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SHORT COMMUNICATION

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Natural molecules for the control of *Paenibacillus larvae*, causal agent of American foulbrood in honey bees (*Apis mellifera* L.)

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Abstract

Aim of study: To evaluate the potential bactericidal activity of natural molecules against *Paenibacillus larvae*. Moreover, we investigated if molecules that exhibit antimicrobial activity were able to inhibit the proteolytic activity of the bacterium.

Area of study: Isolates S1 and S2 were from Balcarce, Buenos Aires province, strain S3 from Rio Cuarto, Cordoba province, strain S4 from Concordia, Entre Rios province, strain S5 and S8 from Necochea, Buenos Aires, strain S6 and S7 from Mar del Plata, Buenos Aires, strain S9 from Modena, Italy and strain S10 from Emilia Reggio, Italy.

Material and methods: Bacterial isolates identification was carried out by amplification of a specific 16S rRNA gene fragment of *P. larvae* using primers PL5 and PL4. Screening of the antimicrobial activity of thirteen molecules against four *P. larvae* isolates was conducted by the agar diffusion technique. The antimicrobial activity of selected molecules was evaluated by broth microdilution method.

Main results: Menadione, lauric acid, monoglyceride of lauric acid and naringenin showed antimicrobial activity against ten *P. larvae* isolates. Menadione and lauric acid showed the strongest activities, with minimum inhibitory concentration mean values ranging 0.78-3.125 µg/mL and 25-50 µg/mL, respectively.

Research highlights: Those concentrations are feasible to be applied at field level, and constitute promissory candidates to be evaluated using *in vivo* larval models.

Additional keywords: antimicrobial activity; metalloproteases.

Abbreviations used: AFB (American foulbrood); ERIC (Enterobacterial Repetitive Intergenic Consensus); MIC (minimum inhibitory concentration); OTC (oxytetracycline hydrochloride); PMSF (phenylmethanesulfonyl fluoride); TCA (trichloroacetic acid).

Authors' contributions: The seven authors participated in all stages of the work, including the conception and design of the research, the revision of the intellectual content and the drafting of the paper. All authors read and approved the final manuscript.

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Introduction

American foulbrood (AFB) is the most severe bacterial disease that affects honey bees, having a nearly cosmopolitan distribution (Genersch, 2010). AFB only kills infected honey bee larvae; however, it eventually leads to the collapse of the entire colony when left untreated. AFB is considered to be very contagious; therefore, it is a notifiable disease in most countries (Djukic *et al.*, 2014). AFB's causative agent

is *Paenibacillus larvae*, a flagellated gram-positive bacterium, whose main characteristic is the formation of highly resistant endospores. Four *P. larvae* genotypes (Enterobacterial Repetitive Intergenic Consensus, ERIC I-IV) have been described, but only two are commonly detected under field conditions (ERIC I and II) (Genersch & Otten, 2003; Alippi *et al.*, 2004).

In some countries the use of antibiotics, particularly oxytetracycline hydrochloride (OTC) is recommended for the prevention and treatment of infected colonies (Hansen & Brødsgaard, 1999; Genersch, 2010). However, in most European countries the use of antibiotics is banned, since chemicals can remain in honey and other bee products affecting their quality for human consumption. Moreover, antibiotic application can affect bee lifespan and can increase the risk of generation of bacterial resistant strains (Martel *et al.*, 2006). To date, the presence of OTC resistant strains has been reported in Argentina, USA, Italy, New Zealand and UK (Alippi *et al.*, 1996; Miyagi *et al.*, 2000).

In this context, the development of alternative and effective methods for the prevention and control of AFB disease is crucial. A promissory strategy is the use of antimicrobial natural bioactive substances. Several studies have been carried out to identify natural molecules with antibacterial activity against *P. larvae*. Those studies included the evaluation of plant extracts (Flesar *et al.*, 2010; Sabaté *et al.*, 2012; Boligon *et al.*, 2013; Damiani *et al.*, 2014; Chaimanee *et al.*, 2017); essential oils (Alippi *et al.*, 1996; Fuselli *et al.*, 2006; Ansari *et al.*, 2016, Tutun *et al.*, 2018); pure compounds extracted from plants, bacteria or fungus (Lokvam *et al.*, 2000; Fuselli *et al.*, 2006; Flesar *et al.*, 2010; Sabaté *et al.*, 2012); and honey bee by-products, such as propolis (Antúnez *et al.*, 2008; Biliková *et al.*, 2013; Isidorov *et al.*, 2017) and royal jelly (Biliková *et al.*, 2013).

Natural molecules for the control and prevention of AFB are a heterogeneous group that includes a number of compounds which share one common characteristic: they are pure natural substances; either commercial, such as fatty acids or that has been obtained from natural sources, such as fungal strains or plants. Shimanuki *et al.* (1992) reported the inhibition of AFB by an ethanolic extract of chalk brood mummies (*Apis mellifera* larvae infected by *Ascosphaera apis* fungus). This fact led scientists to seek the identity of the active compound.

On the other hand, Hornitzky (2003) evaluated the antibacterial activity of fifteen fatty acids on European foulbrood, most exhibited activity at the highest tested concentration (250 µg), except for myristoleic and lauric acids, which also showed reduced activity with diffusion disks containing 25 µg of the molecule.

The aim of this study was to evaluate the potential bactericidal activity of natural molecules such as saturated and unsaturated fatty acids, flavonoids, phenolics acid, vitamins and organic acids against *P. larvae*. Moreover, we investigated whether these molecules that exhibit antimicrobial activity were able to inhibit the proteolytic activity of the bacterium.

Material and methods

Chemicals

Mueller-Hinton broth, agar, brain-heart infusion, yeast extract, glucose and peptone were provided by Britania S.A. (Buenos Aires, Argentina); NaCl (99.99%) by Alun (San Martin, Buenos Aires); K_2HPO_4 (98%) by Cicarelli (San Lorenzo, Santa Fe, Argentina); sodium pyruvate (≥ 99%) by Biopack (Zárate, Buenos Aires); acetonitrile, ethanol and methanol (HPLC grade), glacial acetic acid (ACS reagent), n-butanol (FCC reagent), resazurin sodium salt (forcell culture), lauric acid, palmitic acid, lauric acid monoglyceride, ellagic acid, cinnamic acid, syringic acid, naringenin, menadione, alpha-ketoglutaric acid, salicylic acid, phloridzin dihydrate, and chlorogenic acid by Sigma-Aldrich (Saint Louis, MO, USA); dimethyl sulfoxide (analytical grade) by Biopack (Zarate, Buenos Aires); stearic acid by Materia (Mar del Plata, Argentina) and oxytetracycline hydrochloride by Bayer (Buenos Aires). Distilled water was sterilized in autoclave FAC (Buenos Aires). Sterilized polystyrene 96-well culture plates were supplied by Deltalab (Barcelona, Spain).

Biological material

Paenibacillus larvae isolates were obtained from honey combs of bee hives exhibiting clinical symptoms of AFB, from Argentina and Italy. Isolates S1 and S2 were from Balcarce, Buenos Aires province (37° 52’00″S-58°15’00″W), strain S3 from Rio Cuarto, Cordoba province (33°08′00″S-64°21′00″W), strain S4 from Concordia, Entre Rios province (31° 23′32″S-58°01′01″W), strain S5 and S8 from Necochea, Buenos Aires (38°33′44″S-58°44′43″W), strain S6 and S7 from Mar del Plata, Buenos Aires (38°00′00″S-57°33′00″W), strain S9 from Modena, Italy (44°38′45″N-10°55′33″E) and strain S10 from Emilia Reggio, Italy (44°42′00″N-10°38′00″E).

Bacterial isolates were grown and maintained on 2% (w/v) MYPGP agar plates (1% Mueller-Hinton broth (w/v)), 1.5% yeast extract, 0.3% K_2HPO_4, 0.2% glucose, 0.1% sodium pyruvate and 2% agar (w/v), incubated at 37 °C and 10% (v/v) O_2 for 48 h (Dingman & Stahly,

1983; Nordström & Fries, 1995). The bacterial inocula were prepared in sterile peptone water (0.1%, peptone w/v) and 0.85% NaCl (w/v) to a final optical density at 600 nm OD_{600} = 0.1, using a UV-VIS spectrophotometer Spectrum SP-1103 (Spectrum Instruments Company Ltd., Shanghai, China) (Nordström & Fries, 1995). Brain-heart infusion (3.7%, w/v) was used as a growth medium for the bacterial isolates when performing the broth microdilution assay. *P. larvae* growth was detected using resazurin sodium salt.

Identification and genotyping of *P. larvae*

Total DNA of the *P. larvae* isolates was prepared from overnight cultures of the bacterial isolates grown in J medium (Hornitzky & Nicholls, 1993). DNA was obtained using a commercial genomic DNA purification Kit (Sigma). The DNA concentration and purity were checked using a NanoDrop 2000 spectrometer 169 (Thermo Fisher Scientific Com., Waltham, USA). Bacterial isolates identification was carried out by amplification of a specific 16S rRNA gene fragment of *P. larvae* using primers PL5 and PL4 (Piccini *et al.*, 2002).

ERIC genotyping of bacterial isolates was performed using primers designed by Versalovic *et al.* (1994) and conditions optimized by Antúnez *et al.* (2007). The PCR reactions were carried out by triplicate. The PCR reactions were carried out with 1 U Taq DNA polymerase (Invitrogen), 100 ng of DNA, 0.0002 mol/L of each of the four dNTPs, 0.005 mol/L $MgCl_2$ and 0.003 mol/L of each primer in a total volume of 25 µL. The PCR conditions were as follows: a single denaturation step at 94 ºC for 5 min, 40 cycles of denaturation of the DNA template at 94 ºC for 1 min, annealing of primers at 40 ºC for 2 min and extension of PCR products at 65 ºC for 8 min. A final extension step was performed at 65 ºC for 16 min. Amplified products and two DNA markers, GeneRuler DNA Ladders 100 bp and GeneRuler DNA Laders 1 kb Thermo Fisher Scientific Co., were separated in a 0.8% agarose gel and stained with Gel Red (Olerup 187 SSP). The gels were photographed under UV light.

Screening of the antimicrobial activity by agar diffusion method

Screening of the antimicrobial activity of thirteen molecules against four *P. larvae* isolates was conducted by the agar diffusion technique (Bonev *et al.*, 2008). The natural molecules tested were: saturated and unsaturated fatty acids (lauric acid, palmitic acid, lauric acid monoglyceride, alpha-ketoglutaric acid and stearic acid); phenolics acids (salycilic acid, syringic acid, ellagic acid and chlorogenic acid); flavonoids (naringenin and phloridzin); a vitamin (menadione) and an organic acid (cinamic acid). Each paper disc contained 200 µg of the molecule tested. Oxytetracycline hydrochloride (30 µg) was used as positive control. The natural molecules that showed a strong activity against *P. larvae* isolates by this method were selected for further analysis.

Determination of the minimum inhibitory concentration of the antimicrobial agent

The antimicrobial activity of selected molecules was evaluated by broth microdilution method (Cugnata *et al.*, 2017) using ten *P. larvae* isolates. Two parameters were estimated, the minimum inhibitory concentration (MIC), the concentration at which *in vitro* bacterial growth inhibition is observed; and the minimum non-inhibitory concentration (MNIC), the concentration at which *in vitro* bacterial growth inhibition is not observed. The repeatability of the method within one day (n=3) and between days (n=3) was determined for each molecule.

Determination of protease activity

Paenibacillus larvae isolates were grown in J Medium (Hornitzky & Nicholls, 1993) aerobically (150 rpm) at 37 °C until the stationary growth phase (72 h, OD_{600} > 1.5). Identical culture volumes (1 mL) were centrifuged for 10 min at 10,000 rpm and the cell-free supernatants were collected. One aliquot was examined on agar plates containing casein to verify the presence of protease activity. The remaining volume (750 µL) was concentrated 4-6x in Centricon filtration units (YM-10) and conserved at 4 °C until analysis.

— *Gelatin zymography.* The cell free supernatants were electrophoresed on polyacrylamide gels (10%, w/v) containing SDS 1% and copolymerized gelatin (without reducing agents and heating) using a modification of the protocol from Laemmli (1970). After electrophoresis, SDS was removed from the gel by washing in 0.05 mol/L Tris-ClH (pH 7.5), and then incubated in the same buffer in absence or presence of the metalloprotease inhibitor ethylenediaminetetraacetic acid (EDTA) (0.005 mol/L) 37 °C for 3 h. Finally, the gel was stained with Coomasie Brilliant Blue to evidence the bands with protease activity.

— *Azocaseinolytic assay*. The reaction mix contained 0.05 mol/L Tris-ClH (pH 7.5), 0.5% azocasein (in 0.025 mol/L NaOH) and 0.1 mL cell free supernatants, as a source of enzyme, in a final volume of 0.5 mL. The samples were incubated at 37 °C for different times and the reaction was stopped by addition of cold

trichloroacetic acid (TCA) 10% (v/v); the tubes were left on ice for 15 min and centrifuged at 2000 rpm for 10 min. To optimize the assay conditions, TCA soluble products absorbance was measured at 335nm on a UV-visible spectrophotometer (GeneQuant 1300 Spectrophotometer, Classic from GE Healthcare Life Sciences, USA). The reaction was linear for at least 3 h, thus, 2 h was chosen as incubation time. Samples were incubated in absence and presence of EDTA (0.005-0.01mol/L) and phenylmethanesulfonyl fluoride (PMSF) (0.001 mol/L).

Results and discussion

In this study, we evaluated the antimicrobial activity of different molecules against *P. larvae*. Firstly, we generated a collection of ten *P. larvae* isolates obtained from colonies with clinical signs of AFB from Argentina and Italy. A unique amplicon of 700 bp characteristic of *P. larvae* was obtained confirming the identity of the bacterial strain isolates S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10. Regarding isolates genotyping using ERIC primers, all *P. larvae* isolates showed the same DNA pattern, regardless their geographical origin. Based on comparison between the obtained DNA fingerprints, those previously published (Genersch & Otten, 2003; Genersch *et al.*, 2006) and reference strains (Alippi & Aguilar, 1998; Antúnez *et al.*, 2007), the isolates (S1to S10) were classified as ERIC I genotype. This is the most commonly detected genotype and is worldwide distributed (Genersch & Otten, 2003; Alippi *et al.*, 2004; Antúnez *et al.*, 2007).

Then, we evaluated the antimicrobial activity of thirteen molecules by the agar diffusion method using four *P. larvae* isolates (S1, S2, S9 and S10). Only four of the molecules (menadione, lauric acid, monoglyceride of lauric acid and naringenin) showed inhibition of the bacteria. The inhibitory halo decreased in the following order: menadione, lauric acid, monoglyceride of lauric acid and naringenin (Table 1).

MIC of menadione and lauric acid was evaluated by the broth microdilution method, using ten *P. larvae* isolates. Table 2 shows the results of the MIC values obtained, which ranged from 0.78 to 3.125 µg/mL for menadione, and from 25 to 50 µg/mL for lauric acid.

Feldlaufer *et al.* (1993) had already found that lauric and tridecanoic acids (isolated from mycelia and spores of *Ascosphaera apis*) were the most active saturated fatty acids against *P. larvae* (2.5 µg per disc), while palmitoleic and linoleic acids were the most active among the unsaturated ones. These authors demonstrated that the introduction of a double bond or multiple double bonds seemed to be necessary to maintain the antibiotic activity once the chain length of the fatty acid exceeds fourteen carbons. Furthermore, Hornitzky (2003) showed that fifteen fatty acids proved to have antibacterial activity against European foulbrood. The majority of the tested acids exhibited activity at the highest amount tested (250 µg), except for myristoleic and lauric acids, which showed activity even with diffusion disks containing 25 µg of the molecule. In our study, lauric acid also showed a good antimicrobial activity; however, stearic and palmitic acids did not present any antimicrobial activity.

Flesar *et al.* (2010) analyzed a total of 26 natural compounds of different chemical classes and 19 crude extracts by the broth microdilution method. The MICs, ranging from 2 to 256 µg/mL, showed that 13 compounds exhibited an antimicrobial effect against *P. larvae*. Out of all plant-derived products tested, the greatest antibacterial activity against *P. larvae* was observed for sanguinarine with a MIC = 4 µg/mL. This molecule presents the most compact spatial conformation of all the analyzed molecules. In this sense, the molecular structure of menadione is similar to the sanguinarine molecule (both present aromatic rings); this spatial conformation seems to confer the best activity against microorganisms among the molecules studied in the present work, and may explain the antibacterial activity against *P. larvae* of menadione.

According to Michielin *et al.* (2009), plant materials can be classified as antimicrobial agents based on the MIC values of its extracts. In this sense, Duarte *et al.* (2007) and Wang *et al.* (2008) classified the extracts

Table 1. Screening of the antimicrobial activity (expressed in cm) of natural molecules determined by the agar diffusion method.

Family	Natural molecules	*P. larvae* strains			
		S1	S2	S9	S10
Fatty acids	Lauric acid	3.5	2.5	2	2
	Palmitic acid	0	0	0	0
	Lauric acid monoglyceride	3	2.5	1.5	1.5
	Stearic acid	0	0	0	0
	Alpha-ketoglutaric acid	0	0	0	0
Flavonoids	Narigenin	2	2	2	2
	Phloridzin	0	0	0	0
Vitamins	Menadione	6	6	6	6
Organic acids	Cinnamic acid	0	0	0	0
Phenolics acids	Salicylic acid	0	0	0	0
	Syringic acid	0	0	0	0
	Ellagic acid	0	0	0	0
	Chlorogenic acid	0	0	0	0

Table 2. Antimicrobial activities of menadione and lauric acid against *P. larvae* by the broth microdilution method.

Paenibacillus larvae strains	Mean MIC (µg/mL) (n=3)	
	Menadione	Lauric acid
Argentina		
S1 (Balcarce, Buenos Aires)	1.56	50
S2 (Balcarce, Buenos Aires)	0.78	50
S3 (Río Cuarto, Córdoba)	1.56	50
S4 (Concordia, Entre Ríos)	1.56	50
S5 (Necochea, Buenos Aires)	1.56	50
S6 (Mar del Plata, Buenos Aires)	1.56	50
S7 (Mar del Plata, Buenos Aires)	1.56	50
S8 (Necochea, Buenos Aires)	3.125	25
Italy		
S9 (Módena)	0.78	25
S10 (Emilia Reggio)	3.125	25

MIC: minimum inhibitory concentration.

as: strong inhibitors for MIC value below 500 µg/mL; moderate inhibitors for MIC between 600 and 1500 µg/mL; weak inhibitors for MIC above 1600 µg/mL. In consonance with this classification and the MIC values obtained for menadione and lauric acid in the present study, these molecules are strong inhibitors of *P. larvae*.

In order to deep-in in the study of the antimicrobial activity of those molecules, we investigated its potential inhibitory effect on the proteolytic activity of *P. larvae*. Previous reports showed that *P. larvae* secrets metalloproteases which are considered virulence factors of this microorganism (Dancer & Chantawannakul, 1997; Antúnez *et al.*, 2009). Then, we first confirmed the capacity of the *P. larvae* isolates obtain in this study to produce these proteins. Protease activity was maximal in the stationary growth phase (60-70 h). Then, the proteolytic profile was analyzed by gelatin zymography. All the isolates presented proteolytic activity, showing the same pattern with at least three gelatinolytic bands ranging from 20 to 38 kDa (Fig. 1). The intensity of these bands was reduced by incubation with EDTA, indicating the presence of metalloprotease/s, in agreement with Antúnez *et al.* (2009). This result was confirmed with the azocaseinolytic assay, which showed 80% inhibition of the protease activity in presence of EDTA and no inhibition with the serine protease inhibitor PMSF. To explore the potential inhibitory effect of natural molecules on the metalloproteases secreted by *P. larvae*, we assayed the extracellular azocaseinolytic activity of two isolates in presence of menadione (0.15, 1.5 and 3 µg/mL) and lauric acid

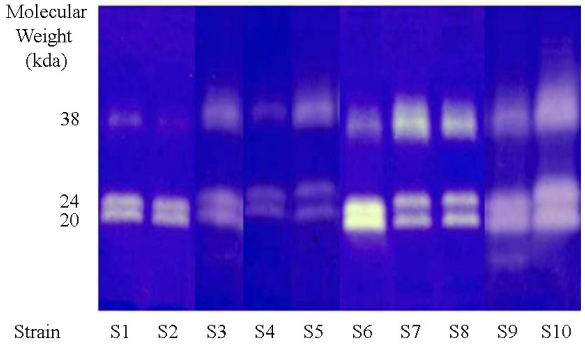

Figure 1. Extracellular proteolytic profile of *P. larvae* isolates. Cultures of ten *P. larvae* isolates were grown in J medium until the stationary phase (OD_{600} = 1.5-2) and the cell-free supernatants were concentrated and examined by gelatin zymography.

(50 µg/mL). However, no inhibition of the protease activity was observed under the concentrations tested.

In conclusion, the present study shows that the natural molecules menadione and lauric acid exhibited *in vitro* bactericidal activity against *Paenibacillus larvae*, at concentrations feasible to be applied in the field. The use of non-toxic compounds represents a natural alternative to synthetic antibiotics for the control of AFB, reducing considerably the controversies of antibiotic residues and bacterial strain resistance. Further research will focus on the efficacy of these compounds and delivery on the field condition and the methods of dosage (sugar solution, candy or aerosol), as well as on their mechanisms of action against *P. larvae*.

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