Ab Intus Revista ***Ab Intus*** **FAV-UNRC**
2022, 10(5): 33-49 ISSN 2618-2734

Effect of the hexanic extract of *Achyrocline satureioides* on *Paenibacillus larvae* growth, pathogen of *Apis mellifera*

Efecto del extracto hexánico de *Achyrocline satureioides* sobre el crecimiento de *Paenibacillus larvae*, patógeno de *Apis mellifera*

DOI: 10.5281/zenodo.7484788

María Fernanda Paletti Rovey[1,3,*], Diana Carolina Pimentel Betancurt[1,3], María de la Paz Moliné[2,3], María de las Mercedes Oliva[1,3]

1-Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto.
2-Centro de Investigación en Abejas Sociales (CIAS), Instituto de Investigaciones en Producción Sanidad y Ambiente, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata.
3-CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas).

ABSTRACT. The health of *Apis mellifera* hives is an important issue due to the ecological and economic role that bees play. *Paenibacillus larvae,* a spore-forming bacillus, is the main bacterial pathogen of bees larvae and the causative agent of American foulbrood. This disease causes considerable economic losses and its control is a challenge for beekeepers around the world. Therefore, there is a need to find effective treatments and natural products derived from medicinal plants are being studied. In the present work, the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the hexanic extract of *Achyrocline satureioides* were determined, obtaining values of 0.30 µg/ml and 1.17 µg/ml, respectively. The growth parameters of *P. larvae 9* (ERIC I) were determined, and then the effect of different concentrations of the hexanic extract (sub-inhibitory, inhibitory, sub-bactericidal and bactericidal) on the bacterial growth was evaluated. The hexanic extract showed a good inhibitory effect regardless of the concentrations tested. Bacterial growth was significantly affected at sub-bactericidal and bactericidal concentrations. Our results demonstrated the effectiveness of the hexanic extract of *A. satureioides* on *P. larvae* growth, constituting a promising alternative for American foulbrood control.

KEYWORDS. American foulbrood, *Paenibacillus larvae, Achyrocline satureioides,* antimicrobial activity, plant extract

Artículo recibido: 15/9/22. Artículo aceptado: 5/12/22

***Corresponding author:** María Fernanda Paletti Rovey. Universidad Nacional de Río Cuarto. Ruta Nac. 36 - Km. 601, X5804BYA Río Cuarto, Córdoba, Argentina. E-mail: mpalettirovey@exa.unrc.edu.ar

Funding: This study was funded by FONCyT (Fondo para la Investigación Científica y Tecnológica - PICT: 2016-1566) and SeCyT-UNRC (Secretaría de Ciencia y Técnica - Universidad Nacional de Río Cuarto - PPI 2020-2022).

Esta obra está bajo licencia Creative Commons Atribución-NoComercial-CompartirIgual 4.0 Internacional http://creativecommons.org/licenses/by-nc-sa/4.0/deed.es_AR

RESUMEN. La salud de *Apis mellifera* es un tema importante debido al rol ecológico y económico que cumplen las abejas. *Paenibacillus larvae*, un bacilo formador de esporas, es el principal patógeno bacteriano de las larvas de abejas melíferas y es el agente causal de Loque Americana. Esta enfermedad provoca pérdidas económicas considerables y su control es un desafío para los apicultores de todo el mundo. Por lo tanto, es necesario encontrar tratamientos efectivos y, en este sentido, se están estudiando productos naturales derivados de plantas medicinales. En este trabajo se determinó la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM) del extracto hexánico de *Achyrocline satureioides*, obteniendo valores de 0,30 µg/ml y 1,17 µg/ml, respectivamente. Se determinaron los parámetros de crecimiento de *P. larvae* 9 (ERIC I) y luego se evaluó el efecto de diferentes concentraciones del extracto hexánico (sub-inhibitoria, inhibitoria, sub-bactericida y bactericida) sobre el crecimiento bacteriano. El extracto hexánico mostró un buen efecto inhibitorio, independientemente de las concentraciones evaluadas. El crecimiento bacteriano fue afectado significativamente a concentraciones sub-bactericidas y bactericidas. Nuestros resultados demostraron la efectividad del extracto hexánico de *A. satureioides* sobre *P. larvae*, constituyendo una alternativa prometedora para el control de Loque Americana.

PALABRAS CLAVE. Loque Americana, *Paenibacillus larvae, Achyrocline satureioides,* actividad antimicrobiana, extracto vegetal

INTRODUCTION

Honeybees play a valuable role in society due to the obtaining of products derived from the hive such as honey, wax, propolis, and pollen. Furthermore, they play an extraordinarily necessary role in nature because they contribute to maintaining the balance and functioning of ecosystems. *Apis mellifera* pollinates approximately 70% of the plant species necessary for the generation of food resources. Therefore, the health of hives is one of the main parameters to consider when the aim is to improve and increase their productivity, and to protect bees. Nowadays, the loss and depopulation of colonies in the world have increased generating a complex situation for the balance established between bees and plants (Potts *et al*., 2010; van der Zee *et al*., 2012; Maggi *et al*., 2016; Cunningham *et al.,* 2018; Gray *et al.*, 2019).

Paenibacillus larvae is the main bacterial pathogen of honey bee larvae and the causative agent of American foulbrood (AFB). It is a Gram-positive bacillus that can produce millions of spores in each infected larva. These structures are the only infective form and are resistant to heat, drying, radiation, and chemical disinfectants what makes them difficult to control, representing a challenge for beekeepers and scientists. AFB is a mandatory reporting disease with worldwide distribution and highly contagious, causing the death of the affected colonies and, consequently, considerable economic losses (Alippi, 1995; Genersch *et al.*, 2006; Genersch, 2010).

Traditionally, antibiotics such as oxytetracycline hydrochloride, sulfathiazole or tetracycline have been used to AFB control, but these products are known to be effective only against vegetative cells and not over spores. The misuse of antibiotics has been associated with a decrease in the vitality and longevity of bees, but mainly with the generation of resistant strains of *P. larvae*. Furthermore, the application of antibiotics has been banned in many countries because residues were found in honey and other products derived from the hive, representing a potential hazard to human health. In Argentina, the use of them was regulated in 2016 by SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria) (2017), which removed from the list of veterinary products approved for beekeeping use all those made with the active ingredient oxytetracycline. Therefore, the burning of hives is the only effective solution for AFB control, despite the economic losses that these practices cause (Hansen and Brødsgaard, 1999; Antúnez *et al,.* 2008; Alippi *et al.*, 2013). The severe consequences caused by this disease and the lack of effective solutions to treat it show the need to develop new strategies for its control. In this way, the study of natural products derived from medicinal plants is gaining attention because they have many biological properties, like the antimicrobial activity (Fuselli *et al.*, 2008; Flesar *et al*., 2010; Sabaté *et al*., 2012; Boligon *et al*., 2013; Ansari *et al*., 2016; Chaimanee *et al*., 2017; Fernandez *et al.*, 2019; Pimentel Betancurt *et al*., 2021).

Our research group has studied the antimicrobial activity against *P. larvae* of natural products obtained from *Achyrocline satureioides*: essential oils,

aqueous extracts, and extracts obtained with solvents of different polarity. All of them were effective inhibitors of this sporulated bacillus, being the hexanic extract (HE) the one that showed the best antimicrobial activity (González and Marioli, 2010; González *et al*., 2015; Pimentel Betancurt *et al.,* 2021). *A. satureioides* (Lam.) DC is a plant species belonging to the *Asteraceae* family, perennial and aromatic, popularly known as "Marcela" and widely used in traditional medicine in the Río de la Plata region. The inflorescences are used in preparations for medicinal purposes (Gattuso and Gattuso, 1998; Gattuso *et al.*, 2008). Several authors have reported that this plant has significant amounts of flavonoids that would be responsible for the biological activities attributed to the plant (De Souza *et al.,* 2007; Retta *et al*., 2012; Retta, 2014; Pimentel Betancurt *et al.,* 2021). Tonello *et al.* (2022) carried out a study of the chemical composition and antimicrobial activity of the HE, isolating and characterizing four main compounds. All of them showed good antimicrobial activity against *P. larvae*, however, the whole extract was the one with the highest activity.

In the search for new antimicrobials, it is essential to understand the behavior and interactions between the compounds and the microorganism. In this way, the study of antimicrobial activity can be studied by performing growth curves analyzing a range of concentrations that allows a better characterization of the temporal evolution of its activity in contact with a bacterial culture (Yourassowsky *et al.*, 1985; Vogelman and Craig, 1986; Ramírez and Castaño, 2009). Therefore, the knowledge of the growth behavior of *P. larvae* and the effect of inhibitory concentrations of the HE could provide useful information to contribute to the design of new alternative strategies for AFB control in the future.

The aim of this research work was to evaluate the effect of different concentrations of the HE of *A. satureioides* on the growth parameters of *P. larvae*.

MATERIALS AND METHODS

Bacterial strain

Paenibacillus larvae 9 (*Pl 9*) was isolated from brood comb of beehives with characteristic symptoms of AFB in the southern area of Río Cuarto Department. It was phenotypically identified in the Laboratory of Microbiology (Universidad Nacional de Río Cuarto (UNRC)). The biochemical identification was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry technique (MALDI-TOF MS) performed in a Bruker Maldi-Tof MS mass spectrometer by the Laboratory of Microbiology (Hospital Privado Universitario de la Ciudad de Córdoba). Genotyping was performed by the diagnostic service of "Unidad de Bacteriología del Centro de Investigaciones en Fitopatología (UB. CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata (UNLP)". For *P. larvae* identification, PL1/PL2 primers (Govan *et al.*, 1999) were used to amplify a specific fragment of the 16S rDNA gene. Subsequently, the rep-PCR technique was performed using ERIC primers (ERIC1R/ERIC2) (Versalovic *et al.*, 1994) for the genotyping. The strain was maintained on MYPGP agar and stored at 4 °C. Long-term maintenance was carried out in J broth (15 g yeast extract l^{-1}, 5 g triptein l^{-1}, 3 g K_2HPO_4 l^{-1}, and 2 glucose l^{-1}) with 20% v/v glycerol and kept at -20 °C.

Plant material

A. satureioides plants were collected in February and March of 2017 in Santa Monica (32° 5' 28.21'' S and 64° 36' 31.66'' W), a low hills rural area in Córdoba province (Argentina). The identification was carried out at the Botany Area (Facultad de Agronomía y Veterinaria, UNRC) and a sample of the specimen was kept in the collection of the UNRC Herbarium. The plant material was dried at room temperature between sheets of paper.

Hexanic extract of *Achyrocline satureioides*

Plant material was prepared and leaves, flowers and thin stems were cut to improve the contact with the extracting solvent. A maceration of these materials was made soaking up 150 g in a mixture of ethanol: water (1:1, final volume 1 L), shaking it softly every day. The extracting solution was periodically renewed, and the macerate was filtered with paper Whatman No. 2 to obtain a hydroalcoholic extract (HAE). The HAE was concentrated under reduced pressure in a rotary evaporator (Rotavapor-IKA RV 10) to evaporate the ethanol and obtain an aqueous extract (AqE). Finally, the AqE was submitted to a liquid-liquid extraction with hexane. The process was carried out for a week or until the absence of color in the solvent was observed, indicating the extraction end. The resulting hexanic extract (HE) was concentrated using a rotary evaporator (Rotavapor-IKA RV 10) and bubbling with nitrogen gas. The HE was kept in a dark bottle at -20 °C until use (González *et al*., 2015).

Antimicrobial activity

Minimum Inhibitory Concentration (MIC)

The broth microdilution technique described by Pellegrini *et al.* (2017a) was used to determine the MIC of the HE. Briefly, 100 µl of BHI broth (37 g l -1) was placed in each well of a 96-well microplate. Then, 100 µl of the HE was added to the first well, and serial dilutions were made, taking 100 µL of the first well and adding it to the next and repeating this process. Finally, 50 µl of the inoculum was added to each well. The microplate was incubated at 37° C for 48 h. After incubation, 10 µl of resazurin (0.01%) was added to each well and incubated for 1 - 2 h under the same conditions. The MIC was determined visually by the indicator color change, from blue to pink, produced by the metabolism of the microorganisms. Positive control consisted of the inoculum without the addition of the HE to verify the viability of the strain, and negative controls consisted of the diluent dimethyl sulfoxide (15% DMSO, non-toxic concentration determined in previous tests), BHI broth, and the HE. These controls were performed to verify that the different components used did not reduce resazurin.

Minimum Bactericidal Concentration (MBC)

The MBC was determined by the microdrop technique. Three drops of 20 µl of the MIC and the previous five dilutions were placed on MYPGP agar plates (10 g Mueller–Hinton broth l^{-1}, 15 g yeast extract l^{-1}, 3 g K_2HPO_4 l^{-1}, 2 g glucose l^{-1}, 1 g sodium pyruvate l^{-1}, and 20 g agar l^{-1}). The plates were incubated at 37°C for 48 h under microaerophilic conditions.

The MBC belonged to the minimum concentration of the HE in which less than 0.01% of the inoculum survived (Finelgold *et al.*, 1992).

Effect of the hexanic extract on *Paenibacillus larvae* growth

An Erlenmeyer containing 30 ml of J broth was inoculated with a *Pl 9* culture grown in MYPGP agar and incubated at 37 °C for 24 h with shaking. Subsequently, the culture was adjusted to 0.01 optical density measured at 600 nm (OD_{600nm}) corresponding to 10^6 CFU/ml and tubes containing 7 ml of this inoculum were made for each measurement time to avoid contamination. Previously growth curves were performed with the addition of DMSO (extract dissolvent) to verify that it did not affect the growth of the microorganism.

Three growth curves were performed at different times and concentrations: **1-** C curve: control, without the addition of the HE), **2-** Ei curve: with the addition of the HE from the beginning of growth (0 h), and **3-** Et curve: with the addition of the HE at the beginning of the stationary phase (46 h). The HE was added at different times and concentrations to evaluate its effect on the growth process: ½ MIC, MIC, 2 ½ MIC, and MBC. Bacterial growth was determined by viable cell count (CFU/ml) and OD_{600nm}. Growth rates (µ) were determined by a linear regression analysis of the exponential phase of the cell counting curves. The generation time (GT) was calculated from the slope value ($GT = \log_{10} 2/\mu$).

Statistical analysis

The experiments were performed in duplicate, and each assay was repeated three times independently. Statistical analysis of the differences between the mean values obtained for the experimental groups was performed by Kruskal-Wallis test using the InfoStat software (InfoStat versión 2018, Di Rienzo *et al.,* 2018). A *p-value* <0.05 was considered statistically significant.

RESULTS

Hexanic extract of *Achyrocline satureioides*

The HE yielding was 1.35% w/w; it presented a viscous appearance, dark green color and intense odor, coinciding with the characteristics reported by Retta (2014), who described the HE of *A. satureioides* with an intense, pleasant, persistent and characteristic aroma.

Antimicrobial activity

The MIC of the HE was determined on *Pl 9* strain (ERIC I genotype) using a microdilution technique. In the assay, different concentrations were evaluated ranging from 8.5 x 10^{-4} to 900 µg/mL, obtaining a MIC value of 0.30 µg/ml. The bactericidal activity of the HE was determined by the microdrop technique obtaining the concentration where no bacterial growth was observed (MBC) at 1.17 µg/ml.

***Paenibacillus larvae* growth parameters**

First, it was confirmed that DMSO did not affect the *P. larvae* growth. It was determined that all growth phases of *Pl 9* ERIC I occurred in a time close to three days (70 h), observing a very short lag phase followed by a long exponential phase, then a short stationary phase, and finally, a death phase confirmed by the decrease in the viable cell count. The growth curves were constructed from the data ob tained from the viable cell counts and the absorbance measurements at different times (Fig. 1a and b). The growth rate was µ=0.0346 h^{-1} and the time required for the population to double, GT, was 8 h 42 min.

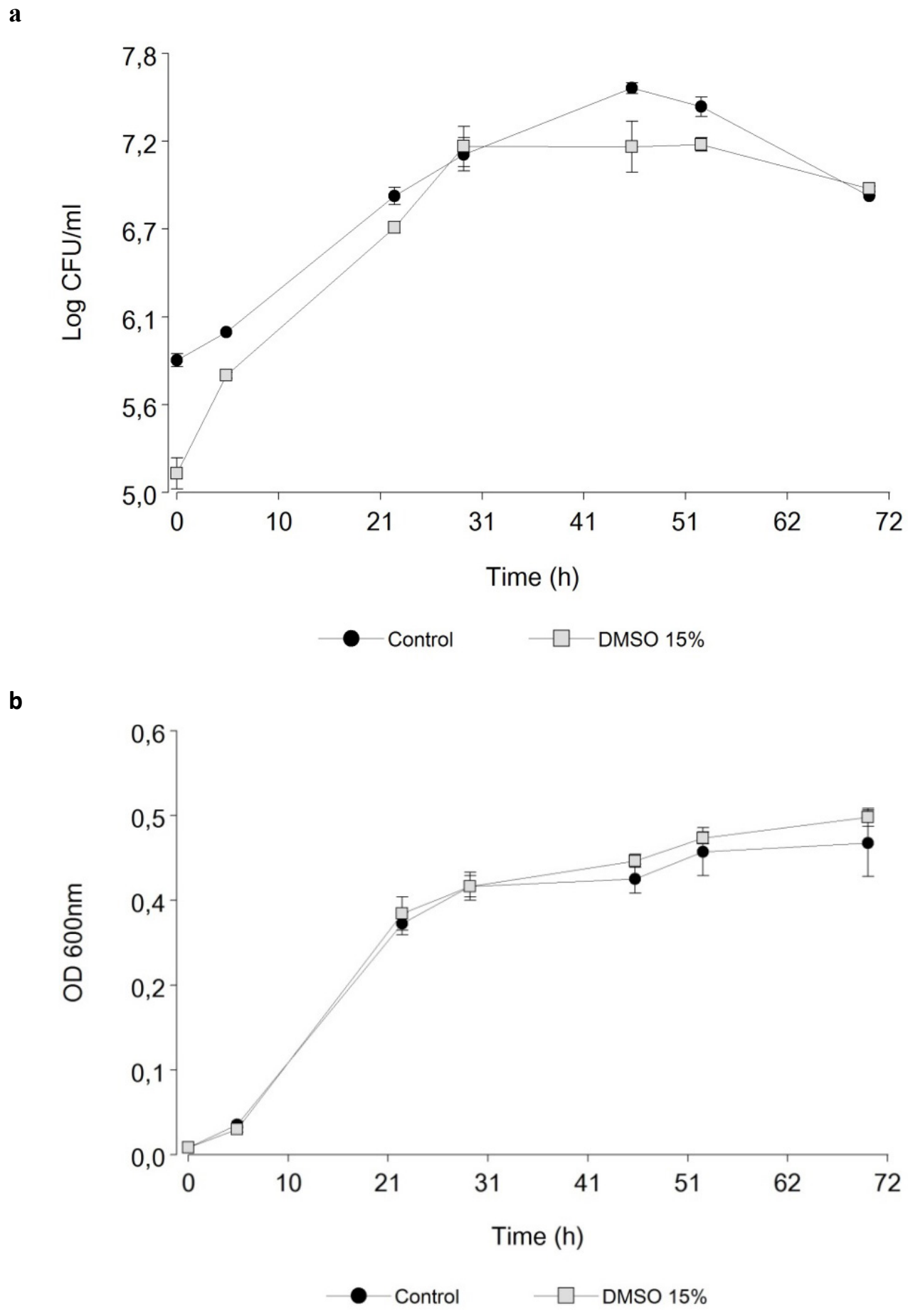


Fig.1 Growth curves of *P. larvae* 9 under optimal conditions (Control) and DMSO 15% (dimetilsulfoxide, dissolvent extract) control. **a.** Viable cell count (CFU/ml). **b.** OD measured at 600 nm (OD 600nm).

Effect of sub-inhibitory concentrations of the HE

A *PI 9* inoculum was exposed to sub-inhibitory concentrations (½ MIC = 0.15 µg/ml) of the HE at two different times: Ei (added at time 0, beginning of the growth) and Et (added at the beginning of the stationary phase), and the viability and absorbance for each time point were measured.

It was observed that the CFU/ml, in the presence of sub-inhibitory concentrations of the HE from the beginning (Ei curve), was reduced in more than one log unit compared to the control; while when the HE was added in the stationary phase (Et curve), a slight decrease in the CFU/ml was observed (Fig. 2a). This meant a decrease in the growth rate of 27.35 % (µ=0.0247 h^{-1}) and an increase in the GT of 40% (12 h 11 min). When OD_{600nm} was analyzed, lower absorbance values were observed in presence of the HE in Ei curve compared to C curve. Although there was an increase in absorbance up to 53 h, the OD_{600nm} began to stabilize, and it always remained below the OD_{600nm} values of C curve. The Et curve presented a similar behavior to C curve until 46 h when the HE was added, showing a decrease in the OD_{600nm} values after this time (Fig. 2b).

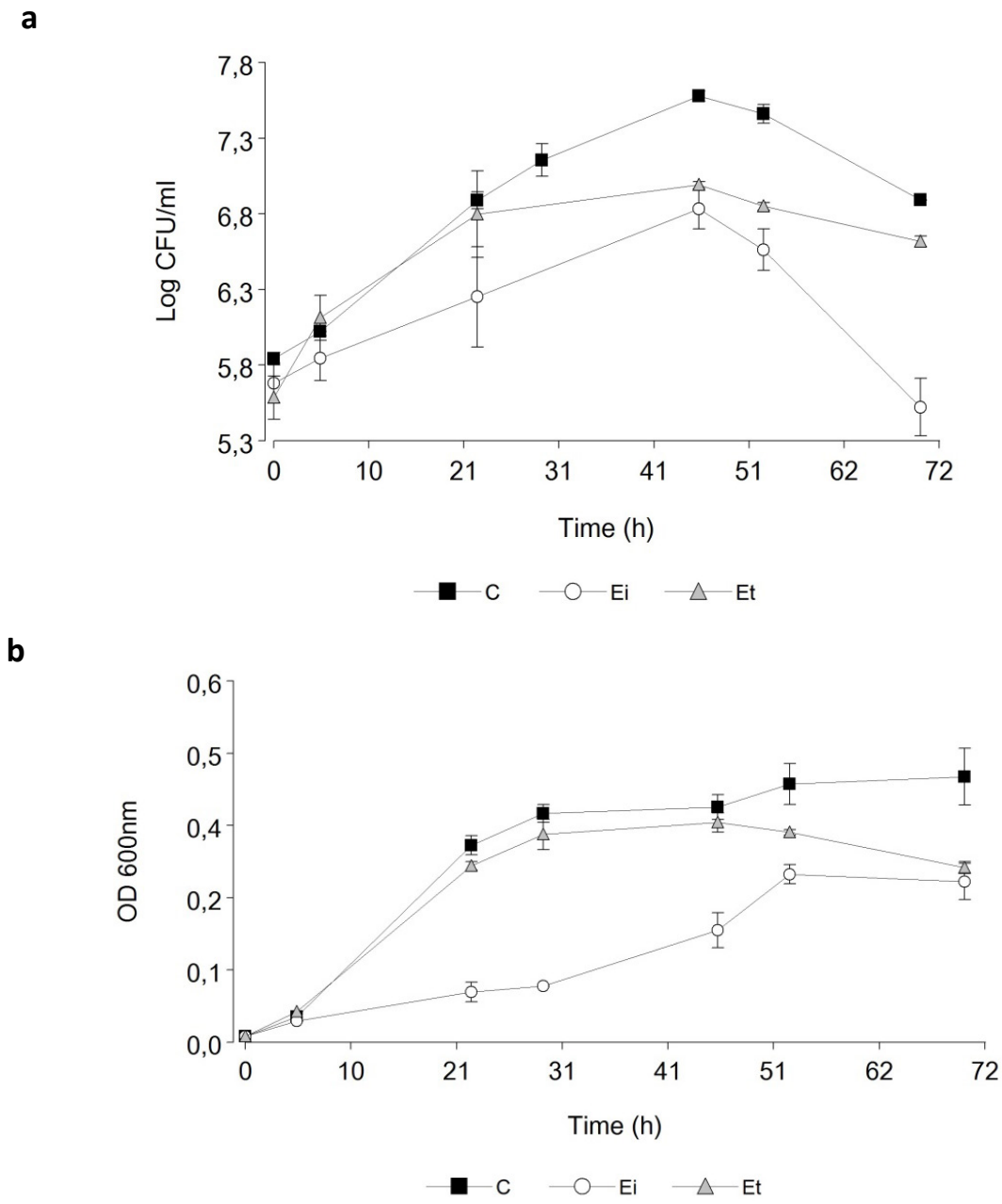


Fig.2 Growth curves of *P. larvae* 9 with the addition of the HE at a concentration of 0.15 mg/mL = ½ MIC. C: control; Ei: addition of the HE at the beginning of growth (0 h); Et: addition of the HE at 46 h. **a.** Viable cell count curves. **b.** OD curves.

Effect of inhibitory concentrations of the HE

The inhibitory concentration (MIC = 0.3 µg/ml) of HE produced a significant reduction of two log units in CFU/ml compared to C curve for *PI 9* throughout the entire experiment for the Ei curve. The µ was 0.0165 h^{-1}, and the GT was 18 h 14 min. It was observed that the addition of the HE in the first times of the experiences produced a notable decrease in *PI 9* growth rate (52.30 %) and a significant increase in the duplication time (109.65 %). These results were showing that the growth of the microorganisms was stopped, which could be explained by the inhibitory effect of the HE, preventing the binary fission of the vegetative cells in the duplication process. In the Et curve, the inhibitory effect on the growth was observed from the moment that the HE was added, decreasing the CFU/ml in one log unit compared with the C curve (Fig. 3a). Figure 3b shows a decrease of OD_{600nm} values in Ei curve compared to the control. It could be noticed that all tubes containing HE, where the bacteria was growing, kept with a limpid appearance throughout the experience, what could be indicating that the duplication ability of the microorganism was inhibited. The Et curve showed a similar effect with differences in growth after the treatment with the HE (46 h), with a decrease in the absorbance compared to the C curve.

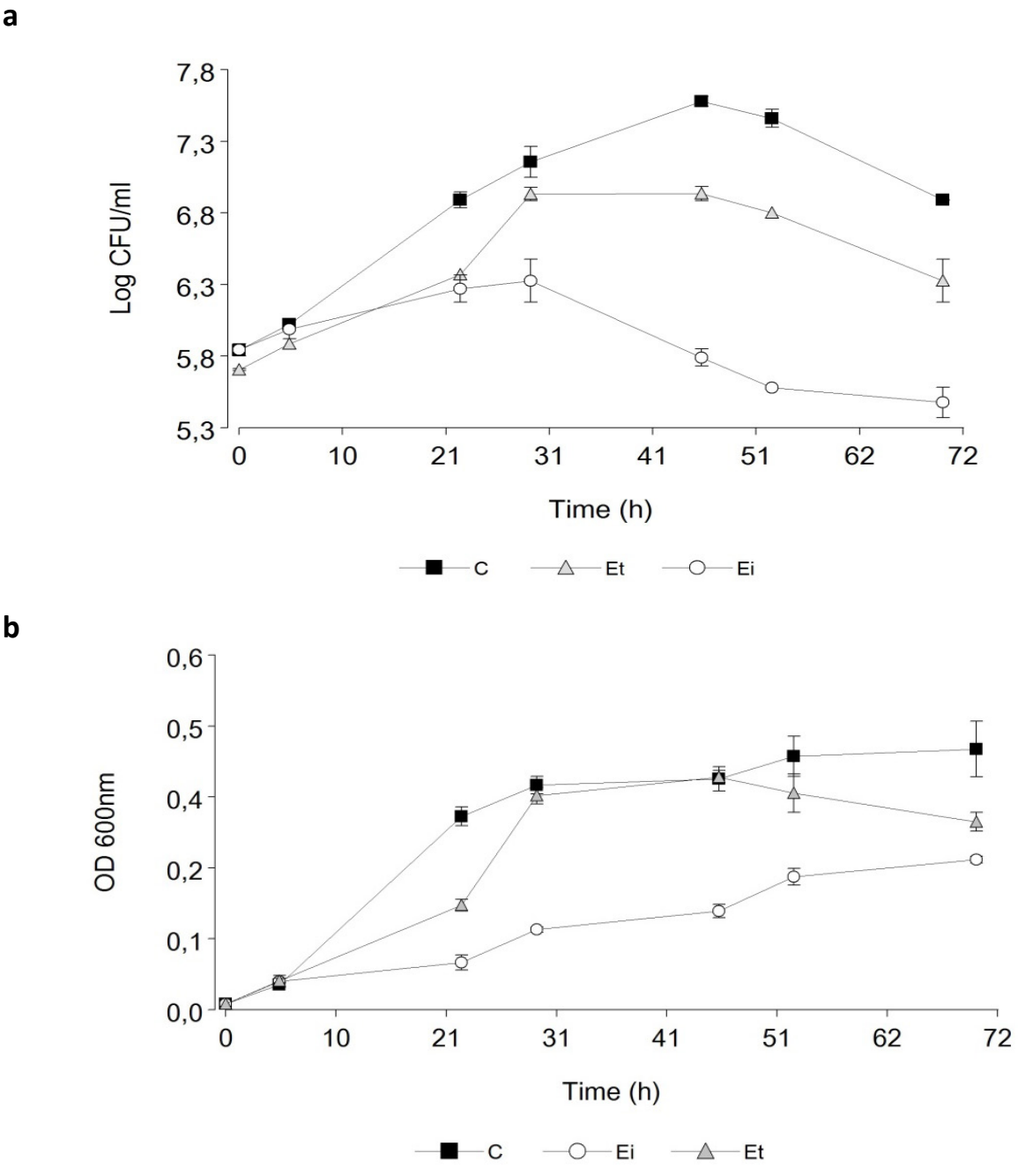


Fig.3 Growth curves of *P. larvae* 9 with the addition of the HE at a concentration of 0.30 mg/mL = MIC. C: control; Ei: addition of the HE at the beginning of growth (0 h); Et: addition of the HE at 46 h. **a.** Viable cell count curves. **b.** OD curves.

Effect of sub-bactericidal concentrations of the HE

When concentrations higher than the MIC but lower than the MBC were tested (2 ½ MIC = 0.75 mg/ml) on *Pl 9* from the beginning (Ei curve) a similar behavior to that obtained with the MIC was observed, determining that at 70 h the growth had decreased in approximately two log units compared to the C curve. The addition of the HE produced a decrease in *Pl 9* growth rate of 38.15 % and an increase in the generation time of 61.7 %. The μ was 0.0214 h^{-1} and the GT was 14 h 4 min. In Et curve, growth decrease was observed in one log unit compared to the C curve, showing a marked falling after the addition of the HE (Fig. 4a). In OD_{600nm} curves, a uniform behavior was observed in Ei curve throughout the whole assay, where the OD_{600nm} values were significantly low (not higher than 0.09) compared to the control. In addition, a similar situation to that of the inhibitory concentration tubes was observed, presenting a limpid appearance throughout the experiment. The Et curve showed similar behavior to the control until 46 h, the time when the HE was added; then, a decrease in the absorbance values began to be observed (Fig. 4b).

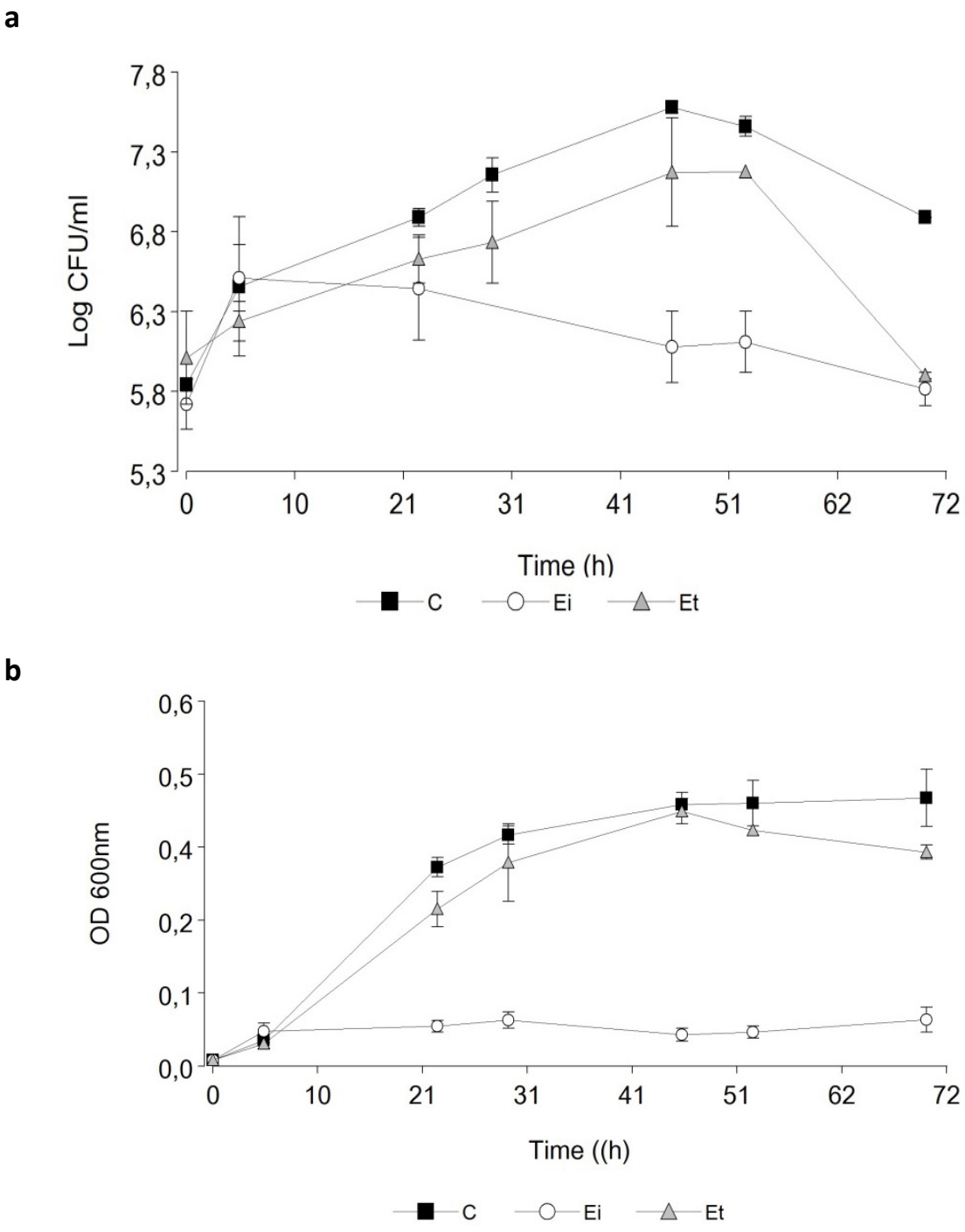


Fig.4 Growth curves of *P. larvae* 9 with the addition of the HE at a concentration of 0.75 mg/mL = 2 ½ MIC. C: control; Ei: addition of the HE at the beginning of growth (0 h); Et: addition of the HE at 46 h. **a.** Viable cell count curves. **b.** OD curves.

Effect of bactericidal concentrations of the HE

The bactericidal concentration produced a strong decrease in the CFU/ml when was added from time 0 (Ei curve). Colonies development was observed until 5 h, while the absence of growth happened at the following times. Therefore, the addition of the HE at the MBC (1.17 mg/ml) caused the death of *Pl 9*, as was expected. In the Et curve, a notable decrease in growth was observed after the addition of HE (46 h), causing the death of the microorganisms (Fig. 5a). When OD_{600nm} was measured, a uniform behavior was observed throughout the whole assay and OD_{600nm} values stayed significantly below the C curve when the HE was present from the beginning of the experiment (Ei curve). In addition, the tubes presented a limpid appearance throughout the experiment, indicating there was no development of microorganisms, which was later corroborated by the absence of growth in MYPGP plates. In Et curve, the behavior was similar to C curve until 46 h, the time when the HE was added. Then, a significant decrease in absorbance values was observed due to the antibacterial effect of the HE. This falling in the OD_{600nm} values could indicate a possible lithic activity produced by the HE (Fig. 5b).

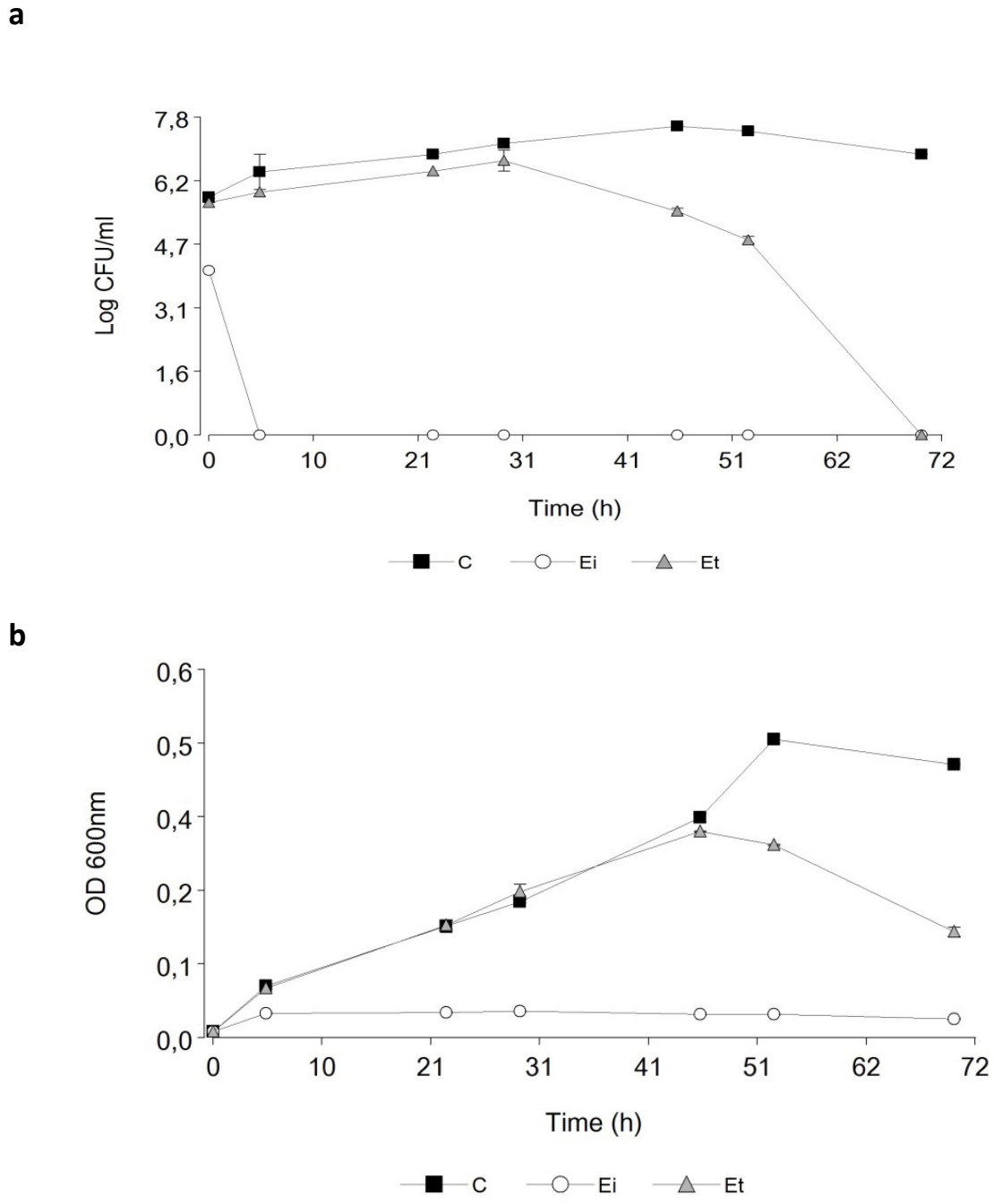


Fig.5 Growth curves of *P. larvae* 9 with the addition of the HE at a concentration of 1.17 mg/mL = MBC. C: control; Ei: addition of the HE at the beginning of growth (0 h); Et: addition of the HE at 46 h. **a.** Viable cell count curves. **b.** OD curves.

It was observed that, independently of the concentration of HE or the growth measurement method (CFU/ml or OD_{600nm}), the curves with the addition of HE from the beginning (Ei) presented a significant decrease in growth compared to the control, especially at concentrations of 0.3 µg/ml (MIC), 0.75 µg/ml (2 ½ MIC) and 1.17 mg/ml (MBC). A decrease in the growth of *P. larvae* was also observed when the HE was added at 46 h (Et), reinforcing the idea of the inhibitory activity of the HE. These results confirm the antibacterial activity of the HE of *A. satureioides*, which was described previously by other authors (González and Marioli, 2010; González *et al*., 2015; Pimentel Betancurt *et al*., 2021); and demonstrate that the HE is effective at all growth stages of this bacillus.

The Kruskal-Wallis test of the variance showed statistically significant evidence ($p<0.05$) that growth was affected by the concentration and time of addition of the HE ($p<0.0001$) (Fig. 6). The comparisons analysis showed significant differences between the curves in which the addition of the HE at all concentrations was from the beginning (Ei curve): 0.15; 0.30; 0.75 and 1.17 µg/ml (½ CIM, CIM, 2 ½ MIC and MBC, respectively) compared to C curves. These results would indicate that the HE had a good antimicrobial effect independently of the concentrations analyzed. When the HE was added at 46 h (Et curve), at concentrations of 0.15; 0.30 and 0.75 µg/ml, growth decrease occurred in the viable cell counts, although statistical analyses did not detect these differences. However, in the curve with the addition of the HE at a concentration of 1.17 µg/ml (MBC), statistically significant differences were observed compared to the control.

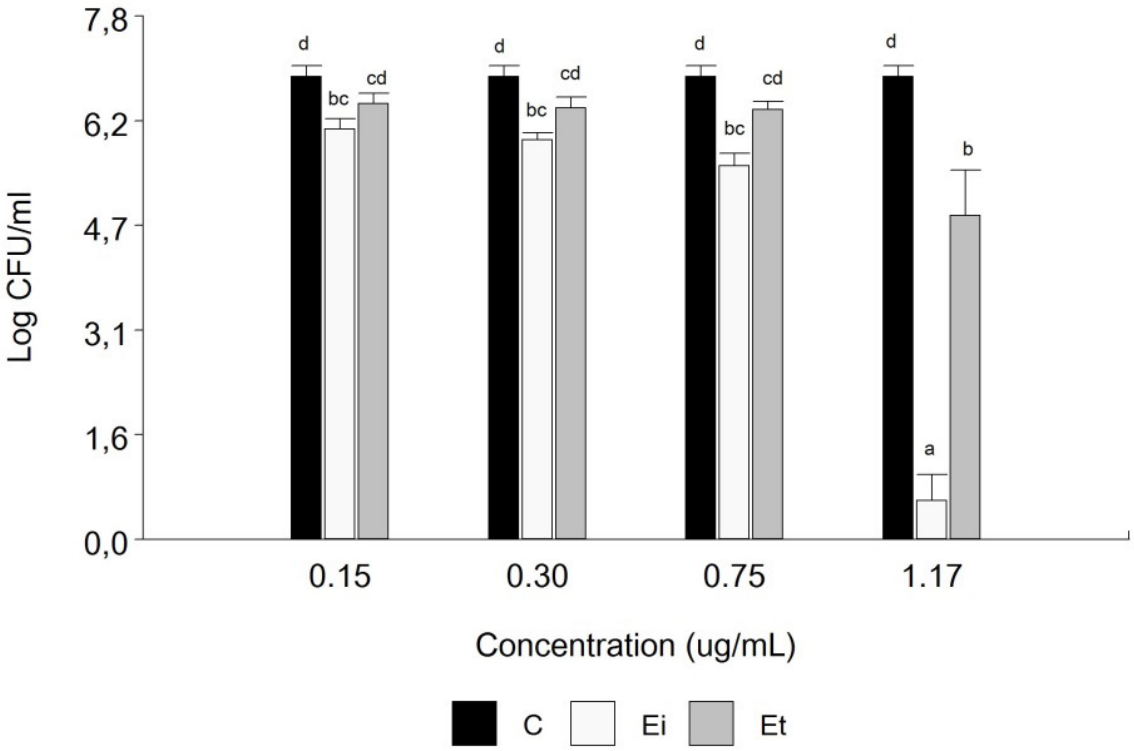


Fig.6 Effect of the HE on *P. larvae* 9 growth: C: control; Ei: addition of the HE at the beginning of growth (0 h); Et: addition of HE at 46 h. *The error bars represent standard error from the mean. Means with a common letter are not significantly different. (p > 0,05).*

It is important to mention that, in our experiments, the tubes with the addition of the HE from the beginning (Ei curve) at MIC, 2 ½ MIC and MBC used for the curves remained limpid throughout the entire experiment. This observation could indicate that there was no development of microorganisms or, more importantly, of their spores, in contrast to the control and the Et curve, where all the tubes presented turbidity. To verify if the spores were present and if they were being inhibited by the presence of the HE, aliquots were taken from

each limpid tube then they were heated, and seeded on MYPGP agar plates observing growth similar to the control. This could indicate that the spores present in the tubes were inhibited by the HE and when they were placed in a fresh medium in the absence of stress (HE), the spores were able to germinate. These results demonstrate that the presence of the HE from the beginning of the experiment presented bacteriostatic and bactericidal activity on *Pl 9* and could also be inhibiting the germination of spores. The importance of these results is based on the fact that, in some way, an effect on the spore is being observed. However, further studies on the spores are needed to confirm these observations.

DISCUSSION

The effect of different concentrations of the HE of *A. satureioides*, an Argentinean autochthonous plant, against *Pl 9* ERIC I strain was evaluated in this research. The results demonstrated that the HE had good antimicrobial activity and affected the growth of the pathogen.

The *Asteraceae* family is characterized by its phytochemical composition rich in phenolic compounds and *Achyrocline satureioides*, a member of this family, has been reported as a species with a high content of flavonoids (Arredondo *et al.,* 2004; De Souza *et al.,* 2007). In previous studies carried out by members of our research group, the presence of these compounds in the HE has been reported (Pimentel Betancurt *et al.,* 2021; Tonello *et al.,* 2022). In addition, Tonello *et al.* (2022) identified four major compounds present in the HE of *A. satureioides*, similar to those previously reported in the literature (Casero *et al.,* 2015). What is more, the four compounds showed antimicrobial activity against *P. larvae* and synergistic relationship when they were combined (Tonello *et al.,* 2022). Therefore in this work the whole HE was used to performing the tests. González *et al.* (2015) analyzed the antimicrobial activity against *P. larvae* of extracts of *A. satureioides* obtained with different solvents that included hexane, finding MIC values for the HE of 60 µg/ml and bactericidal activity. Pimentel Betancurt *et al.* (2021) studied the antimicrobial activity of the HE of *A. satureioides* obtaining MIC values of 0.40 µg/ml, representing a closer concentration to the one obtained in this study; however, they did not observe bactericidal activity.

Nowadays, there is not a unique classification criteria for defining the antimicrobial activity of natural products derived from plants. Holetz *et al.* (2002) classified those extracts with MIC values below 100 µg/ml as compounds with good antimicrobial activity, values between 100 and 500 µg/ml as moderate antimicrobial activity, values ranging from 500 to 1000 µg/ml as compounds of weak antimicrobial activity, and values greater than 1000 µg/ml were considered as extracts without activity. Other classification criteria were proposed by Duarte *et al.* (2007) who proposed that the activity of plant extracts could be defined as strong inhibitors for extracts with MIC values below 500 µg/ml, moderate inhibitors for MIC values between 600 and 1500 µg/ml and weak inhibitors for MIC values greater than 1600 µg/ml. Based on both classifications, the HE of *A. satureioides* used in this study could be classified as a strong inhibitor with good antimicrobial activity (MIC = 0.30 µg/ml).

Another item to consider is the activity of this extract compared to substances commonly used for the treatment of hives. Sabaté *et al.* (2012) studied the concentrations of oxytetracycline on *P. larvae* obtaining MIC values between 0.5 and 1 µg/ml, which represented higher values than the MIC of *A. satureioides* observed in this work (0.30 µg/ml). These results demonstrated a better effect of this natural product than the conventional antibiotic substances used for the AFB control and which are forbidden. Therefore, the HE could be a candidate to be considered in the future as a therapeutic agent or a food additive since it represents a natural and effective alternative to be used and it has not been described the development of bacterial resistance, as it was observed for antibiotics (Alippi, 1996; Miyagi *et al.*, 2000).

Several studies have been conducted on natural alternatives, such as the use of essential oils, plant extracts, propolis extracts, purified individual components and, bee venom, which have been described as antimicrobial agents that inhibited the growth of *P. larvae* (Antúnez *et al.,* 2008; Bastos *et al*., 2008; Fuselli *et al*., 2008; Flesar *et al.*, 2010; Gende *et al.*, 2010; Boligon *et al*., 2013; Fernández *et al*., 2014; Gende *et al*., 2014; Anjum *et al*., 2015; Chaimanee *et al*., 2017; Pellegrini *et al*., 2017a; Pellegrini *et al.*, 2017b). The antimicrobial activity of natural products on *P. larvae* growth was also analyzed by some research groups. Ansari *et al.* (2016) performed growth curves to determine the death of this pathogen using oils of *Pimenta dioica* (162 µg/ml), *Litsea cubeba* (186 µg/ml) and *Trachyspermum ammi* (224.8 µg/ml), finding that these compounds inhibited the growth of *P. larvae*, causing the death at

24, 36 and 48 h of exposure, respectively. De Almeida Vaucher *et al.* (2015) studied the effect of two concentrations, MIC (0.39%) and 2 MIC (0.78%), of nanoemulsions of essential oils of *Carapa guaianensis* and *Copaifera officinalis* . They observed that independently of the essential oil concentration used, the number of viable cells was reduced, with a significant decrease between 48 and 72 h after treatment exposure, which is in agreement with the results obtained in study. Lamei *et al.* (2019) studied the effect of the secretome of lactic acid bacteria isolated from honey bees on the growth of *P. larvae* through the analysis of growth curves. They observed that a maximum growth peak (OD_{600nm} ≈ 0.5) occurred in the control curves followed by a decrease in cell density as the culture entered the sporulation phase, while in the growth curves supplemented with the cell free supernatant (CFS) this growth peak was absent, reaching lower final cell densities than in the control curves (OD not higher than 0.1). This result was similar to that observed in our work with treatments at sub-bactericidal and bactericidal concentrations of the HE. Furthermore, in the study, the authors determined that the addition of CFS reduced bacterial proliferation and perhaps prevented sporulation triggers, consistent with what was observed in our assays. Brittes Benitez *et al.* (2012) analyzed the effect of an antimicrobial factor isolated from *Bacillus amyloliquefaciens* on the growth of *P. larvae*. In the study, growth curves were performed to establish the mode of action of the antimicrobial factor using concentrations of 1,600 AU ml^{-1}. The compound was added after 4.5 h of incubation producing a strong decrease (approximately 4 log units) in the number of viable cells (initially 10^6 CFU/ml) with respect to the control. The authors reported that the decrease in *P. larvae* cell count occurred simultaneously with a decrease in OD_{600nm} that would indicate a bactericidal effect with cell lysis. These results are consistent with what was observed in our assays, where *P. larvae* at the MBC of the HE resulted in a marked decrease in the viable cells count (until death) and in the OD values, both for the treatment from the beginning (Ei) and the treatment after 46 h of incubation (Et), confirming the bactericidal action and suggesting a possible lytic effect.

In the search for new antimicrobials, it is important to understand the effect and the interaction between the compound and the microorganism. Under optimal growth conditions, a microorganism follows the characteristic phases and the growth curve is highly reproducible. Instead, when an antimicrobial substance is added, growth is in some way disrupted, so the growth curve becomes altered. The addition of a compound with antimicrobial activity during the exponential phase can be used to evaluate its bacteriostatic or bactericidal effect. For this, concentrations between ½ and 4 CIM are usually studied. Therefore, the study of growth curves analyzing different concentrations of an antimicrobial substance allows a more precise characterization of the time course of the activity of the compound in contact with a bacterial culture. Furthermore, studying in vitro bacterial growth curves can provide information on what might occur in vivo, particularly when different concentrations of the antimicrobial substance are studied in vitro to simulate the pharmacokinetics of the compound in vivo (Mattie, 1981; Vogelman and Craig, 1986; García Rodríguez *et al*., 2000; Arredondo and Voltolina, 2007; Ramirez and Castaño, 2009).

In recent years it has been observed that the use of synthetic pesticides for the control of honey bee diseases has led to environmental disturbances, the resurgence of pests, resistance to pesticides, lethal effects for organisms in agricultural ecosystems and direct toxicity to users (Maggi *et al.,* 2016; Mariani 2016; Fernández *et al.,* 2019). On the other hand, the use of antibiotics for AFB control generates negative effects on the commensal microbiota of honey bees and the ecosystem of the hive. An incorrect use of antibiotics favors the generation of resistant *P. larvae* strains and its effectiveness is limited due to the ability of the pathogen to form spores. Furthermore, they leave residues in honey, becoming a serious problem in public health and in the export sector (Alippi *et al.,* 2004; Lodesani and Costa, 2005; Alippi *et al*., 2007; Murray *et al*., 2007; Alippi *et al.*, 2014). Considering the harmful effects of these compounds, there is an urgent need to search for new alternatives that help in bee pathogens control.

CONCLUSIONS

The results obtained in this work demonstrated the effectiveness of a natural extract obtained from a medicinal plant on *P. larvae* growth, an extremely aggressive pathogen of honey bees. The HE of *A. satureioides*, a native Argentinean plant, opens up a new possibility for the development of safer antimicrobial agents and the prevention and/or the treatment of American foulbrood, a mandatory reporting disease, which control involves the loss of living and inert material by burning of beehives or the use

of unauthorized chemicals in most countries. Studies are currently being carried out that complement the results obtained in the present work in relation to the possible mechanisms of action of the HE on bacterial cells and spores of *P. larvae*.

ACKNOWLEDGEMENTS

The authors gratefully appreciate the support from UNRC (Universidad Nacional de Río Cuarto) and CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas).

Competing Interests: No potential conflict of interest was reported by the authors.

REFERENCES

Alippi, A. M. (1995). Detection of *Bacillus larvae* spores in Argentinian honeys by using a semi-selective medium. Microbiología – Madrid. 11: 343-350.

Alippi, A. M. (1996). Caracterización de aislamientos de *Paenibacillus larvae* mediante tipo bioquímico y resistencia a oxitetraciclina. Revista Argentina de Microbiología. 28: 197–205.

Alippi, A. M., Reynaldi, F. J., López, A. C., De Giusti, M. R., Aguilar, M. (2004). Molecular epidemiology of *Paenibacillus larvae larvae* and incidence of American foulbrood in Argentinean honeys from Buenos Aires province. Journal of Apicultural Research. 43 (3): 135-143. https://doi.org/10.1080/00218839.2004.11101124

Alippi, A. M., López, A. C., Reynaldi, F.J., Grasso, D. H., Aguilar, O. M. (2007). Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees. Veterinary Microbiology. 125: 290–303. https://doi.org/10.1016/j.vetmic.2007.05.018

Alippi, A. M., Reynaldi, F. J., López, A. C. (2013). Evaluación del método epsilométrico Etest para la determinación de la sensibilidad a tetraciclina en *Paenibacillus larvae*, agente causal de la loque americana de las abejas. Revista Argentina de Microbiología. 45(4): 257-261. https://doi.org/10.1016/S0325-7541(13)70033-6

Alippi, A. M., León, I. E., López, A. C. (2014). Tetracycline-resistance encoding plasmids from *Paenibacillus larvae*, the causal agent of American foulbrood disease, isolated from commercial honeys. International Microbiology. 17(1): 49-61. https://doi.org/10.2436/20.1501.01.207

Anjum, S. I., Ayaz, S., Shah, A. H., Khan, A., Khan, S. N. (2015). Controlling honeybee pathogen by using neem and Barbaka plant extracts. Biotechnology & Biotechnological Equipment. 29 (5): 901-906. https://doi.org/10.1080/13102818.2015.1051493

Ansari, M., Al-Ghamdi, A., Usmani, S., Al-Waili, N., Nuru, A., Sharma, D. (2016). In vitro evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood. Biotechnology & Biotechnological Equipment. 30 (1): 49-55. https://doi.org/10.1080/13102818.2015.1086690

Antúnez, K., Harriet, J., Gende, L., Maggi, M., Eguaras, M., Zunino, P. (2008). Efficacy of natural propolis extract in the control of American Foulbrood. Veterinary Microbiology. 131: 324–331. https://doi.org/10.1016/j.vetmic.2008.04.011

Arredondo, M. F., Blasina, F., Echeverry, C., Morquio, A., Ferreira, M., Abin-Carriquirv, J. A., et al (2004). Cytoprotection by *Achyrocline satureioides* (Lam) D.C. and some of its main flavonoids against oxidative stress. Journal of Ethnopharmacology. 91: 13-20. https://doi.org/10.1016/j.jep.2003.11.012

Bastos, E. M., Simone, M., Jorge, D. M., Soares, A. E., Spivak, M. (2008). In vitro study of the antimicrobial activity of Brazilian propolis against *Paenibacillus larvae*. Journal of Invertebrate Pathology. 97 (3): 273–281. https://doi.org/10.1016/j.jip.2007.10.007

Boligon, A. A., Faccim de Brum, T., Zadra, M., Piana, M., Filippi dos Santos Alves, C., Pedroso Fausto, V., dos Santos Barboza, J. V., de Almeida Vaucher, R., Vianna Santos, R. C., Athayde, M. L. (2013). Antimicrobial activity of *Scutia buxifolia* against the honeybee pathogen *Paenibacillus larvae*. Journal of Invertebrate Pathology. 112: 105-107. https://doi.org/10.1016/j.jip.2012.11.009

Brittes Benitez, L., Voltolini Velho, R., de Souza da Motta, A., Segalin, J., Brandelli, A. (2012). Antimicrobial factor from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American foulbrood. Archives of Microbiology. 194:177–185.

Casero, C., Machín, F., Méndez-Álvarez, S., Demo, M., Ravelo, A. G., Pérez-Hernández, N., Joseph-Nathan, P., Estévez-Braun, A. (2015). Structure and Antimicrobial Activity of Phloroglucinol Derivatives from *Achyrocline satureioides*. Journal of Natural Products. 78: 93−102. https://doi.org/10.1021/np500735f

Chaimanee, V., Thongtue, U., Sornmai, N., Songsri, S., Pettis, J. S. (2017). Antimicrobial activity of plant extracts against the honeybee pathogens, *Paenibacillus larvae* and *Ascosphaera apis* and their topical toxicity to *Apis mellifera* adults. Journal of Applied Microbiology. 123: 1160-1167. https://doi.org/10.1111/jam.13579

Cunningham, C., Tyedmers, P., Sherren, K. (2018). Primary data in pollination services mapping: potential service provision by honey bees (*Apis mellifera*) in Cumberland and Colchester, Nova Scotia. International Journal of Biodiversity Science. 14 (1): 60-69. https://doi.org/10.1080/21513732.2017.1417331

De Almeida Vaucher, R., Giongo, J. L., Perger Bolzan, L., Saldanha Correa, M., Pedroso Fausto, V., Filippi dos Santos Alves, C., Quintana Soares Lopes, L., Agusti Boligon, A., Linde Athayde, M., Pereira Moreira, A., et al (2015). Antimicrobial activity of nanostructured Amazonian oils against *Paenibacillus* species and their toxicity on larvae and adult worker bees. Journal of Asia-Pacific Entomology. 18: 205–210. https://doi.org/10.1016/j.aspen.2015.01.004

De Souza, K., Bassani, V., Schapoval, E. (2007). Influence of excipients and technological process on anti-inflammatory activity of quercetin and *Achyrocline satureioides (Lam.)* D.C. extracts by oral route. Phytomedicine. 14: 102-108. https://doi.org/10.1016/j.phymed.2005.10.007

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M., Robledo, C. W. InfoStat versión 2018. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.infostat.com.ar

Duarte, M. C. T., Leme, E. E., Delarmelina, C., Soares, A. A., Figueira, G. M., Sartoratto, A. (2007). Activity of essential oils from Brazilian medicinal plants on *Escherichia coli*. Journal of Ethnopharmacology. 111: 197–201. https://doi.org/10.1016/j.jep.2006.11.034

Fernández, N. J., Porrini, M. P., Podaza, E. A., Damiani, N., Gende, L. B., Eguaras, M. J. (2014). A scientific note on the first report of honey bee venom inhibiting *Paenibacillus larvae* growth. Apidologie 45: 719–721. https://doi.org/10.1007/s13592-014-0289-y

Fernández, N. J., Damiani, N., Podaz, E. A., Martucci, J. F., Fasce, D., Quiroz, F., Meretta, P. E., Quintana, S., Eguaras, M. J., Gende, L. B. (2019). *Laurus nobilis L.* Extracts against *Paenibacillus larvae*: antimicrobial activity, antioxidant capacity, hygienic behavior and colony strength. Saudi Journal of Biological Sciences. 26: 906-912. https://doi.org/10.1016/j.sjbs.2018.04.008

Finelgold, S., Baron, E., Braily, S. (1992). Diagnóstico microbiológico, aislamiento e identificación de microorganismos patógenos. Ed. Médica Panamericana, Bs. As. 36: 514-533.

Flesar, J., Havlik, J., Kloucek, P., Rada, V. (2010). In vitro growth-inhibitory effect of plant-derived extracts and compounds against *Paenibacillus larvae* and their acute oral toxicity to adult honey bees. Veterinary Microbiology. 145: 129–133. https://doi.org/10.1016/j.vetmic.2010.03.018

Fuselli, S., García de la Rosa, S., Eguaras, M., Fritz, R. (2008). Chemical composition and antimicrobial activity of Citrus essences on honeybee bacterial pathogen *Paenibacillus larvae*, the causal agent of

American foulbrood. World Journal of Microbiology and Biotechnology. 24: 2067–2072. https://doi.org/10.1007/s11274-008-9711-9

García Rodríguez, J. A., Cantón, R., Sánchez, J. E., Gomez-Lus, M. L., Martínez Martínez, L., Rodríguez-Avial, C., Vila, J. (2000). Métodos especiales para el estudio de la sensibilidad a los antimicrobianos, in: Picazo, J. J. (Ed.), Procedimientos en Microbiología Clínica. Recomendaciones de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica, Madrid, pp. 1-54.

Gattuso, S. J., Gattuso, M. A. (1998). Caracteres anatómicos y exomorfológicos distintivos de *Achyrocline satureioides (Lam*.) DC (*Asteraceae - Inuleae*). Acta Farmacéutica Bonaerense. 17 (4): 255-261.

Govan VA, Allsopp MH, Davidson S (1999) A PCR detection method for rapid identification of *Paenibacillus larvae*. Applied and Environmental Microbiology. 65: 2243-2245. https://doi.org/10.1128/AEM.65.5.2243-2245.1999

Gattuso, M., Cortadi, A., Rodriguez, M. V., Mc Cargo, J., Retta, D., Bandoni, A., Ferraro, G., Gattuso, S. (2008). Caracteres florales en la identificación de *Achyrocline satureioides*, *Achyrocline flaccida* y *Gnaphalium gaudichaudianum* (Asteraceae-Inuleae). Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas. 7 (5): 247 – 256.

Gende, L. B., Fernandez, N., Buffa, F., Ruiu, L., Satta, A., Fritz, R., Eguaras, M. J., Floris, I. (2010). Susceptibility of *Paenibacillus larvae* isolates to a tetracycline hydrochloride and Cinnamon (*Cinnamomum zeylanicum*) essential oil mixture. Bulletin of Insectology. 63 (2): 247-250.

Gende, L., Mendiara, S., Fernández, N. J., Van Baren, C., Di Leo Lira, A., Bandoni, A., Fritz, R., Floris, I., Eguaras, M. J. (2014). Essentials oils of some *Mentha* spp. and their relation with antimicrobial activity against *Paenibacillus larvae*, the causative agent of American foulbrood in honey bees, by using the bioautography technique. Bulletin of Insectology. 67 (1): 13–20.

Genersch, E., Forsgren, E., Pentikainen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., Fries, I. (2006). Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. International Journal of Systematic and Evolutionary Microbiology. 56: 501–511. https://doi.org/10.1099/ijs.0.63928-0

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. Journal of Invertebrate Pathology. 103: S10-S19. https://doi.org/10.1016/j.jip.2009.06.015

González, M. J., Marioli, J. M. (2010). Antibacterial activity of water extracts and essential oils of various aromatic plants against *Paenibacillus larvae*, the causative agent of American Foulbrood. Journal of Invertebrate Pathology. 104: 209–213. https://doi.org/10.1016/j.jip.2010.04.005

González, M. J., Beoletto, V. G., Agnese, A. M., Audisio, M. C., Marioli, J. M. (2015). Purification of Substances from *Achyrocline satureioides* with Inhibitory Activity *Against Paenibacillus larvae*, the Causal Agent of American Foulbrood in Honeybees' Larvae. Applied Biochemistry and Biotechnology. 175: 3349–3359. https://doi.org/10.1007/s12010-015-1506-5

Gray, A., Brodschneider, R., Adjlane, N., Ballis, A., Brusbardis, V., Charrière, J. D., Chlebo, R., Coffey, M. F., Cornelissen, B., Amaro da Costa, C., Csáki, T., Dahle, B., Danihlík, J., Maja Dražić, M., Evans, G., Fedoriak, M., Forsythe, I., de Graaf, D. (2019). Loss rates of honey bee colonies during winter 2017/18 in 36 countries participating in the COLOSS survey, including effects of forage sources. Journal of Apicultural Research. 58 (4): 479-485. https://doi.org/10.1080/00218839.2019.1615661

Hansen, H., Brødsgaard, C. J. (1999). American foulbrood: a review of its biology, diagnosis and control. Bee World. 80: 5–23. https://doi.org/10.1080/0005772X.1999.11099415

Holetz, F. B., Pessini, G. L., Sanches, N. R., Garcia Cortez, D. A., Nakamura, C. V., Dias Filho, B. P. (2002). Screening of Some Plants Used in the Brazilian Folk Medicine for the Treatment of Infectious Diseases. Memórias do Instituto Oswaldo Cruz. 97(7): 1027-1031. http://dx.doi.org/10.1590/S0074-02762002000700017

Lamei, S., Stephan, J. G., Riesbeck, K., Vasquez, A., Olofsson, T., Nilson, B., de Miranda, J. R., Forsgren, E. (2019). The secretome of honeybee-specific lactic acid bacteria inhibits *Paenibacillus larvae* growth. Journal of Apicultural Research. 58 (3): 405-412. https://doi.org/10.1080/00218839.2019.1572096

Lodesani, M., Costa, C. (2005). Limits of chemotherapy in beekeeping: development of resistance and the problem of residues. Bee World. 86(4): 102-109. http://dx.doi.org/10.1080/0005772X.2005.11417324

Maggi, M., Antúnez, K., Invernizzi, C., Aldea, P., Vargas, M., Negri, P., Brasesco, C., De Jong, D., Messwage, D., Teixeira, E. W. (2016). Honeybee health in South America. Apidologie. 47: 835–854. https://doi.org/10.1007/s13592-016-0445-7

Mariani, V. (2016). Manual de buenas prácticas apícolas con manejo orgánico. Ministerio de Agroindustria Recuperado de https://magyp.gob.ar/sitio/areas/ss_alimentos_y_bebidas/_pdf/Manual%20de%20Buenas%20Practicas%20Apicolas%20con%20Manejo%20Organico.pdf. (11 de Diciembre de 2017).

Mattie, H. (1981). Kinetics of Antimicrobial Action. Reviews of Infectious Diseases. 3(1): 19-27. https://doi.org/10.1093/clinids/3.1.19

Miyagi, T., Peng, C. Y. S., Chuang, R. Y., Mussen, E. C., Spivak, M. S., Doi, R. H. (2000). Verification of oxytetracycline-resistant American Foulbrood pathogen *Paenibacillus larvae* in the United States. Journal of Invertebrate Pathology. 75: 95–96. https://doi.org/10.1006/jipa.1999.4888

Murray, K. D., Aronstein, K. A., de León, J. H. (2007). Analysis of pMA67, a predicted rolling-circle replicating, mobilizable, tetracycline-resistance plasmid from the honey bee pathogen, *Paenibacillus larvae*. Plasmid. 58 (2): 89-100. https://doi.org/10.1016/j.plasmid.2007.02.001

Pellegrini, M. C., Zalazar, L., Fuselli, S. R., Ponce, A. G. (2017a). Inhibitory action of essential oils against proteases activity of *Paenibacillus larvae*, the etiological agent of American Foulbrood disease. Spanish Journal of Agricultural Research. 15: 1-13. e0504. https://doi.org/10.5424/sjar/2017154-10785

Pellegrini, M. C., Alonso-Salces, R. M., Umpierrez, M. L., Rossini, C., Fuselli, S. R. (2017b). Chemical Composition, Antimicrobial Activity, and Mode of Action of Essential Oils against *Paenibacillus larvae*, Etiological Agent of American Foulbrood on *Apis mellifera*. Chemistry and Biodiversity. 14: 1-18. e1600382. https://doi.org/10.1002/cbdv.201600382

Pimentel Betancurt, D. C., Tonello, N. V., Padilla Alvarez, F., Paletti Rovey, M. F., Oliva, M. M., Marioli, J. M. (2021). Oral and contact toxicity of the extract obtained with hexane from *Achyrocline satureioides* on larvae and adult honey bees. Spanish Journal of Agricultural Research. 19 (3): 1-9. e0504. https://doi.org/10.5424/sjar/2021193-16302

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. Trends in Ecology and Evolution. 25 (6): 345-353. https://doi.org/10.1016/j.tree.2010.01.007

Ramirez, L. S., Castaño, D. (2009). Metodologías para evaluar in vitro la actividad antibacteriana de compuestos de origen vegetal. Scientia et Technica. XV 42: 263-268.

Retta, D., Dellacassa, E., Villamil, J. (2012). Marcela, a promising medicinal and aromatic plant from Latin America: A review. Industrial Crops and Products. 38: 27-38. https://doi.org/10.1016/j.indcrop.2012.01.006

Retta, D. (2014). Determinación de calidad de "marcela" *Achyrocline satureioides* (*Lam*.) DC. (*Asteraceae*). Parámetros fitoquímicos. Dominguezia. 30(2): 5-17.

Sabaté, D., González, M., Porrini, M., Eguaras, M., Audisio, M., Marioli, J. (2012). Synergistic effect of surfactin from *Bacillus subtilis* C4 and *Achyrocline satureioides* extracts on the viability of *Paenibacillus larvae*. World Journal of Microbiology and Biotechnology. 28: 1415 – 1422. https://doi.org/10.1007/s11274-011-0941-x

SENASA. (2017). Recuperado de Sanidad Apícola. Se recuerda que no está permitido el uso de antibióticos en las colmenas. Recuperado de http://www.senasa.gob.ar/senasa-comunica/noticias/se-recuerda-que-no-esta-permitido-el-uso-de-antibioticos-en-las-colmenas (11 de Septiembre de 2018).

Tonello, N., Pimentel Betancurt, D., Huallpa, C. L., Marioli, J. M., Moressi, M. B., Oliva, M. M., D'Eramo, F. (2022). Fractionation of hexane extracts from *Achyrocline satureioides* and their biological activities against *Paenibacillus larvae.* Brazilian Journal of Microbiology. https://doi.org/10.1007/s42770-022-00736-y

van der Zee, R., Pisa, L., Andonov, S., Brodschneider, R., Charrière, J. D., Chlebo, R., Coffey, M. F., Crailsheim, K., Dahle, B., Gajda, A., Gray, A., Drazic, M. M., Higes, M., Kauko, L., Kence, A., Kence, M., Kezic, N. (2012). Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008–9 and 2009–10. Journal of Apicultural Research. 51 (1): 100-114. https://doi.org/10.3896/IBRA.1.51.1.12

Versalovic, J., Schneider, M., de Bruijn, F. J., Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology 5: 25-40.

Vogelman, B., Craig, W. A. (1986). Kinetics of antimicrobial activity. The Journal of Pediatrics. 108 (5 pt 2): 835-840. https://doi.org/10.1016/s0022-3476(86)80754-5

Yourassowsky, E., Van der Linden, M. P., Lismont, M. J., Crokaert, F., Glupczynski, Y. (1985). Correlation Between Growth Curve and Killing Curve of *Escherichia coli* After a Brief Exposure to Suprainhibitory Concentrations of Ampicillin and Piperacillin. Antimicrobial Agents and Chemotherapy. 28(6): 756-760. https://doi.org/10.1128/aac.28.6.7566