Apidologie

DOI: 10.1007/s13592-021-00868-4

Original article

Immune-related gene expression in honey bee larva (*Apis mellifera*) exposed to plant extracts from *Humulus lupulus* with antimicrobial activity against *Paenibacillus larvae*

Pablo Giménez-Martínez[1,2], Fabian Zuñiga[3], Celina Junges[4,5], Matias Maggi[1,2], Sandra Fuselli[2,6], Jessica Martínez[3]

[1]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[2]Instituto de Investigaciones en Producción Sanidad y Ambiente (IIPROSAM), CONICET-UNMdP, Centro de Asociación Simple CIC PBA, Mar del Plata, Argentina
[3]Centro de Medicina Regenerativa, Facultad de Medicina-Clínica Alemana, Universidad del Desarrollo, Santiago, Chile
[4]Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (FCV-UNL), Esperanza, Argentina
[5]Laboratorio de Ecología de Enfermedades (LEcEn), ICiVet LITORAL (UNL-CONICET), Esperanza, Argentina
[6]Comisión de Investigaciones Científicas (CIC), La Plata, Argentina

Received 11 January 2021 – Revised 29 March 2021 – Accepted 28 April 2021

Abstract – American foulbrood caused by *Paenibacillus larvae* brings with it several problems on beekeepers for the use of synthetic antibiotics, likewise led to the appearance of resistant strains and the presence of chemical residues in the commercial products of the hive. In recent years, the use of natural compounds for the control of *P. larvae* has increased. Herein, we propose to determine the value of the Minimal Inhibitory Concentration (MIC) of a hydroalcoholic extract of *Humulus lupulus* (hop) leaves of Victoria variety on *P. larvae* strain, and to evaluate its toxicity and effect on the expression of three genes of the immune system on honey bee larvae. For the extract, the MIC value was 83.79 μg/mL. For larvae, the extract did not show toxicity, on the other hand presented a down-regulation in the gene expression for the three genes evaluated. The results obtained here are a first report on the evaluation in the effect of natural extracts on the expression of genes related to the immune system in bee larvae.

secondary metabolites / hops / *Apis mellifera*, *Paenibacillus larvae*, antimicrobial peptides

1. INTRODUCTION

American foulbrood (AFB), caused by the Gram-positive bacillus *Paenibacillus larvae* (Shimanuki 1997), brings with it several problems on beekeepers: (1) it is the most widespread disease of bee breeding around the word (Matheson 1993), (2) the use of synthetic antibiotics such as oxytetracycline to control disease led to the appearance of resistant strains (Reynaldi et al. 2008), and (3) the use of antibiotics generate the appearance of residues in the commercial products of the hive (Kochansky et al. 2001; Kumar et al. 2020). As an alternative to this problem, the use of plant extracts has begun to be studied because plants are a rich source of bioactive compounds (Friedman 2015). Some hydroalcoholic botanical extracts and their main components have shown good in vitro antibacterial activity against *P. larvae* (Fuselli et al. 2018; Domínguez et al. 2019; Fangio et al. 2019; Giménez-Martínez et al. 2019, 2020).

In the beer industry, female *Humulus lupulus* (hops) flowers are one of the main inputs used as a

Corresponding author: P. Giménez-Martínez, beedarian@gmail.com
Manuscript editor Klaus Hartfelder

Published online: 19 May 2021

natural preservative due to the high concentrations of bioactive compounds capable of inhibiting the growth of Gram-positive bacteria (Knez Hrnčič et al. 2019). The *H. lupulus* cones have a concentration up to ten times higher than secondary metabolites compared with leaves, due to the presence of lupulin glands, where the biosynthesis of these compounds is performed (Chadwick et al. 2006). Due to this, most of the research carried out on the antibacterial activity of hops is based on the use of this part of the plant (Bartmańska et al. 2018; Knez Hrnčič et al. 2019). Abram et al. (2015) after conducting an analysis of the antimicrobial activity against *Staphylococcus aureus* of leaf and flower extracts of two hop varieties (Aurora and Magnun) obtained promising results in the application of leaf extracts.

There are few studies on the safety and selectivity of products based on plant extracts on *A. mellifera*. Potrich et al. (2020) found that different plant extracts had an effect on the survival rates of *A. mellifera*, although it is lower compared to the effect of synthetic chemical insecticides (Henry et al. 2012; Christen et al. 2016; Thompson et al. 2019). In turn, there is little information regarding the bactericidal activity against *P. larvae* of the hydroalcoholic extracts of hop leaves, and even less information about the benefits of secondary metabolites found in natural plant extracts on bees in terms of activation in the regulation of gene expression involved in longevity, immune function, pesticide detoxification, stress tolerance, and the production of certain antimicrobial peptides (AMPs) that help control infections caused by various pathogens (Manson et al. 2009; Alaux et al. 2011; Liao et al. 2017).

Here, we propose to investigate the following objectives: (1) to evaluate the antimicrobial activity of a hydroalcoholic extract of leaves of the variety of Victoria hops by obtaining Minimum Inhibitory Concentration (MIC) against different strains of *P. larvae*; (2) to evaluate the toxicity of a diet supplemented with hydroalcoholic extract of hop leaves on larvae of *A. mellifera* reared in vitro, and (3) to analyze the effect of hydroalcoholic extracts of hop leaves on the expression of certain AMP genes on bee larvae, since it is in them where the bacterium is activated and generates more damage.

2. MATERIAL AND METHODS

2.1. Biological material

Leaves of hop variety Victoria were collected in Santa Paula, Mar del Plata (National Route 226, Km 10, Argentina) (37° 55′ 48″ S–57° 40′ 59″ O), belonging to the Centro de Investigación en Abejas Sociales (CIAS - IIPROSAM) from FCEyN, UNMdP. The hop plant did not receive any fertilizer treatment during the development. The collection of the leaves was carried out on the morning once the harvest season was over (February–March); it was carried out manually, taking leaves of the individuals of the same variety at random.

Botanical material was determined by the Botany chair of the Department of Biology, University of Mar del Plata, using taxonomic keys according to Dimitri (1978) *Humulus lupulus L.* (Linnaeus). The samples were stored and cataloged into the Herbarium of Vascular Plants of the Institute of Marine and Coastal Research (MDQ). The voucher number is Victoria; IIMyCher : MDQ : 00460.

Paenibacillus larvae strains were isolated from honey combs of beehives exhibiting clinical symptoms of American foulbrood, located in the provinces of Buenos Aires, Córdoba, Río Negro, and Entre Ríos in Argentina. Strains C1 and C2 were from Balcarce of Buenos Aires province (37° 52′ S-58° 15′ W), strain C6 from Rio Cuarto of Cordoba province (33° 08′ 00″ S–64° 21′ 00″ O), strain C8 from Viedma of Rio Negro province (40° 48′ S–63° 00′ O), and strain C9 from Concordia of Entre Rios province (31° 23′ 32″ S–58° 01′ 01″ O). Bacterial strains were grown and maintained in accord with Nordström and Fries (1995). All strains used are genetically identical to the ERIC I profile (Giménez-Martínez et al. 2019).

The *A. mellifera carnica* larvae used for toxicological assays were collected in the apiary of Universidad Nacional del Desarrollo, Santiago de Chile (Chile).

2.2. Extraction and quantification of secondary metabolites

The extraction of fresh leaves was performed by using solvent mixtures: ethanol-water (50:50) and

carried out according to Kowalczyk et al. (2013). The final concentration was set to 2 mg/mL.

The content of the total phenolic compounds was determined by the colorimetric method of Singleton and Rossi (1965). The results were expressed as μg equivalents of gallic acid (GAE)/mg extract. The values are presented as the average of analyzes performed in triplicate ± standard deviation.

The total flavonoid content was determined by the method of Kumazawa et al. (2004) with some modifications. The total flavonoid content was calculated as equivalent μg of quercetin (QE)/mg extract. The values are presented as the average of analyses performed in triplicate ± standard deviation.

For the quantification of saponins, oleanoic acid was used as a reference. The measured results were calculated as μg saponins/g extract (Le et al. 2018).

2.3. Determination of Minimal Inhibition Concentration

To obtain the Minimum Inhibitory Concentration (MIC), the agar diffusion analysis was performed. The diameter of the inhibition zone reflects the susceptibility of the strain, and its size depends on the nature of the diffusion of the extract through the agar layer characterized by two diffusion concepts proposed by Bonev et al. (2008):

Free diffusion model: is based on the assumption that the antimicrobial drug diffuses freely in the solid agar.

Dissipative diffusion model: takes account of extract loss through interactions with the agar matrix.

For the assay, four concentrations of the hop extract were prepared to obtain the MIC (2000, 1000, 500, and 250 μg/mL). The assay was made triplicate, and when obtaining the MIC value, the average value of the measurement of the inhibition halos was taken.

Ethanol:water was used for the negative control, because it was used to make the dilutions of the hop extracts.

2.4. Effect on the survival of *A. mellifera* larvae

To evaluate the toxicity of Victoria extract *H. lupulus* on *A. mellifera* larvae, an in vitro bioassay was performed. Bee larva was placed into 96-well plates (DELTALAB S L, Barcelona, España; the size of plate is 127.7 × 85.8 mm, with a size well of nine mm; the plates were come sterile vacuum packed) and was fed with a diet containing 50% of royal jelly, obtained from a commercial supplier, and 50% of sugar solution composed by yeast extract, D-glucose (Sigma-Aldrich, St. Louis, MO, USA), and D-fructose (Fluka, St. Gallen, Switzerland). Each larva was fed daily with the volumes and compositions according to Aupinel et al. (2005). All larvae used were standardized in the larval stage L1, and received a total of 160 μL diet during the 6-day rearing period. On day 4 (D4) of the test, the *H. lupulus* extract was administered with the diet at a concentration corresponding to the MIC value and then continued feeding with a normal diet until the end of the trial. A group of 32 larvae were fed with the standard diet (solvent control); another group of 32 larvae were fed with the standard diet supplemented with the extract at the MIC value. The incubation conditions were 34 to 35 °C, 95% HR (OECD Test No. 237 2013) (Figure 1), to evaluate the toxicity of the hydroalcoholic extracts of hop leaves at the MIC value obtained on the *A. mellifera* larvae, a survival analysis, and the Mantel-Cox Test.

2.5. Molecular analysis: effects on bee larvae AMP genes

The effect of the hydroalcoholic extract of hop leaves of the Victoria variety at the concentration equivalent to the MIC obtained was evaluated on the expression of three AMP genes (abaecin, defensin-2, and hymenoptaecin). Once fed, 6 larvae were taken at 24, 48, and 72 h after the exposure (D4) and placed individually in Trizol and stored at − 4 °C until use.

2.5.1. RNA extraction

Total RNA was extracted from larval samples using trizol (TRIzol® reagent, Ambion by life technologies, USA) according to the supplier's instructions. The RNA was resuspended in 30 μL of RNase-free water and stored at − 80 °C until use.

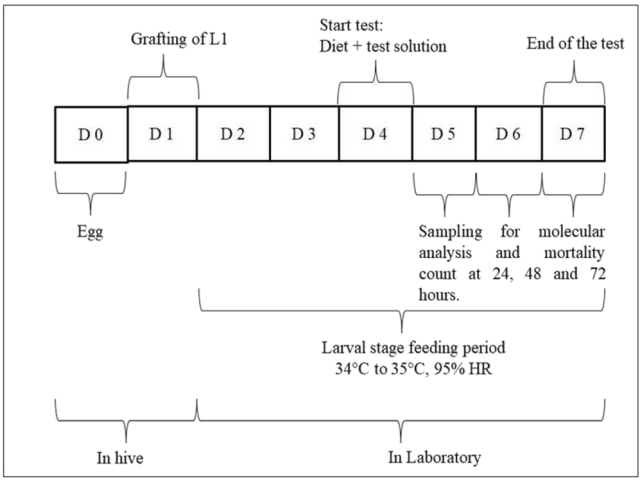

Figure 1. This timeline indicates the age of honey bee larvae. The larvae used for the toxicity test (D1, D2, D3, D4, D5, D6, D7 = indicates days 1, 2, 3, 4, 5, 6, 7 of incubation, respectively). Taken and modified from OECD Test No. 237 (2013).

2.5.2. RNA quantification

The samples were quantified in units of μg/μL of total RNA using the NANODROP 2000 kit.

2.5.3. DNase treatment

DNA was removed from samples using DNase I, Amp Grade (Ambion, life technologies, USA). 1 μg/μL of RNA, 16 μL of RNase-free water, 2 μL 10X DNase I reaction buffer (200 mM Tris-HCl (pH 8.4), 20 mM MgCl2, 500 mM), and 1 μL of DNase I AMp Grade were added (1 Unit/μL). The enzymatic reaction was carried out at 25 °C for 15 min, and then the temperature dropped to 4 °C where 2 μL of 25 mM EDTA (pH 8.0) was added and finally it was incubated at 65 °C for 10 min. The samples were stored at − 20 °C until later use.

2.5.4. Reverse transcript

For a final volume of 20 μL, 5 μL of total RNA treated with DNase I was added, and then a first mix was added consisting of 5 μL of nuclease-free water and 2 μL of oligo (dT) 12-18 500 μg/mL. It was incubated at 70 °C for 10 min and then the second mix consisting of 4 μL of 5X First-Strand buffer (250 mM Tris-HCL (pH 8.3), 375 mM KCl, 15 mM MgCl2), 2 μL of 10 mM dNTPs, 1 μL of RNasin® Ribonuclease Inhibitor, Promega (40 units/μL), and 1 μL (200 units/μL) of M-MLV RT (Invitrogen, USA), finally incubated at 42 °C for 50 min and then at 70 °C for 15 min. The cDNA was stored at 4 °C until further use.

2.5.5. Real-time PCR for the detection of the β actin, abaecin, defensin-2, and hymenoptaecin genes

The reaction mixture with a final volume of 10 μL consisted of 1 μL of cDNA, mixed with 5.8 μL of H2O, PCR-grade, 1.2 μL of 25 mM MgCl2, 0.5 μL of each primer (Table I) at a concentration of 10 μM, and 1 μL of 10X Master SYBR Green I in LigthCycler thermocycler kit (Roche, Indianapolis, IN). Amplification was performed with a pre-incubation cycle for 10 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 15 s, and a final cycle at 65 °C for 15 s. The amplicons were characterized according to their melting temperature, determined in the LightCycler equipment, and the sizes verified by agarose gel electrophoresis.

2.5.6. Quantitative real-time PCR

The quantification of the relative expression of the mRNA was performed using comparative Ct

Table I. Sequence of primers used in molecular analysis assays

Gen	Sense (5′– >3′)	Antisense (3′– > 5′)	Reference
Abaecin	CAGCATTCGCATACGTACCA	GACCAGGAAACGTT GGAAAC	Evans et al. (2006)
Defensin-2	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACC GAAACG	Yang and Cox-Foster (2005)
Hymenoptaecin	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCAT TCCATT	Evans et al. (2006)
β actin	ATGCCAACACTGTCCTTTCT GG	GACCCACCAATCCA TACGGA	Yang and Cox-Foster (2005)

method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak 2008). The Ct, from the English cycle threshold, obtained for the genes of interest were normalized using the Ct obtained for *β-actin* (constitutive gene), after verifying the amplification efficiency of each gene with respect to *β-actin* by performing a standard curve. The amplification efficiency was determined with the following formula: $E = 10^{(-1/m)}$ where E corresponds to the amplification efficiency and m to the slope of each standard curve.

2.6. Statistics

To analyze the effect of hydroalcoholic hop extract on gene expression, we performed the Shapiro-Wilk normality test data distribution and the Mann-Whitney U-test, a non-parametric test, was used to determine significant differences in pairwise comparison. For all the statistical analyses, the software GraphPadPrism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com was utilized.

3. RESULTS

The composition of total polyphenols, total flavonoids, and saponins from Victoria *H. lupulus* extract is presented in Table II.

To calculate MIC values for Victoria extract, the models proposed by Bonev et al. (2008) were used. The average value of MIC for the five strains of the hop variety Victoria extract was 83.79 μg/mL (Table III).

In the survival analysis test in *A. mellifera* larvae fed with hydroalcoholic extracts of hop leaves at MIC values, the mortality was 9.375% during the 3 days of the trial. For the control group, mortality was 6.25%, 12.5%, and 12.5% at 24, 48, and 72 h of exposure, respectively. The values obtained did not show significant differences in the survival with respect to the control group (Mantel-Cox Test, p-value: 0.7082) (Figure 2). The gene expression of the three genes studied on larvae fed with the hydroalcoholic extracts of hop leaves MIC was significantly reduced compared to the control after 24 h of feeding (Figure 3a). After 48 h of feeding, there was less expression of the abaecin and hymenoptaecin genes (Figure 3b) and at 72 h, there was less expression of the abaecin and defensin-2 genes (Figure 3c) with respect to the control group; these differences were significant (p-value <0.05).

4. DISCUSSION

The use of natural extracts for the control of bee pathologies has been studied for years as a promising alternative to avoid the negative effects such as resistance and residues generated by synthetic ones (Miyagi et al. 2000; Fuselli et al. 2006, 2009; Flesar et al. 2010; Pellegrini et al. 2014, 2017, Chaimanee et al. 2017; Isidorov et al. 2018; Giménez-Martínez et al. 2019, 2020).

On the antimicrobial assay, the formation of inhibition halos depends on the nature of the diffusion of the extract through the agar layer characterized by two diffusion concepts. The free diffusion model is based on the assumption that the antimicrobial drug diffuses freely in the solid matrix. By contrast, the dissipative diffusion model takes account of drug loss through interactions with the agar matrix, aggregation of drug

Table II. Bioactive compounds of hop leave extract of the Victoria variety

Total polyphenols (GAE μg/mg extract)	Total flavonoids (Q μg/mg extract)	Saponinns (μg/g extract)
8.928 ± (0)	1.059 ± (0.11)	0.342

molecules, or drug inactivation to some extent. Though plots of both the free and dissipative diffusion model indicated linearity, the corresponding R^2 values of linear regression were higher for the dissipative diffusion model. Thus, diffusion of hop leave extract through the solid agar overlay can be considered a dissipative diffusion process which yields the best suit model for the determination of MICs.

Until now, few studies have used hop extracts as bactericides against *P. larvae*. Flesar et al. (2010) analyzed several extracts, including hop flower extracts, obtaining MIC values in the range of 2–4 μg/mL; compared with our results (MIC = 83.79 μg/mL), the values obtained for Flesar and collaborators show the cited afore in terms of the greater bactericidal power of the flowers against the hop leaves. Many of the compounds belonging to the secondary metabolism of plants present significant antibacterial activity, which gives them a leading role in the development of natural alternatives to synthetic preservatives in food (Bouarab et al. 2019). Flavonoids are those with the highest antimicrobial activity (Cushnie and Lamb 2011). Also, saponins are considered part of the defense system of plants against pathogens and herbivores with known fungicidal (Lee et al. 2001), antimicrobial (Avato et al. 2006), and

Table III. MIC values obtained for the Victoria variety against five *P. larvae* strains. (The values are expressed in μg/mL)

Strain	Model of diffusion	MIC and R^2 values	
C1	Free diffusion model	MIC	8
		R^2	0.96
	Dissipative diffusion model	MIC	**103.908**
		R^2	1
C 2	Free diffusion model	MIC	24.803
		R^2	0.72
	Dissipative diffusion model	MIC	**166.289**
		R^2	0.75
C 6	Free diffusion model	MIC	0.038
		R^2	0.72
	Dissipative diffusion model	MIC	**6.801**
		R^2	0.75
C 8	Free diffusion model	MIC	2.08
		R^2	0.896
	Dissipative diffusion model	MIC	**38.076**
		R^2	0.899
C 9	Free diffusion model	MIC	7.813
		R^2	0.96
	Dissipative diffusion model	MIC	**103.908**
		R^2	1
	Average MIC		83.79

The values in bold in Table III indicate the MIC values chosen to perform the analyzes, since they present the highest R2 value

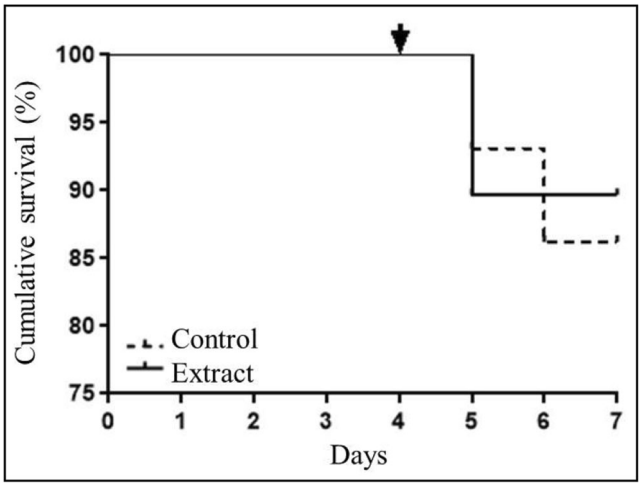

Figure 2. Survival record of *A. mellifera* larvae from day 1 to 7. The arrow indicates the day of initiation of exposure to the extract. A survival analysis and the Mantel-Cox Test to compare the results with the control group were carried out.

insecticidal (Kuzina et al. 2011; Nielsen et al. 2010) activity. The most studied effect of this group is the ability to cause disturbances in cell membranes (Baumann et al. 2000; Chwalek et al. 2006). Future studies will focus on analyzing the mode of action of these phytomolecules on the integrity of the *P. larvae.*

Despite having less bactericidal power than flowers, the hydroalcoholic extracts of hop leaves presented a strong antimicrobial activity, according to the classification provided by Duarte et al. (2007) and Wang et al. (2008), where they classify the bactericidal power of the extracts in strong inhibitors (<500 μg/mL), moderate inhibitors (600–1500 μg/mL), and weak inhibitors (> 1600 μg/mL).

In the case of the survival analysis in *A. mellifera* larvae, we proceed to evaluate the action of the MIC value obtained in stage L4 because it is here where the larva of *A. mellifera* begins to receive, in addition to royal jelly, a dose of raw pollen since it is at this age when it is naturally exposed to a toxic in the hive. After they were fed a diet supplemented with the MIC average value obtained for the hydroalcoholic extract of hop leaves of the Victoria variety, there were no significant differences with respect to the group control.

On the other hand, regarding the expression of the AMP genes, significant differences were observed at 24, 48, and 72 h after feeding the larvae. AMPs are the major and the most important component in humoral immune system present in insects. If a bacterial or fungal pathogen invades the hemocoel, its presence can be detected by pattern recognition receptors, which in turn induces a more systemic response and a greater regulation of AMPs in fatty bodies and hemolymph (Broderick et al. 2009). Gram-positive bacteria and fungi mainly trigger the Toll pathway which induces the production of AMPs related to the control of these microbes (James and Xu 2012).

Simone et al. (2009) report a negative regulation expression of genes of the immune system due to the action of natural components. These authors compared the expression of various AMP genes between two groups of hives: (1) one group received a propolis cover; (2) a control group without cover. observed that the treated hives registered a significant decrease in gene expression levels with respect to the control group. Our results could be compared with those obtained by Simone et al. (2009), due to when administering a supplemented diet with bioactive components with known antimicrobial activity, there was a down-regulation of the three AMP genes analyzed.

On the other hand, Mao et al. (2015) analyzed the effect on honey bees of diets supplemented

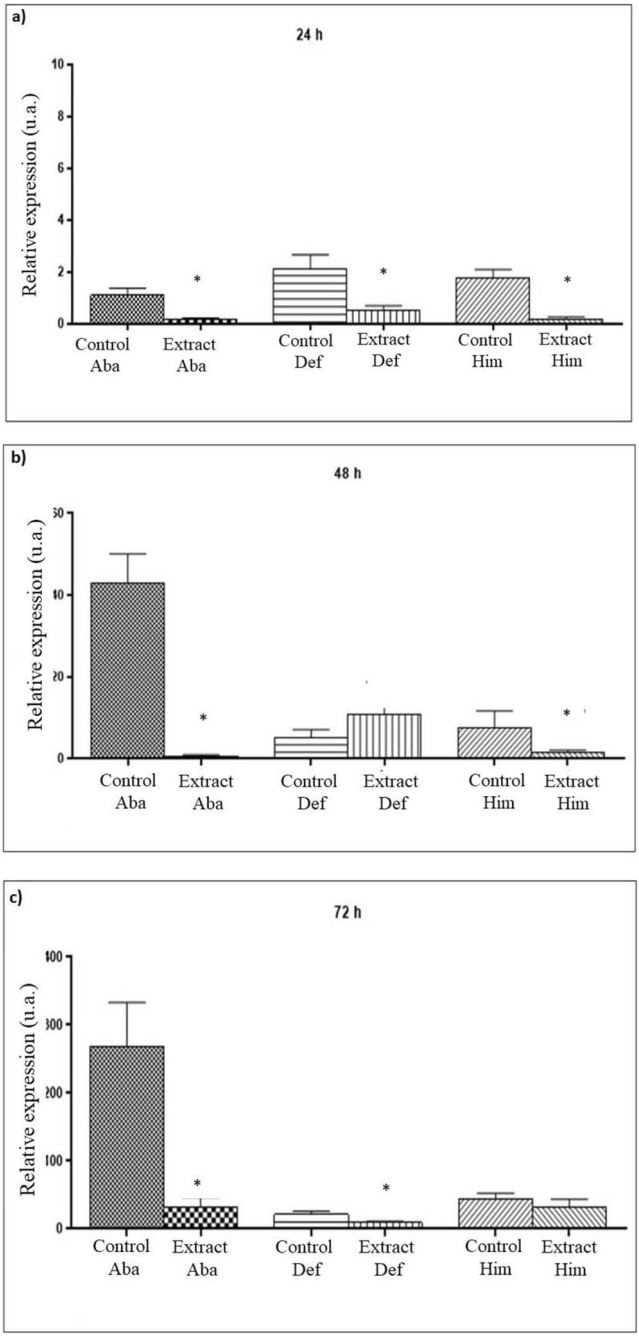

Figure 3. a–**c** Relative expression of antimicrobial peptide genes in *A. mellifera* larvae fed with the hydroalcoholic extract of hop leaves to MIC concentration obtained. The asterisk indicates significant difference (p-value < 0.05) (Aba = Abaecin; Def = Defensin-2; Hym = hymenoptaecin). To analyze the gene expression, the Shapiro-Wilk normality test data distribution and Mann-Whitney U-test were performed, and a non-parametric test was used to determine significant differences in pairwise comparison.

with p-coumaric acid (a secondary metabolite component of pollen and honey) on the Hippo signaling pathway, which is responsible for organ and tissue development in larvae (Li et al. 2019; Schneck 2004) observing that these signaling pathways were positively regulated.

Therefore, our results and those obtained by Simone et al. (2009) could suggest that bees that have their hive enriched with resin or are fed a diet supplemented with secondary metabolites could invest less energy in the regulation and expression of genes related to the immune system, and this energy could be redirected to other signaling pathways such as those for the development and growth of *A. mellifera*.

The changes in immunological expression observed in our studies are a reflection of changes in the initial immunity levels in bees. One possibility is that when bees are exposed to different pathogens, larger differences are noticed. This hypothesis is supported by the laboratory study conducted with *Formica paralugubris* ants, which showed that when individual ants housed in a propolis resin petri dish were challenged with a pathogen, they had higher survival rates than those without resin (Chapuisat et al. 2007).

Because the *A. mellifera* genome is completely sequenced, and because in insects the activation and maintenance of the immune system entail a very great physiological cost (Rolff and Siva-Jothy 2003; Siva-Jothy et al. 2005), future studies will focus on analyzing other expression pathways, as well as its effect at the colony level.

5. CONCLUSION

In recent years, there has been an increase in the study of the use of natural products for the control of different diseases in bees since they do not present high toxicity in both adult and larval individuals. In our study, it was demonstrated that precisely the hop leaf extract is not toxic to *A. mellifera* larvae; and on the other hand, it negatively regulates the expression of AMPs genes studied, possibly generating metabolic savings for the control of microbial diseases. These results are a first step to elucidate the possible effects of natural extracts in regulating the expression of immunity genes in bees.

ACKNOWLEDGEMENTS

Pablo Giménez Martínez wishes to thank the CONICET and UNMdP, for his Ph. D. Grant.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Analyses were conducted in accordance with standard usages of Graphpad Prism 7.00 software and packages (see Section 2 above). Interested parties may contact the corresponding author to obtain the specific files.

AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Pablo Giménez-Martínez and Fabian Zuñiga; Celina Junges contributed additional ideas; Jessica Martínez, Matias Maggi, and Sandra Fuselli participated in acquisition of funding, study design, interpretation and discussion of results, and edited the manuscript. The first draft of the manuscript was written by Pablo Giménez-Martínez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.Funding

This research was funded by Project IDEA CIC /19 and Project REDES 180154 of bilateral collaboration Chile-Argentina (Agencia Nacional de Investigación y Desarrollo de Chile (ANID)). The authors would like to thank Comisión de Investigaciones Científicas (CIC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Universidad Nacional de Mar del Plata (UNMdP) for financial support.

DECLARATIONS

Ethics approval Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

Expression de gènes liés au système immunitaire chez les larves d'abeilles *(Apis mellifera)* exposées à des extraits végétaux de *Humulus lupulus* ayant une activité antimicrobienne contre *Paenibacillus larvae*

Métabolites secondaires / houblon / *Apis mellifera* / *Paenibacillus larvae* / peptides antimicrobiens

Immunabhängige Genexpression bei Honigbienenlarven (*Apis mellifera*), die Pflanzenextrakten von *Humulus lupulus* mit antimikrobieller Aktivität gegenüber *Paenibacillus larvae* ausgesetzt sind

Sekundäre Metaboliten / Hopfen / *Apis mellifera* / *Paenibacillus larvae* / antimikrobielle Peptide

REFERENCES

Abram, V., Čeh, B., Vidmar, M., Hercezi, M., Lazić, N., Bucik, V., Poklar Ulrih, N. (2015) A comparison of antioxidant and antimicrobial activity between hop leaves and hop cones. Ind. Crop. Prod. **64**, 124-134. https://doi.org/10.1016/j.indcrop.2014.11.008

Alaux, C., Dantec, C., Parrinello, H., Le Conte, Y. (2011) Nutrigenomics in honey bees: Digital gene expression analysis of pollen's nutritive effects on healthy and Varroa-parasitized bees. BMC Genomics **12**, 496. https://doi.org/10.1186/1471-2164-12-496

Aupinel, P., Fortini, D., Dufour, H., Tasei, J., Michaud, B., Odoux, J., Pham-Delegue, M. (2005) Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae. Bull. Insectol. **58** (2), 107

Avato, P., Bucci, R., Tava, A., Vitali, C., Rosato, A., Bialy, Z. (2006) Antimicrobial activity of saponins from *Medicago* sp.: structure-activity relationship. Phytother. Res. **20** (6), 454-457

Bartmańska, A., Wałecka-Zacharska, E., Tronina, T., Popłoński, J., Sordon, S., et al. (2018) Antimicrobial properties of spent hops extracts, flavonoids isolated therefrom, and their derivatives. Molecules **23** (8). https://doi.org/10.3390/molecules23082059

Baumann. E., Stoya, G., VoÈlkner, A., Richter, W., Lemke, C., Linss, W. (2000) Hemolysis of human erythrocytes with saponin affects the membrane structure. Acta Histochem. **102** (1), 21-35

Bonev, B., Hooper, J., Parisot, J. (2008) Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. J. Antimicrob. Chemother. **61** (6), 1295-1301

Bouarab, C.L., Degraeve, P., Ferhout, H., Bouajila, J., Oulahal, N. (2019) Plant antimicrobial polyphenols as potential natural food preservatives. J. Sci. Food Agric. **99** (4), 1457-1474.

Broderick, N., Welchman, D., Lemaitre, B. (2009) Recognition and response to microbial infection in Drosophila. In (pp. 13-33)

Chadwick, L.R., Pauli, G.F., Farnsworth, N.R. (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. Phytomedicine **13** (1-2), 119-131. https://doi.org/10.1016/j.phymed.2004.07.006

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

Chapuisat, M., Oppliger, A., Magliano, P., Christe, P. (2007) Wood ants use resin to protect themselves against pathogens. Proc. R. Soc. Lond. B **274**, 2013–2017

Christen, V., Mittner, F., Fent, K. (2016) Molecular effects of neonicotinoids in honey bees (*Apis mellifera*). Environ. Sci. Technol. **50** (7), 4071–4081

Chwalek, M., Lalun, N., Bobichon, H., Plé, K., Voutquenne-Nazabadioko, L. (2006) Structure–activity relationships of some hederagenin diglycosides: Haemolysis, cytotoxicity and apoptosis induction. Biochim. Biophys. Acta Gen. Subj. **1760** (9), 1418-1427. https://doi.org/10.1016/j.bbagen.2006.05.004

Cushnie, T., Lamb, A. (2011) Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents **38**, 99-107. https://doi.org/10.1016/j.ijantimicag.2011.02.014

Dimitri, M. (1978) Enciclopedia Argentina de agrícultura y jardinería. 3. Editorial Acme. Buenos Aires.

Domínguez, E., Moliné, M.D.L.P., Churio, S., Arce, V., Mártire, D., Mendiara, S., Damiani, N. (2019) Bioactivity of gallic acid–conjugated silica nanoparticles against *Paenibacillus larvae* and their host, *Apis mellifera* honeybee. Apidologie. https://doi.org/10.1007/s13592-019-00675-y

Duarte, M.C.T., Leme, E., Delarmelina, C., Soares, A.A., Figueira, G.M., Sartoratto, A. (2007) Activity of essential oils from Brazilian medicinal plants on *Escherichia coli*. J. Ethnopharmacol. **111** (2), 197-201. https://doi.org/10.1016/j.jep.2006.11.034

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol. Biol. **15**, 645-656

Fangio, M.F., Orallo, D.E., Gende, L.B., Churio, M.S. (2019) Chemical characterization and antimicrobial activity against *Paenibacillus larvae* of propolis from Buenos Aires province, Argentina, J. Apic. Res. **58** (4), 626-638. https://doi.org/10.1080/00218839.2019.1601318

Flesar, J., Havlik, J., Kloucek, P., Rada, V., Titera, D., Bednar, M., Stropnicky, M., Kokoska, L. (2010)

In vitro growth-inhibitory effect of plant-derived extracts and compounds against *Paenibacillus larvae* and their acute oral toxicity to adult honey bees. Vet. Microbiol. **145** (1-2), 129-133. https://doi.org/10.1016/j.vetmic.2010.03.018

Friedman, M. (2015) Antibiotic-resistant bacteria: prevalence in food and inactivation by food-compatible compounds and plant extracts. J. Agric. Food Chem. **63** (15), 3805-3822. https://doi.org/10.1021/acs.jafc.5b00778

Fuselli, S.R., de la Rosa, S.B.G., Gende, L.B., Eguaras, M.J., Fritz, R. (2006) Antimicrobial activity of some Argentinean wild plant essential oils against *Paenibacillus larvae larvae*, causal agent of American foulbrood (AFB). J. Apic. Res. **45** (1), 2-7. https://doi.org/10.1080/00218839.2006.11101304

Fuselli, S.R., Maggi, M., de la Rosa, S.B.G., Principal, J., Eguaras, M.J., Fritz, R. (2009) *In vitro* antibacterial and antiparasitic effect of citrus fruit essential oils on the honey bee pathogen *Paenibacillus larvae* and the parasitic mite *Varroa destructor*. J. Apic. Res. **48** (1), 77-78. https://doi.org/10.3896/IBRA.1.48.1.15

Fuselli, S.R., Cugnata, N.M., Giménez Martínez, P., Alonso-Salces, R.M. (2018) Control and prevention of American foulbrood in honey bees. Hauppauge: Nova Science Publishers, Inc.

Giménez-Martínez, P., Cugnata, N., Alonso-Salces, R.M., Arredondo, D., Antunez, K., De Castro, R., Fuselli, S.R. (2019) Short communication: Natural molecules for the control of *Paenibacillus larvae*, causal agent of American foulbrood in honey bees (*Apis mellifera* L.). Span. J. Agric. Res. **17**, 3. https://doi.org/10.5424/sjar/2019173-14740

Giménez-Martínez, P., Ambrosi, M., Alonso-Salces, R.M., Gallo, B., Berrueta, L., Maggi, M., Fuselli, S. (2020) Antimicrobial activity of phenolic extract of apple pomace against *Paenibacillus larvae* and its toxicity on *Apis mellifera*. J. Apic. Sci. https://doi.org/10.2478/jas-2020-0016

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P., Jean, A., Sylvie, T., Decourtye, A. (2012) A common pesticide decreases foraging success and survival in honey bees. Science **336** (6079), 348–350

Isidorov, V.A., Buczek, K., Segiet, A., Zambrowski, G., Swiecicka, I. (2018) Activity of selected plant extracts against honey bee pathogen *Paenibacillus larvae*. Apidologie **49**, 687-704. https://doi.org/10.1007/s13592-018-0586-y

James, R.R., Xu, J. (2012) Mechanisms by which pesticides affect insect immunity. J. Invertebr. Pathol. **109** (2), 175-182. https://doi.org/10.1016/j.jip.2011.12.005

Knez Hrnčič, M., Španinger, E., Košir, I.J., Knez, Ž., Bren, U. (2019) Hop Compounds: Extraction Techniques, Chemical Analyses, Antioxidative, Antimicrobial, and Anticarcinogenic Effects. Nutrients **11** (2), 257

Kochansky, J., Knox, D., Feldlaufer, M., Pettis, J.S. (2001) Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*. Apidologie **32** (3), 215-222

Kowalczyk, D., Świeca, M., Cichocka, J., Gawlik-Dziki, U. (2013) The phenolic content and antioxidant activity of the aqueous and hydroalcoholic extracts of hops and their pellets. J. Inst. Brew. **119** (3), 103-110

Kumar, A., Gill, J.P.S., Bedi, J.S., Chhuneja, P.K., Kumar, A. (2020) Determination of antibiotic residues in Indian honeys and assessment of potential risks to consumers. J. Apic. Res. **59** (1), 25-34

Kumazawa, S., Hamasaka, T., Nakayama, T. (2004) Antioxidant activity of propolis of various geographic origins. Food Chem. **84** (3), 329-339

Kuzina, V., Nielsen, J.K., Augustin, J.M., Torp, A.M., Bak, S., Andersen, S.B. (2011) Barbarea vulgaris linkage map and quantitative trait loci for saponins, glucosinolates, hairiness and resistance to the herbivore *Phyllotreta nemorum*. Phytochemistry **72** (2-3), 188-198

Le, A., Parks, S., Nguyen, M., Roach, P. (2018) Improving the Vanillin-Sulphuric Acid Method for Quantifying Total Saponins. Technologies **6**, 84. https://doi.org/10.3390/technologies6030084

Lee, M.W., Kim, S.U., Hahn, D., Bulletin, P. (2001). Antifungal activity of modified hederagenin glycosides from the leaves of *Kalopanax pictum* var. chinense. Biol. Pharm. Bull. **24** (6), 718-719

Li, N., Tong, X., Zeng, J., Meng, G., Sun, F., Hu, H., Song, J., Lu, C., Dai, F. (2019) Hippo pathway regulates somatic development and cell proliferation of silkworm. Genomics **111**, 391-397. https://doi.org/10.1016/j.ygeno.2018.02.014

Liao, L.H., Wu, W.Y., Berenbaum, M.R. (2017) Impacts of Dietary Phytochemicals in the Presence and Absence of Pesticides on Longevity of Honey Bees (*Apis mellifera*). Insects **8** (1). https://doi.org/10.3390/insects8010022

Manson, J., Otterstatter, M., Thomson, J. (2009) Consumption of a Nectar Alkaloid Reduces Pathogen Load in Bumble Bees. Oecologia **162**, 81-89. https://doi.org/10.1007/s00442-009-1431-9

Mao, W., Schuler, M.A., Berenbaum, M.R. (2015) A dietary phytochemical alters caste-associated gene expression in honey bees. Sci. Adv. **1**, e1500795. https://doi.org/10.1126/sciadv.1500795

Matheson, A. (1993) World bee health report. J. Bee World **74** (4), 176:212

Miyagi, T., Peng, C., Chuang, R., Mussen, E., Spivak, M., Doi, R. (2000) Verification of Oxytetracycline-Resistant American Foulbrood Pathogen *Paenibacillus larvae* in the United States. J. Invertebr. Pathol. **75**, 95-96. https://doi.org/10.1006/jipa.1999.4888

Nielsen, N.J., Nielsen, J., Staerk, D. (2010) New resistance-correlated saponins from the insect-resistant crucifer *Barbarea vulgaris*. J. Agric. Food Chem. **58** (9), 5509-5514

Nordström, S., Fries, I. (1995) A comparison of media and cultural conditions for identification of *Bacillus larvae*

in honey. J. Apic. Res. **34** (2), 97-103. https://doi.org/10.1080/00218839.1995.11100894

OECD (2013) Test No. 237: Honey Bee (*Apis Mellifera*) Larval Toxicity Test, Single Exposure

Pellegrini, M.C., Alvarez, M.V., Ponce, A.G., Cugnata, N.M., De Piano, G., Fuselli, S.R. (2014) Anti-quorum sensing and antimicrobial activity of aromatic species from South America. J. Essent. Oil Res. **26** (6), 458-465

Pellegrini, M.C., Zalazar, L., Fuselli, S.R., Ponce, A.G. (2017) Inhibitory action of essential oils against proteases activity of *Paenibacillus larvae*, the etiological agent of American Foulbrood disease. Span. J. Agric. Res. **15** (4)

Potrich, M., Silva, R.T., Maciel, R.M.A., Costa-Maia, F.M., Lozano, E.R., Rossi, R.M., Martins, J.R., Dallacort, S. (2020) Are plant extracts safe for honey bees (*Apis mellifera*)?. J. Apic. Res. 1-8

Reynaldi, F.J., Albo, G.N., Alippi, A.M. (2008) Effectiveness of tilmicosin against *Paenibacillus larvae*, the causal agent of American Foulbrood disease of honeybees. Vet. Microbiol. **132** (1), 119-128. https://doi.org/10.1016/j.vetmic.2008.04.034

Rolff, J., Siva-Jothy, M. (2003) Invertebrate Ecological Immunology. Science **301** (5632), 472. https://doi.org/10.1126/science.1080623

Schmittgen, T., Livak, K. (2008) Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. **3,** 1101–1108. https://doi.org/10.1038/nprot.2008.73

Schneck, L. (2004) How a hippo came to work in an Alaska medical practice. An innovative approach to HIPAA compliance. J MGMA connexion 4

Shimanuki, H. (1997) Bacteria. In Flottum (Ed.), Honey Bee Pests, Predators, and Disease (third ed. ed., pp. 33-54)

Simone, M., Evans, J.D., Spivak, M. (2009) Resin collection and social immunity in honey bees. Evolution **63**, 3016-3022. https://doi.org/10.1111/j.1558-5646.2009.00772.x

Singleton, V.L., Rossi, J.A. (1965) Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. J. Enol. Vitic. **16** (3), 144

Siva-Jothy, M.T., Moret, Y., Rolff, J. (2005) Insect Immunity: An Evolutionary Ecology Perspective. In S. J. Simpson (Ed.), Advances in Insect Physiology (Vol. 32, pp. 1-48): Academic Press

Thompson, H., Overmyer, J., Feken, M., Ruddle, N., Vaughan, S., Scorgie, E., Bocksch, S., Hill, M. (2019) Thiamethoxam: Longterm effects following honey bee colony-level exposure and implications for risk assessment. Sci. Total Environ. **654**, 60–71

Wang, Y.S., He, H.P., Yang, J.H., Di, Y.T., Hao, X.J. (2008) New Monoterpenoid Coumarins from Clausena anisum-olens. Molecules **13** (4). https://doi.org/10.3390/molecules13040931

Yang, X., Cox-Foster, D.L. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. Proc. Natl. Acad. Sci. **102**, 7470-7475