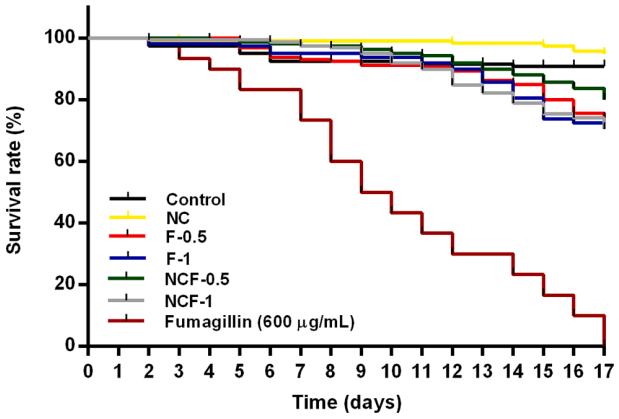

Fig. 3. Survival rate between experimental groups.

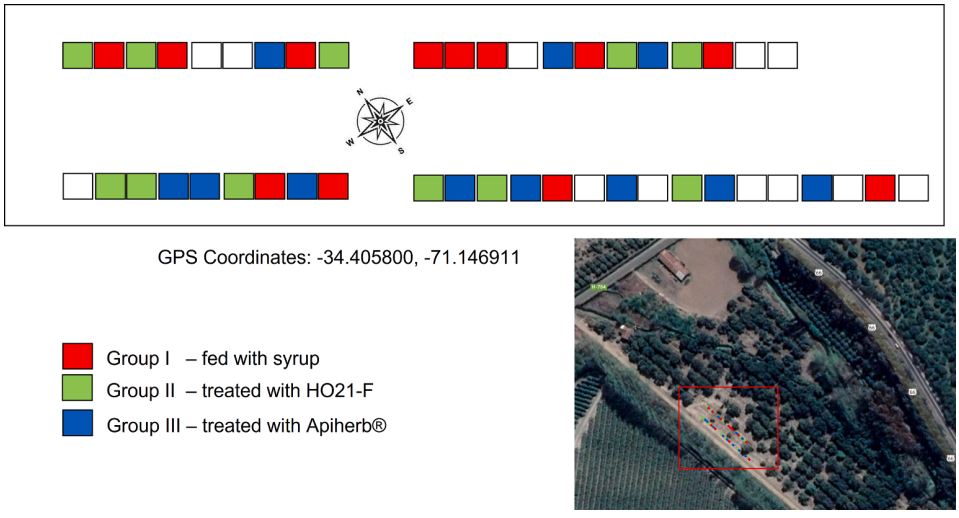

Fig. 2. Location of naturally infected colonies in the field experiment. Experimental groups are group I: infected honeybees fed with fructose syrup, group II: infected honeybee treated with HO21-F, and group III: infected honey bees treated with Apiherb®. White boxes correspond to uninfected colonies that were not been treated. Fumagillin was not available as control for field experiments according to current regulations in Chile.

3. Results

3.1. HO21-F is non-toxic for honey bees

In Fig. 3 we observed the effect of HO21-F on the survival percentage of each experimental group on a daily basis. No significant differences were observed between groups Control and NC, that reached a survival percentage of 91.6 % and 96.6 % at 7 dpi ($p = 0.0577$) and 90.3 % and 94.1 % at 14 dpi respectively ($p = 0.1955$). Survival of the group treated with fumagillin was 43.3 % at 7 dpi and 0 % at 14 dpi.

The groups of F-0.5; F-1, NCF-0.5 and NCF-1 were all between 92.9 and 97.8 % survival rate at 7 dpi and between 73.7 and 82.5 % at 14 dpi (Table 1). There are no significant differences in survival between F-0.5 and F-1 with respect to Control group ($p = 0.2348$; 0.6569) at 7 dpi. However, a significant difference in survival percentage was observed at 14 dpi between F-0.5 and F-1 concerning the Control group ($p = 0.0007$; 0.0002). If we compare groups F-0.5 and F-1 with their corresponding infected groups NCF-0.5 and NCF-1, there were no significant differences at 7 dpi ($p = 0.1791$; 0.9699) and 14 dpi ($p = 0.1699$; 0.7686). In contrast, at 14 dpi there are significant differences in survival rate between NCF-0.5 and NCF-1 ($p = 0.0439$) (Table S1). Regarding daily consumption, the average registered volume of all groups was between 21.13 and 24.18 μL / bee (Fig. 4) and there are no significant differences between groups ($p = 0.0820$), nor between consumption of syrup with and without HO21-F (Table S2). Besides, the daily dose per bee ingested by F-0.5/NCF-0.5 and F-1/NCF-1 groups were 21.3 ± 2.7 and 41.7 ± 7.1 μg HO21-F /bee respectively at the end of the experiment (Fig. 5). The group fed with fumagillin 600 μg/mL had a consumption average of 18.98 ± 4.8 μL and a accumulated dose of 159.4 ug fumagillin per honey bee in 14 days of syrup consumption (Table S2).

3.2. HO21-F reduces the level of N. ceranae spores at the laboratory level.

It was observed at 7 dpi a significant reduction of infection level in NCF-0.5 (50.6 %) and NCF-1 (51.2 %) group compared to NC group (Fig. 6) ($p = 0.0005$). The greatest reduction was observed at 14 dpi 80.6 % and 83.6 % respectively ($p < 0.0001$) (Fig. 7, Table 2). There was no significant difference in spore reduction between groups NCF-0.5 and NCF-1 at 7 and 14 dpi. Regarding the NC group, a highly dispersed distribution of spore count was detected between each individual from 5.38×10^5 and up to 1.63×10^6 spores/bee at 7dpi and from 9.88×10^5 and up to 1.98×10^7 spores/bee at 14 dpi. No infection were detectable in group treated with fumagillin.

3.3. HO21-F reduces the level of N. ceranae spores in field tests and increases colony strength

We evaluated the therapeutic effect of HO21-F in naturally infected colonies, and there was a significant reduction in infection level of 88.1 % for group II (HO21-F) comparing days 0 and 28 ($p < 0.0001$) (Fig. 8). We also observed that group I (Control) has a reduction of infection level of 56.5 % between days 0 and 28 ($p = 0.0116$). There is a significant difference between groups I and II at day 28 ($p = 0.0033$), indicating that HO21-F has a positive effect on controlling *N. ceranae* infection level. Group III fed with the nutritional product Apiherb® shows a reduction of infection level up to 68.3 % ($p < 0.0001$). The level of infection on day 28 in group II was considerably lower when compared to group I, and similar reduction level to group III (Table 3).

Regarding colony strength, an increase of honey bee population was observed in group II with HO21-F in number of frames (25.2 %) meanwhile a decrease was observed in groups I with syrup (−39 %) and III with Apiherb® (−86 %). Honey production was observed by counting the number of frames per colony, where a slight increase of 12.5 % can be observed in group II treated with HO21-F and a decrease was observed in groups I (−39 %) and III (−346 %). The third parameter assessed was the number of frames covered with honeycomb cells, where an increase of 13 % in rearing can be observed in group II, while group I and III decreases (−95 % and –23 %) (Fig. 9). As we observe the percentage change, colonies of the groups fed with HO21-F shows the majority of increases in N° of frames, while Control and Apiherb® colonies shows the majority of decreases in N° of frames (Supplementary material – Table S5). The studied parameters demonstrates that the treatment HO21-F can increase colony strength significantly for the N° of frames with adult bees ($p = 0.0152$) and with frames with honey ($p = 0.0379$) (Supplementary material – Table S6).

Table 1
Survival rate between experimental groups at days 7 and 14 post-infection.

Group	Survival rate (%)	
	7dpi	14dpi
Control	91.6	90.3
Fumagillin	43.3	0
NC	96.6	94.1
F-0.5	92.9	73.7
F-1	97.8	74.7
NCF-0.5	97.5	82.5
NCF-1	92.5	74.7

4. Discussion

Chronic HO21-F consumption does not affect the survival of *A. mellifera* in laboratory conditions at concentrations of 0.5 and 1 g/L at 7 dpi (F-0.5 and F-1), however, a slight decrease was observed in survival rate at 14 dpi (day 17 of life). This could be explained by the stress caused for honey bees developing in cages, that has been reported before by the alteration of genes related to physiological and oxidative stress (Alburaki et al., 2019; De Smet et al., 2017). The survival observed in NCF-0.5 and NCF-1 at 14 dpi could be explained by other factors reported in honey bees experiments with artificial infection of *N. ceranae* that can alter survival rate, these are colony and queen conditions where worker bees were extracted, differences among honey bee species, other virulence factors associated with collected spores of *N. ceranae* used for artificial infection and the possibility that dead honey bee had higher infection levels (Azzouz-Olden et al., 2018; Fries et al., 2013). Other previous laboratory experiments reported 95 % of mortality in infected honey bees at day 7 dpi (day 12 of life) (Higes et al., 2007), but the results presented here agree with other studies where no significant mortality was reported up to day 12 (Paxton et al., 2007; Vidau et al., 2011). HO21-F is safer than other previously reported plant-based formulations and extracts with a lower survival rate between 40 and 42 %, indicating a considerable degree of toxicity (Chaimanee et al., 2021). The group fed with fumagillin 600 μg/mL had lower survival rate and all honey bees were dead at 14 dpi (day 17 of life), which is higher compared with other reported studies (Chaimanee et al., 2021; van den Heever et al., 2016). It is well known that Fumagillin can affect survival of honey bees since acts as non-specific inhibitor of methionine aminopeptidase type-2 present in *Nosema* spp, bees and also humans, and for this reason the application has been restricted in the European Union.

The effect of HO21-F on the level of infection can be already observed at 7dpi, when both concentrations reduced the level of infection above 50 % in comparison with the infected control and there was no dose-dependence response observed in the studied concentrations, with these results we could infer that the concentrations applied are above the minimal inhibitory concentration against *N. ceranae* spores. Other studies with plant extract derived from *Laurus nobilis* have reported antimicrosporidial activity from 1×10^4 μg/mL, which is composed of active ingredients essential oils, and polyphenols (Porrini et al., 2017). HO21-F is composed of natural plant extract with a high concentration of oleuropein, luteolin, quercetin, and its glycoside derivatives as active ingredients, that has a widely reported antifungal

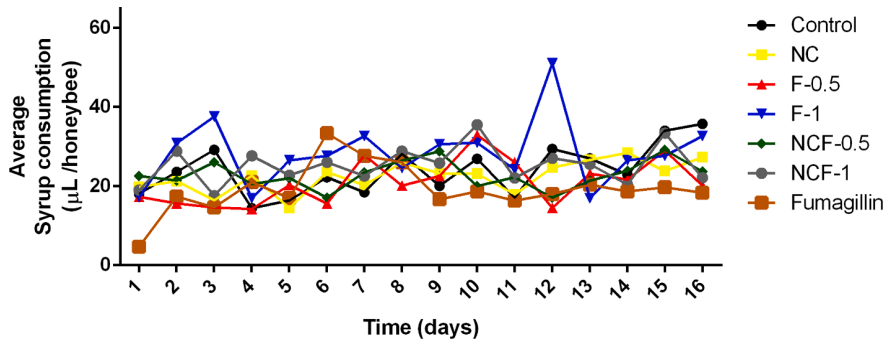

Fig. 4. Average daily consumption of syrup of different experimental groups. HO21-F and Fumagillin was administered from day 3.

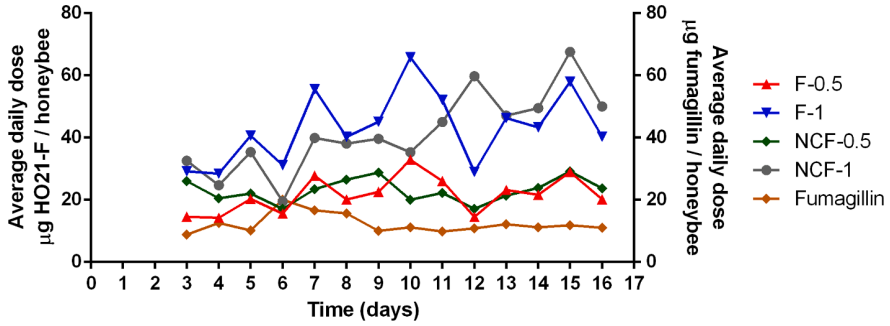

Fig. 5. Average daily dose of HO21-F and fumagillin of experimental groups. Consumption of HO21-F and fumagillin starts at day 3.

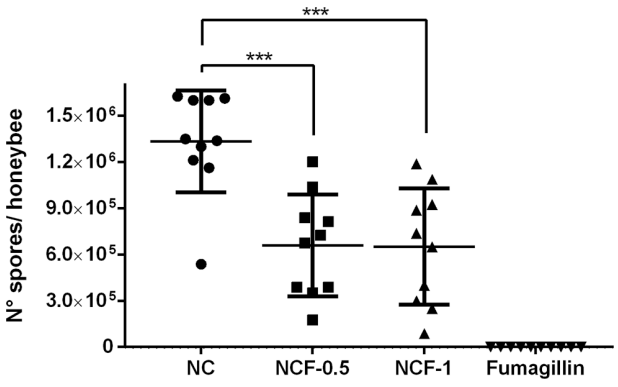

Fig. 6. Spore count of *N. ceranae* per honey bee of artificially infected groups that were exposed and non-exposed to HO21-F and exposed to fumagillin (Positive control). Day 7 post-infection (p-value: *** = 0,0005).

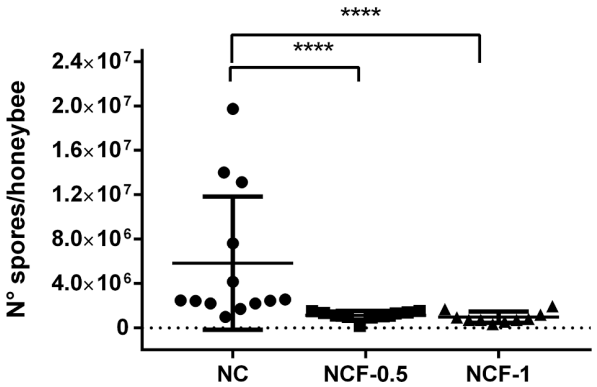

Fig. 7. Spore count of *N. ceranae* per honey bee of artificially infected groups that were exposed and non-exposed to HO21-F. Day 14 post-infection (p-value: **** < 0.0001).

activity in *in-vitro* studies (Báidez et al., 2006; Burnham et al., 2020; Chen et al., 2021; Zorić et al., 2016). The highly dispersed spore count between individuals in a group could be an effect of differential consumption of HO21-F in each cage, considering that it was only measured as the average daily consumption per cage. At 14 dpi, the reduction of 80 % in contrast with the NC group, indicates that HO21-F is still being effective in controlling the infection, suggesting that could be an alternative for nosemosis control for the whole lifetime of adult bees (Fig. 7). It's relevant to consider that HO21-F is effective in reducing the level of infection at 0.5 and 1 g/L similarly at 7 and 14 dpi. This indicates that 0.5 g/L is more than enough for application and to achieve a significant reduction in the level of infection of *N. ceranae*. The average accumulated dose of the plant extract contained in HO21-F as an active ingredient between healthy and infected experimental groups of bees was 147 and 295 µg /bee at 14 dpi. These results are consistent and more effective compared with other plant extracts, where they observed a reduction of the level of infection by 52 – 74 %, but with accumulated doses over 346–500 µg /bee at day 7, and with highly variable data depending on the plant extract administered and with the dispersed level of spore count within each experimental groups (Arismendi et al.,

Table 2
Average spores per bee and percentage of reduction of infection level per group.

Group	Average spores/bee. (× 10^5)		Reduction (%)	
	7 dpi	14 dpi	7 dpi	14 dpi
Fumagillin	0	–	100	–
NC	13.30 ± 3.3	58.20 ± 6.0	–	–
NCF-0.5	6.59 ± 3.3	11.30 ± 3.9	50.6	80.6
NCF-1	6.51 ± 3.8	9.55 ± 5.1	51.2	83.6

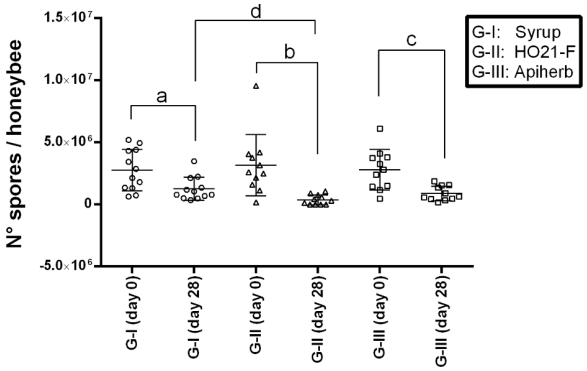

Fig. 8. Spore count of *N. ceranae* per honey bee of field tests that were exposed and non-exposed to HO21-F (p values: a = 0.0116, b < 0.0001, c = 0.0032, d = 0.0033).

2018; Burnham et al., 2020; Porrini et al., 2011).

In the field experiment, the reduction in the infection level observed also in group I (control) could be related to other factors that in combination can help colonies to recover from nosemosis and reduce the infection levels like agro-ecological conditions, relative humidity, pollen availability, change in the generation of population, relative hygienic behavior of colonies and artificial feeding (Formato et al., 2022). It has been reported that variations in environmental conditions can influence the infection prevalence and intensity, as well as the viability of *N. ceran*ae spores. (Chen et al., 2012; Gisder et al., 2011; Michalczyk and Sokół, 2018; Ptaszyńska et al., 2018). All these factors can produce variations in the level of infection that had been reported to oscillate between weeks in non-treated colonies, but at last, it could increase the level of infection in non-treated colonies 2 weeks after the intervention of the apiary (Nanetti et al., 2014b).

Reported field experiments of novel treatments have compared the effect of natural products with the application of fumagillin and reported a reduction of infection level of only 64 % (fumagillin), 46 % (Apiherb®), and 13 % (thymol) respectively (Nanetti et al., 2014b), a considerably lower reduction of spore load compared with the observed results in this study. Other authors have screened several plant extracts and reported spore load reductions from 20 to 60 % (Botías et al., 2013; Bravo et al., 2017; Chaimanee et al., 2021; Cilia et al., 2020). However they experienced seasonal variations in consumption rates from 38 to 100 % that can affect the effectivity of the treatments (Botías et al., 2013). This is an important factor that must be kept in consideration, which is directly related to the palatability of the formulations. HO21-F was designed to increase palatability to obtain a constant consumption rate subjected to changes in environmental conditions and it's equivalent to fructose syrup in this study.

Besides, other aspects must be kept into consideration, as well as the behavior of colonies, the dynamic of the population, honey production, toxicity levels, and the consumption rate of honey bees (Delaplane et al., 2013; Zheng et al., 2014). Several studies have reported that colony strength parameters are a key component to prevent nosemosis and colony collapse in the most extreme conditions (Barroso-Arévalo et al., 2019; Burnham, 2019; Jovanovic et al., 2021). Field tests performed in this study were designed in a way to assess the real conditions with the natural infection of colonies and their effects on strength. Since the spore loads at the midgut level have been recommended not to be considered as the only indicator of the severity of *N. ceranae* infection in *A. mellifera* (Zheng et al., 2014), there are other aspects related to colony strength that has been studied (covered honeycombs, population in frames, and production of honey). HO21-F can significantly increase the population of adult honey bees in colonies after 28 days of weekly application. This can directly influence the production of honey, and pollen availability to counteract the physiological stress caused by nosemosis, in contrast with colonies only fed by fructose syrup. This is a plus for a product that can act as a dual effect nutritional and pharmaceutical for this pathology.

Besides, the observed effects of the formulation in terms of survival rate and innocuity, it has been considered safer than other plant extract and essential oils reported and more effective in reducing the level of infection (Braglia et al., 2021; Ebert et al., 2015). For the selection of an

Table 3
Average spores per colony of experimental groups in field.

Groups		spores/bee (x 10^6) (day 0)	spores/bee (x 10^6) (day 28)	Reduction (%) (day 28)	P value (significance)
G-I	Syrup	2.697 ± 1.7	1.172 ± 9.3	56.5	a = 0.0116
G-II	HO21-F	3.173 ± 2.5	0.3784 ± 4.0	88.1	b < 0.0001
G-III	Apiherb®	2.797 ± 1.6	0.891 ± 0.5	68.1	c = 0.0032

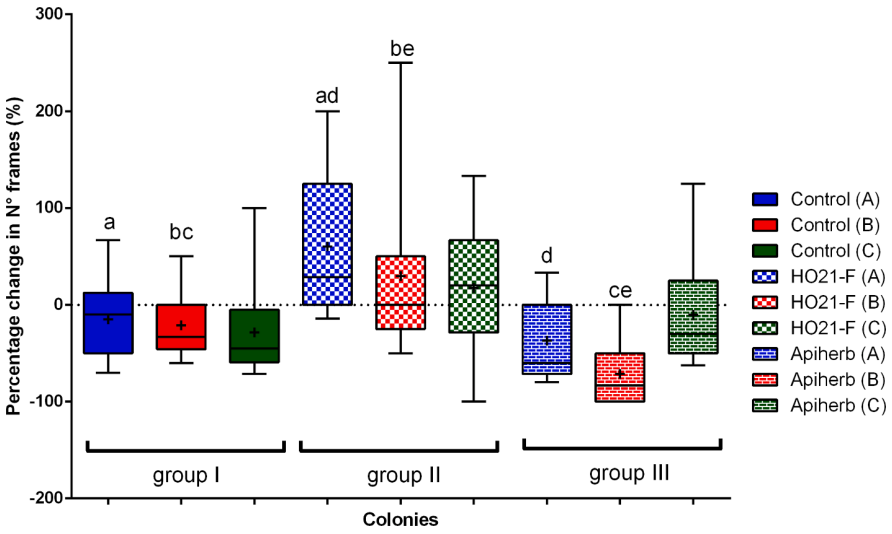

Fig. 9. Percentage change of strength parameters of honey bee colonies of each experimental group. A: N° of frames covered with honeybees, B: N° of frames with honey, C: N° of frames with covered honeycombs. Group I: infected honeybees fed with fructose syrup, group II: infected honeybee treated with HO21-F, group III: infected honeybees treated with Apiherb®. Different letters indicate pairwise significant differences in average frame N° between treatments (statistical details in Table S6).

effective treatment for nosemosis, it should demonstrate non-toxicity throughout all the life cycle of honey bees. However, other commercial products and active compounds to control nosemosis have reported slight sublethal toxicity in larvae stages and the cells of the midgut, increasing DNA fragmentation and altering the HSP70 immunolabeling pattern (Borges et al., 2020; Tadei et al., 2020). Therefore, further studies need to be done to determine the effect of this HO21-F in the larvae stage of *A. mellifera*.

To understand the mechanism of action of active ingredients of HO21-F, it's important to point out that it could be by 2 alternatives: i) inhibition of germination; the content of total polyphenols as well its composition could disrupt the natural germination process of *N. ceranae* spores and proliferation in the midgut. Several studies have demonstrated the antifungal activity of plant extracts and preparation of its main phenolic components, evidencing that can affect the viability of spores by inactivating membrane proteins associated with germination (Aouidi et al., 2012; Báidez et al., 2006; Battinelli et al., 2006; Li et al., 2011; Nanetti et al., 2021; Zorić et al., 2016), as well as other compounds that can modulate *N. ceranae* multiplication by inducing humoral immunity responses (Borges et al., 2020; Valizadeh et al., 2020). It has been proposed that the diversity of compounds available in plant extracts could act against microsporidia spores, this is with the total polyphenols content of HO21-F that has previously demonstrated wide antimicrobial activity (data not published) and ii) immunostimulant activity in honey bees: HO21-F could have an immunostimulant effect, inducing the expression of antimicrobial peptides that could prevent nosema infection. There are reported studies that indicate that plant extracts and pollen diets can influence the production of antimicrobial peptides and immune response-related enzymes (Danihlík et al., 2018, 2015; Ptaszyńska et al., 2016; Vezza et al., 2017).

HO21-F is a prototype formulation designed for controlling nosemosis and could inhibit *N. ceranae* spore germination and improve honey bee humoral immunity by modulating antimicrobial peptides synthesis. Further studies are necessary to understand the effect of HO21-F on the physiological and immune response of honey bees at different days post-infection, considering pre and post-germination stages in the midgut of honey bees. The reported antifungal activity of HO21-F against *N. ceranae*, with a sustained control of spore proliferation in worker bees, suggests the promising commercial application use of this product against nosemosis, and it will encourage new research studies to understand the mechanism of action, whether related to the spore-inhibition effect and/or a stimulating effect in natural response of colonies to counteract the disease.

Funding information

This work was financially supported by Fundación para la Innovación Agraria FIA Grant Project PYT-2019-0215.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank M. Henríquez and C. Sepúlveda for technical support and assistance in hive handling where samples were taken.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107801.

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