



Inhibitory action of essential oils against proteases activity of *Paenibacillus larvae*, the etiological agent of American Foulbrood disease

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Abstract

American foulbrood (AFB) is a disease affecting the larva of *Apis mellifera*. The etiological agent is *Paenibacillus larvae*, which releases metalloproteases involved in the degradation of larval tissues. Through quorum sensing (QS) mechanism, bacteria are able to activate specific genes such as virulence factors. The exoproteases regulation of *P. larvae* could be associated with QS. A promising mechanism of AFB control is to block QS mechanism with essential oils (EO). The aim of this study was to investigate the potential presence of QS signals in the regulation of *P. larvae* proteases and the effect of seven EOs on the exoproteases activity of *P. larvae*. From growth curves and evaluation of the presence of proteases by milk agar plates assay, it was observed protease activity during the late exponential phase of growth. Early production of protease activity (15 hours earlier than control) was observed when a low density culture was incubated with late exponential spent medium (SM) suggesting the presence of factor(s) inducing this activity. SM was obtained by the ultrafiltration of *P. larvae* cultures on late growth phase and was free of proteases. Proteolytic activity was quantified on *P. larvae* cultures in presence of sublethal concentration of EO by azocasein method. The EOs, except *S. chilensis* EO, reduced significantly protease activity (more than 50%). We report for the first time evidence on the possible role of QS on *P. larvae* and the antiproteolytic activity of EOs (except for *S. chilensis*) on exoproteases, an interesting therapeutic strategy to control AFB.

Additional keywords: virulence factor; proteases; spent medium; gram positive; bee infection.

Abbreviations used: AFB (American Foulbrood); C (control); EO (essential oils); MSC (maximum sublethal concentration); OD (optical density); QS (quorum sensing); SM (spent medium).

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Introduction

Currently, American Foulbrood (AFB) is considered the most serious illness plaguing apiculture with a nearly cosmopolitan distribution. It is a highly contagious disease affecting the larval stage in the development of honey bees (*Apis mellifera* L.). The etiological agent is the spore forming gram positive

bacterium *Paenibacillus larvae* (Genersch *et al.*, 2006). Antúnez *et al.* (2009, 2010, 2011a,b) determined that *P. larvae* produces and secretes different proteins with proteolytic activity during vegetative replication, such as metalloproteases and enolase. These proteins are produced within the cell and secreted into the external medium, where they remain on the surface of the spores producing an immune response on *A. mellifera* and

probably being involved in larval tissue degradation. It has been proposed that metalloproteases released by *P. larvae* are involved in the inhibition of the immune system through the degradation of antibacterial polypeptides and in the degradation of larval tissues.

In recent years, the detection of quorum sensing (QS) signals in bacteria has added a new dimension to study the process of infection. QS or cell-to-cell communication is employed by diverse groups of bacteria to communicate with each other by producing the signaling molecules or autoinducers. Through the mechanism of QS, bacteria are able to activate specific genes in response to population density (Kleerebezem *et al.*, 1997; Fuqua *et al.*, 2001; Henke & Bassler, 2004; Waters & Bassler, 2005; Williams *et al.*, 2007). QS can regulate a number of activities such as the expression of virulence factors, bioluminescence, sporulation, biofilm formation and conjugation (Cámara *et al.*, 2002; Williams, 2007; Boyer & Wisniewski-Dyé, 2009). Many bacteria coordinate expression of multiple virulence factors such as toxins, redox active compounds, siderophores, exoproteases, lipases, and biofilm formation. This coordination on microbial attack maximizes the chances of bacterial infection and allows their spread (Antúnez *et al.*, 2010; Castillo-Juárez *et al.*, 2015).

The signals of QS are produced while the bacterial population grows until it reaches a threshold concentration perceived by the bacteria and resulting in the activation or repression of specific genes. Accumulation of a stimulatory amount of such molecules can occur only when a specific, sufficient number of cells, referred to as a quorum, is present. These autoinducer molecules have been identified as acylated homoserine lactones in gram negative bacteria and oligopeptides, thiolactone/lactone peptide, lanthionines, isoprenyl groups (Waters & Bassler, 2005) and even acylated homoserine lactones in gram positive bacteria (Biswa & Doble, 2013; Monnet & Gardan, 2015). Similar signaling mechanisms have not been demonstrated yet in *P. larvae*. It is possible that the larval infection by *P. larvae* is influenced by QS regulated phenotypes such as proteases exported by bacteria into their environment. The concept of QS has encouraged the development of a novel non-antibiotic, anti-bacterial therapy by using QS inhibitor compounds (Hentzer *et al.*, 2003; Rasmussen & Givskov, 2006).

The increase in multidrug resistance of bacteria to traditional drugs, drastically reduces the efficiency of conventional antibiotics. This multi-resistance is now recognized as a world global problem (Olivero-Verbel *et al.*, 2014). Therefore, the development of a new therapeutic strategy to prevent such multidrug is necessary. A promising mechanism is to block

cell to cell communication, establishing a strategy called quorum quenching (Zhang, 2003). While traditional antimicrobial agents result in cell death of the pathogen, the use of systems that alter QS sensors adopts a less aggressive strategy (Otero Casal *et al.*, 2005). There are several sources of QS inhibitors (quorum quenchers), but so far the most diverse and abundant are those derived from natural sources such as algae and plants. Cases of inhibitors of QS in bacteria, fungi, algae, bryozoans, corals, sponges (Tang & Zhang, 2014), plant extracts (Gala & Desai, 2014; Ghosh *et al.*, 2014), essential oils (Khan *et al.*, 2009), compounds isolated from bacteria (Chu *et al.*, 2013) and furanones (Bhardwaj *et al.*, 2013), among others, are recorded.

Essential oils (EOs) extracted from plants, such as *Cymbopogon citratus*, *Cymbopogon martini*, *Rosmarinus officinalis*, *Mentha piperita*, *Pelargonium odoratissimum* and *Negundo vitex*, were actually used in the treatment of various diseases, and different natural compounds, such as citral, geraniol, thymol and linalool have been used to evaluate their protease inhibitory activity, constituting one of the virulence factors of bacteria that can be regulated by QS (Sivamani *et al.*, 2012).

The aim of this study was to investigate the potential presence of QS signals in the regulation of *P. larvae* proteases and the effect of *Artemisia annua*, *Heterothalamus alienus*, *Lepechinia floribunda*, *Satureja odora*, *Solidago chilensis*, *Tagetes minuta* and *Wedelia glauca* EOs on the exoproteases activity of *P. larvae*.

Material and methods

Strains and culture conditions

Paenibacillus larvae strains were isolated from honey combs of a hive exhibiting clinical symptoms of AFB. The hives were located in the province of Buenos Aires, Argentina. Isolation and strains identification were conducted using previously described techniques (Alippi, 1991) and confirmed afterwards according to biochemical and physiological tests (Neuendorf *et al.*, 2004). The *P. larvae* strains were stored at -20°C on MYPGP agar (Mueller-Hinton broth 19.6% w/v, yeast extract 29.4% w/v, glucose 3.9% w/v, sodium pyruvate 1.96% w/v, PO_4HK_2 5.9% w/v and agar-agar 39.2% w/v), with 20% v/v of glycerol until used. Frozen culture was inoculated into J-broth (yeast extract 60% w/v, tryptone 20% w/v, PO_4HK_2 12% w/v and glucose 8% w/v) (9 mL) and incubated 48 hours at 37°C . Then the culture was isolated in MYPGP agar plate.

Relative protease production and cell growth

Cultures of *P. larvae* were prepared in 30 mL of J-broth with a final concentration of $8 \cdot 10^6$ CFU/mL and incubated at 37°C with shaking (150 rpm). Aliquots were taken at different times (0, 4, 10, 16, 20, 22, 24, 30, 35, 42, 46, 48 and 50 hours). Cell growth was monitored by the increase in optical density (OD) at 600 nm and by quantifying CFU/mL. The aliquots were centrifuged at 10,000 rpm for 10 min and the supernatant was used to quantify total proteins by Bradford (1976) and determine the presence of caseinolytic proteases by inoculating skim milk agar plates. The assays were conducted on two isolates of *P. larvae*, in triplicate.

Caseinolytic protease activity

Skimmed milk agar plate assays allow principally for qualitative determinations of protease activity. To detect the caseinolytic activities of extracellular proteases of *P. larvae*, aliquots of 50 µL were introduced into wells made on skim milk agar plates (skim milk 0.62 % w/v, yeast extract 0.25 % w/v, glucose 0.1 % w/v and agar-agar 0.14 % w/v). The plates were incubated at 37°C and the diameter of radial digestion of casein (proteolytic halos) was recorded after 24 hours.

Spent medium preparation

In order to collect those molecules that are produced in the late exponential phase of *P. larvae* culture, which could be acting as inducers of QS, spent medium (SM) was prepared. To obtain a SM, the technique described by Paggi *et al.* (2003) was used with some modifications.

Cultures of *P. larvae* were prepared in 30 mL of J-broth with a final concentration of $8 \cdot 10^6$ CFU/mL and incubated at 37°C with shaking (150 rpm) for 44 hours until late exponential phase ($OD_{600}=2.5$). Cells were harvested by centrifugation at 10,000 rpm for 10 min and the supernatant was ultra-filtrated (YM 30, Amicon) to eliminate the extracellular proteases that may be present in the medium. The ultrafiltration performed had that cut-off point (30 kDa) because previous studies in which zymographs were performed (data not shown) the proteases released by *P. larvae* were approximately 60 kDa. Those results coinciding with previous studies of Antúnez *et al.* (2011b) in *P. larvae* strains from Uruguay and Argentina. The absence of proteases on the filtrate and the presence of proteases in the fraction retained were checked by inoculating skim milk agar plates and the observation of absence / presence of proteolytic halos was analyzed (data not shown). The SM was stored at 4°C and used in the induction assay.

Induction assay

To study the effect of SM on the exoproteases of *P. larvae*, a low density culture ($OD_{600} = 0.2-0.3$) of *P. larvae* was centrifuged at 10,000 rpm and the cells were suspended in the same volume of either fresh medium (control) or late exponential conditioned medium (90% SM-10% fresh medium). Both cultures were incubated at 37°C with shaking (150 rpm) and aliquots were taken at different times (0, 4, 10, 16, 20, 22, 24, 30, 35, 42, 46, 48 and 50 hours). Cell growth was monitored by the increase in OD_{600} and by quantifying CFU/mL. Aliquots were centrifuged at 10,000 rpm during 10 min and the supernatant was used to quantify total protein content by Bradford (1976) and determine the presence of caseinolytic protease activity. The assays were conducted on two isolates of *P. larvae*, in triplicate.

Protein precipitation and SDS-PAGE

In order to compare the protein profile between control culture and SM culture, an SDS-PAGE was done. Samples of control broth (CT_0), control at 22 hours (CT_{22h}), SM control (SMT_0) and SM at 22 hours (SMT_{22h}) were precipitated. Briefly, trichloroacetic acid (TCA, 15%) was added to the medium, mixed vigorously and placed on ice for 30 min. The aggregated proteins were precipitated by centrifugation (10,000 rpm), washed twice in cold 80% acetone and once in cold 100% acetone. After the final wash the samples were dried and solubilized in Laemmli sample buffer. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions, as described by Laemmli (1970) and stained with 0.22% v/v Coomassie brilliant blue G-250. Intensity of proteins bands resolved by SDS-PAGE gel, was analyzed and quantified by using IMAGEJ1.47 free software (National Institute of Health, Bethesda, MD, USA; <http://imagej.nih.gov/ij/>).

Plant material, preparation and analysis of essential oils

To study the effect of EOs on the activity of proteases released by *P. larvae*, seven native plants of Argentina belonging to two different botanical families were collected in the Province of San Luis (Argentina) (Table 1). All EOs were obtained by hydrodistillation of the fresh material with *in situ* steam generation in a Clevenger apparatus type. EOs were stored at 5°C in dark containers.

Gas chromatography with flame ionization detection

EOs (10 mg) were diluted in dichloromethane (1 mL) and analyzed by gas chromatography (GC)

Table 1. Argentinian native aromatic plant species.

Botanic family	Plant species	Local common name	Part of the plant ^a	Biological activity ^b	References
Asteraceae	<i>Solidago chilensis</i>	Vara de oro	Leaves and flowers	Antioxidant	Gastaldi <i>et al.</i> , 2016
				Antifungal	Vila <i>et al.</i> , 2002
	<i>Artemisia annua</i>	Ajenjo	Leaves and flowers	Antimicrobial	Demo <i>et al.</i> , 2005; Pellegrini <i>et al.</i> , 2014
				Antimicrobial (<i>P. larvae</i>)	Fuselli <i>et al.</i> , 2008
				Insecticidal	Palacios <i>et al.</i> , 2009
	<i>Tagetes minuta</i>	Manzanilla o suico	Leaves and flowers	Antifungal	Zygadlo <i>et al.</i> , 1994; Larran <i>et al.</i> , 2001; Camiletti <i>et al.</i> , 2016
				Insecticidal	Gillij <i>et al.</i> , 2008; Palacios <i>et al.</i> , 2009; López <i>et al.</i> , 2011
				Acaricidal	Eguaras <i>et al.</i> , 2005; Ruffinengo <i>et al.</i> , 2005; Chamorro <i>et al.</i> , 2008
				Antimicrobial (<i>P. larvae</i>)	Eguaras <i>et al.</i> , 2005; González & Marioli, 2010
	<i>Wedelia glauca</i>	Sunchillo	Leaves	Antimicrobial (<i>P. larvae</i>)	Fuselli <i>et al.</i> , 2008
				Acaricidal	Ruffinengo <i>et al.</i> , 2005
	<i>Heterothalamus alienus</i>	Romerillo	Leaves and stems	Antiviral	Duschatzky <i>et al.</i> , 2005
Antimicrobial (<i>P. larvae</i>)				Ruffinengo <i>et al.</i> , 2006; Fuselli <i>et al.</i> , 2008	
Acaricidal				Ruffinengo <i>et al.</i> , 2006	
Antifungal				Ruffinengo <i>et al.</i> , 2006	
Lamiaceae	<i>Satureja odora</i>	Muña-muña	Leaves and flowers	Antimicrobial (<i>P. larvae</i>)	Fuselli <i>et al.</i> , 2008
				Antimicrobial	Pellegrini <i>et al.</i> , 2014
	<i>Lepechinia floribunda</i>	Salvia blanca	Leaves and flowers	Antimicrobial (<i>P. larvae</i>)	Fuselli <i>et al.</i> , 2008
				Insecticidal	Palacios <i>et al.</i> , 2009
				Antimicrobial	Pellegrini <i>et al.</i> , 2014

^a Part of the plant used for obtaining the essential oil. ^b Biological activity of the Argentinian EOs reported in literature.

using a Hewlett-Packard 5890 Series II instrument equipped with a flame ionization detector (FID). Data were analyzed using the Hewlett-Packard GC Chem Station Software (1990-1998). Separation was carried out on a Elite-5 capillary column (30 m × 0.25 mm id, 0.25 µm film thickness) provided by Altech Inc. (Texarkana, TX 75501, USA). The carrier gas was hydrogen at 1 mL/min, and the inlet pressure, 9 psi.

The oven temperature program was as follows: 40°C for 2 min, increased to 240°C at 5°C/min, held at 240°C for 1 min, increased to 310°C at 10°C/min, and held at 310°C for 5 min. Injector and detector temperatures were 220°C and 250°C respectively. Injections were performed in split mode (40:1), and the injection volume was 1 µL. Analyses were performed in triplicate. The relative concentration of

each compound was calculated and expressed as the percentage of the corresponding peak area related to the total chromatogram peak area.

Gas chromatography coupled to mass spectrometry

The identification of the individual compounds was carried out using a Shimadzu 2010 GC coupled to a Shimadzu QP2010 plus mass spectrometer (MS). Data were analyzed using Shimadzu Corporation GC-MS Solution v2.50 (1999-2006). Analyses were run on a DB5MS column (30 m × 0.25 mm id, 0.25 µm film thickness) provided by Macherey-Nagel (Düren, Germany). The carrier gas was helium at 1 mL/min. The oven temperature program was as follows: 40°C for 2 min, increase to 240°C at 5°C/min for 1 min, increase to 300°C at 10°C/min for 2 min. Injector and detector temperatures were 250°C. Injections were performed in split mode (30:1), and the injection volume was 1 µL. MS parameters were: electron impact ionization at 70 eV ionization potential, m/z 40-350. The identification of the EO components was done by comparison of their retention indices with those reported by Adams (2007) and Pherobase database (www.pherobase.com), and of their fragmentation patterns with those contained in NIST 05 (<http://webbook.nist.gov/>) and SHIM 2205 mass spectrometer libraries.

Antimicrobial activity of essential oils on liquid cultures of *P. larvae*

In order to calculate the maximum concentration of EO that allows the *P. larvae* growth in liquid cultures (maximum sublethal concentration, MSC), cultures of *P. larvae* were prepared in 30 mL of J-broth with a final concentration of 8·10⁶ CFU/mL and different volumes of the EOs. The cultures were incubated at 37°C with shaking (150 rpm) for 48 hours. Cell growth was monitored by the increase in OD₆₀₀. The assays were conducted on two isolates of *P. larvae*, in quadruplicate.

Evaluation of the release of proteases in the presence of sublethal concentrations of essential oil

In order to analyze whether the EOs have antipathogenic activity against *P. larvae* exoproteases, cultures of *P. larvae* were prepared in 30 mL of J-broth with a final concentration of 8·10⁶ CFU/mL and the MSC of each EOs. The cultures were incubated at 37°C with shaking (150 rpm) for 48 hours. Finally, aliquots were taken in late exponential growth phase and the following parameters were measured: *i*) cell growth by the increase in OD₆₀₀, *ii*) presence of caseinolytic protease activity in supernatants obtained from

aliquots centrifuged at 10,000 rpm (10 min), and *iii*) quantification of proteases in supernatants obtained from aliquots centrifuged at 10,000 rpm for 10 min by the method of azocasein (Charney & Tomarelli, 1947). For the negative control, cultures of *P. larvae* were prepared under the same conditions but without the addition of EOs. The assays were conducted on two isolates of *P. larvae*, in quadruplicate.

Evaluation of the effect of essential oils on the proteases released by *P. larvae*

To test whether EOs have a direct effect on the proteases released (isolated) by *P. larvae*, the following tests were made: from cultures of *P. larvae* on J-broth on late exponential phase, aliquots were centrifuged and the supernatant (with proteases) was placed in contact with the volume of EO corresponding to the MSC, considering that the final volume was 100 µL (*S. odora*: 0.02 µL, *H. allienus*: 0.0046 µL, *T. minuta*: 0.0066 µL, *A. annua*: 0.01 µL, *S. chilensis*: 0.00067 µL, *W. glauca*: 0.0033 µL, *L. floribunda*: 0.00333 µL). The samples were incubated at 37°C for 30 min. Subsequently, caseinolytic protease activity was analyzed and proteolytic activity was quantitated by azocasein. The tests were conducted on two isolates of *P. larvae*, in quadruplicate.

Proteolytic activity quantified by azocasein

Azocasein prepared in our laboratory as described by Charney & Tomarelli (1947) was used as substrate. The reaction mixture containing 0.025 mL of 10% (w/v) azocasein on 25 mM NaOH, 0.375 mL NaHCO₃ buffer (pH 7) and 0.1 mL of cell-free culture medium (of culture with or without EOs) was incubated at 37°C for 1 hour. Incubations were stopped by adding 1 volume of cold 5% TCA and the assay tubes were left at 4°C for 30 min and then centrifuged at 2,000 rpm for 10 min. Acid-soluble products were detected in the supernatant by measuring OD₃₃₅. One unit of activity (U) was defined as the amount of enzyme that produced an increase of 1 in OD₃₃₅ per hour under the conditions described above.

Statistical analysis

The data of the cell growth and quantification of total proteins are presented as mean ± standard deviation of three parallel measurements (StatMost, vers. 2.5). An analysis of variance (ANOVA) was applied in the percentage of azocasein to determine statistical differences between treatments. Data were compared using Tukey's test at a significance level

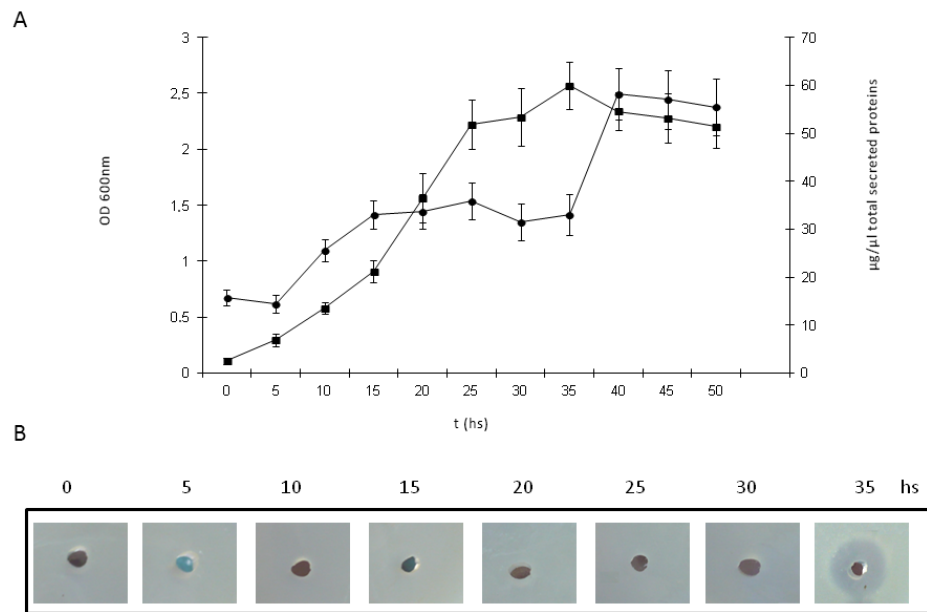


Figure 1. (A) Cell growth and total secreted proteins of *P. larvae* grown in fresh J-broth. Cell growth was monitored by the increase in optical density at 600 nm (■) and the extracellular proteins were measured by Bradford (●). (B) Protease activity of *P. larvae* grown in fresh J-broth. Protease activity was monitored by the production of proteolytic halos on skim milk agar plates.

of 5% ($p < 0.05$) using the Statistica program (v. 6.1 StatSoft, Inc., Tulsa, USA).

Results

Relative protease production and cell growth. Induction assay

In order to determine the bacterial growth phase associated with extracellular protease production, cell growth of *P. larvae* isolates (Fig. 1A) and the presence of proteolytic halos were observed. Extracellular protease release in *P. larvae* started in the late growth phase (Fig. 1B). These observations led us to propose that protease production may be induced in response to high cell density which may be indicative of quorum sensing signalling. To examine this possibility, *P. larvae* low density cultures were suspended in fresh medium (control medium) or in a cell-free culture medium obtained from a filtration of a culture of cells grown to late exponential phase ($OD_{600} = 2.5$) (SM) and the time course of extracellular protease activity was examined. The culture containing SM showed higher bacterial growth in a forward stage than the control (Fig. 2A). In addition, the exponential phase of the culture began almost immediately; meaning that there would be no lag phase. The detection of protease activity in the culture containing SM was done 15 hours earlier than in control medium (Fig. 2B). Releasing SM culture proteases not only corresponds to an increase in

growth bacterial, but also, at similar OD_{600} (0.85 and 0.9, $t = 20$ hours) (Figs. 2A,B), the proteins released in SM culture exhibit caseinolytic activity whereas this does not occur in the control. Furthermore, the increase in the concentration of total protein in SM would suggest the presence of an increased concentration of molecules which could be induced by the SM culture.

To investigate the presence of potential autoinducers in *P. larvae* culture media, SDS-PAGE was performed (Figs. 3A,B). Densitometry between both control (CT_{22h}) and SM treatment (SMT_{22h}) was done by comparing the intensity of the viewable protein bands. There were bands that were down-regulated in SMT_{22h} and others up-regulated compared with CT_{22h} .

Essential oils

The volatile compositions of the EOs of *A. annua*, *H. alienus*, *L. floribunda*, *S. odora*, *S. chilensis*, *T. minuta* and *W. glauca* collected in Argentina are presented in Table S1 [suppl.]. The main families of volatile compounds found in these EOs were monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. One hundred and two individual volatiles were determined in the EOs studied.

The MSC values (EO $\mu\text{g/mL}$ J-broth) for the EOs tested are presented in Table 2. Supernatants of the cultures of *P. larvae* at MSC of EOs in the late growth phase were taken and caseinolytic protease activity

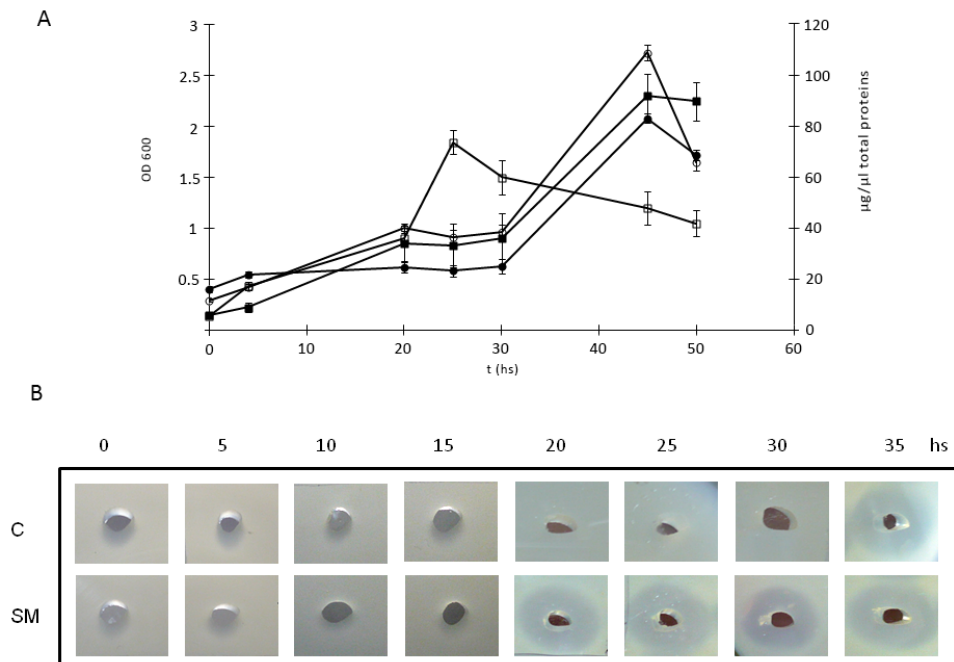


Figure 2. (A). Cell growth and total secreted proteins of *P. larvae* grown in fresh J-broth (C) and in spent medium (SM). *P. larvae* low density culture ($OD_{600}=0.1$) was centrifuged and the cells were resuspended in the same volume of fresh J-broth. Cell growth was monitored by the increase in optical density at 600 nm of the control (■) and of the SM (□). The extracellular proteins of the control (●) and of the SM (○) were measured by Bradford. (B) Protease activity of *P. larvae* grown in fresh J-broth (C) and in SM. Protease activity was monitored by the production of proteolytic halos on skim milk agar plates.

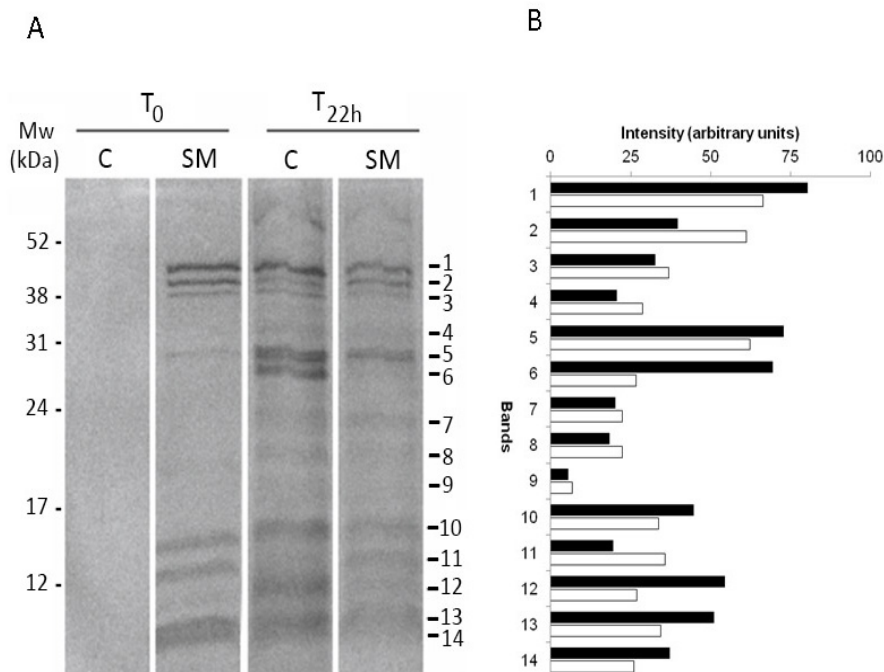


Figure 3. (A) SDS-PAGE (15%) analysis of *P. larvae* cultures. A representative gel is shown. CT₀, culture control (T=0h); CT_{22h}, culture control (T=22h); SMT₀, SM culture (T=0h); SMT_{22h}, SM culture (T=22h). The positions of molecular mass markers (Mw, kDa) are indicated on the left side of the gel. (B) Scanning densitometry. The intensity of the bands was quantified in both Control and SM samples. Black bar: CT_{22h}; white bar: SMT_{22h}.

Table 2. Maximum sublethal concentrations (MSCs) for the EOs against *Paenibacillus larvae*.

Essential oil	MSC $\mu\text{g/mL}$
<i>Artemisia annua</i>	45.50
<i>Hetherothalamus alienus</i>	19.60
<i>Lepechinia floribunda</i>	30.33
<i>Satureja odora</i>	89.00
<i>Solidago chilensis</i>	6.47
<i>Tagetes minuta</i>	30.67
<i>Wedelia glauca</i>	33.00

(Fig. 4A) and quantification of proteases by the azocasein method (Fig. 4B) were recorded. Proteolytic activity was observed for all the EOs and none of them showed complete inhibition of caseinolytic activity (Fig. 4A). The quantification of proteolytic activity by the method of azocasein indicated that all the EOs

except *S. chilensis* EO showed significant differences ($p < 0.05$) compared to the control. That is, the EOs of *A. annua*, *H. alienus*, *L. floribunda*, *S. odora*, *T. minuta* and *W. glauca* had an effect on the activity of proteases of *P. larvae*. In all cases, the EOs reduced protease activity (Fig. 4B).

Studies on the activity of EOs directly on the proteases released by *P. larvae* were also conducted (Figs. 5A,B). Proteolytic activity was observed on agar skim milk plates and none of EOs showed inhibition of caseinolytic activity (in all cases the same size halo was observed) (Fig. 5A). Proteolytic activity was quantified by the method of the azocasein (Fig. 5B). None of the EOs had a direct effect on the proteases (isolated) released by *P. larvae* ($p > 0.05$).

Discussion

In many bacteria, the production of extracellular hydrolytic enzymes (including proteases) is regulated by

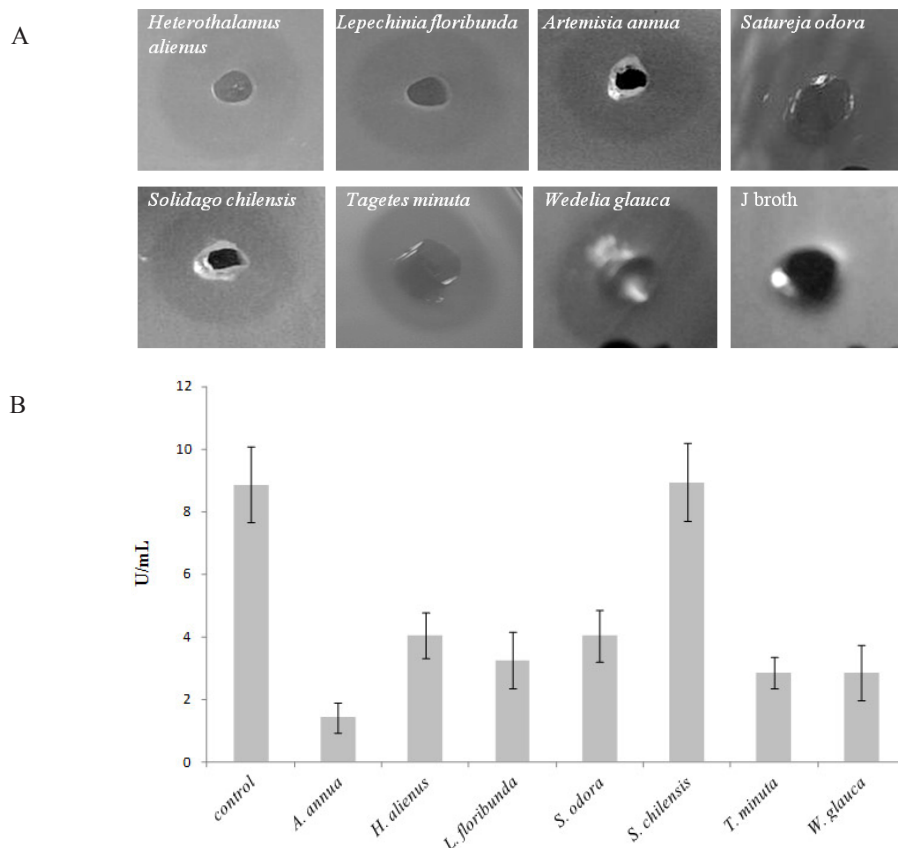


Figure 4. Proteolytic activity of proteases released by *Paenibacillus larvae* in cultures with essential oils at a maximum sublethal concentration on skim milk agar plates (A) and proteolytic activity quantified by azocasein (B). Error bars indicate the standard deviation of three replicates. (*) represents significant differences ($p < 0.05$) compared to control.

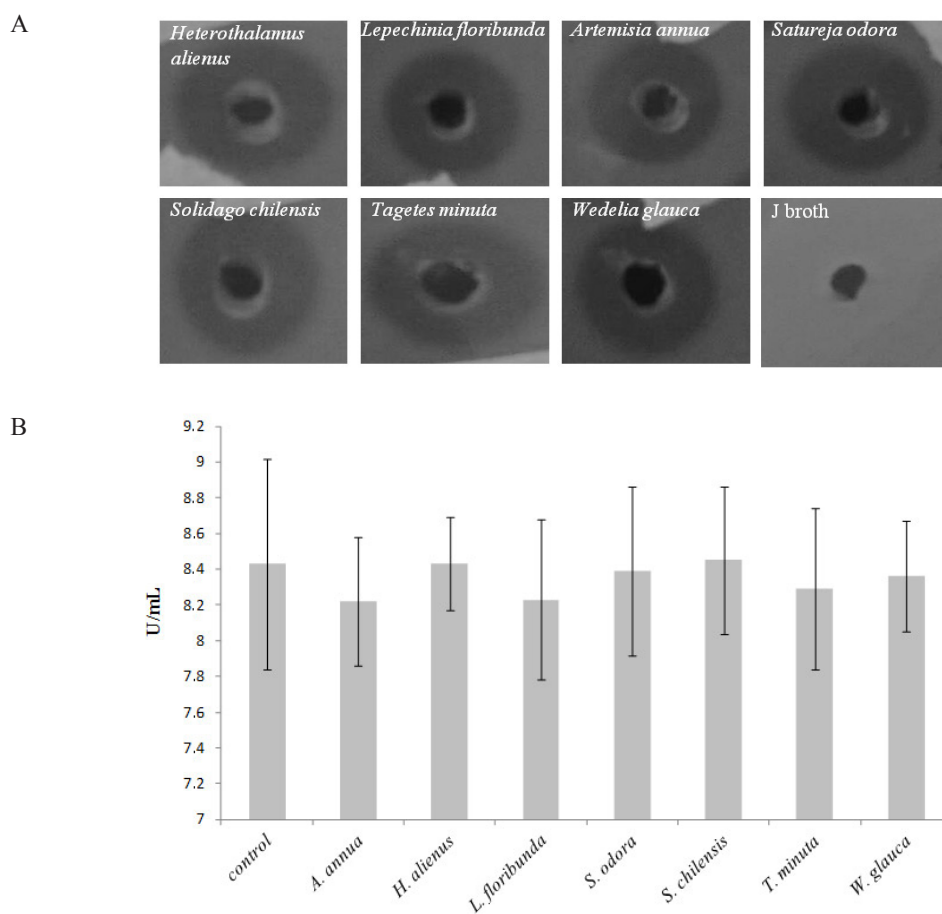


Figure 5. Proteolytic activity of proteases released by *Paenibacillus larvae* in direct contact with essential oils tested at maximum sublethal concentration on skim milk agar plates (A) and proteolytic activity quantified by azocasein (B). Error bars indicate the standard deviation of three replicates.

QS (Novick *et al.*, 1993; Choi *et al.*, 2014; Tran *et al.*, 2014; Weng *et al.*, 2014). This signalling mechanism is mediated by molecules called autoinducers that accumulate in the external medium as the culture reaches a high cell density.

Given that the release of proteases in *P. larvae* was produced in the late exponential phase and due to the presence of new proteins that were found in low intensity in the control sample by SDS-PAGE, it could be established that the release of proteases may be regulated by quorum sensing. For the first time we report preliminary evidence on the occurrence of potential autoinducer molecules in *P. larvae*.

All the EOs presented in this work, except for the EO of *S. chilensis*, have been previously studied as *P. larvae* antimicrobials (Eguaras *et al.*, 2005; Ruffinengo *et al.*, 2006; Fuselli *et al.*, 2008; González & Marioli, 2010). The antimicrobial control strategy is most commonly used to eliminate a pathogen from a host, however, the use of EOs as antipathogenic, i.e. as agents that block or alter the activity of the virulence

factors of *P. larvae* (proteases), has not been studied so far.

Since all of the EOs except for the EO of *S. chilensis* showed significant differences in proteolytic activity (measured by azocasein) compared with the control by decreasing the activity of proteases, the EOs possibly act as inhibitors of QS if it is found that the virulence factors (proteases) are regulated by this mechanism.

No studies of EOs of *A. annua*, *H. alienus*, *L. floribunda*, *S. chilensis*, *S. odora*, *T. minuta* and *W. glauca* as protease inhibitors of *P. larvae* or against other microorganism were recorded on bibliography; except for those on EO of *A. annua* from Iran which showed protease inhibitor activity on lepidopteran larvae of *Helicoverpa armigera* using the azocasein technique (Mojarab-Mahboubkar *et al.*, 2015). There are studies that describe antiproteolytic activity of EO of *Lippia alba*, rich in linalool, on dermatophyte fungi (Costa *et al.*, 2014). The EO of *Piper corcovadensis* showed a decrease in the proteolytic activity of enzymes from intestines of dengue mosquito, by

zymography technique (da Silva *et al.*, 2016). Extracts of *Helianthemum canum*, *Erica arborea*, *Arbutus unedo* and *Arctostaphylos uva-ursi* showed antiproteolytic activity on rumen bacteria sheep (Selje *et al.*, 2007) and the aromatic compound acetate eugenyl showed antiproteolytic activity on *Acinetobacter baumannii*, by halos of proteolysis on milk agar plates (Musthafa & Voravuthikunchai, 2016).

Studies on the activity of EOs were carried out directly on the proteases released by *P. larvae*. Since the EOs had no direct effect on proteases released by *P. larvae*, we could conclude that the EOs have no effect once proteases are released from the cell of *P. larvae*, as its proteolytic activity was evidenced by proteolysis halos and quantitation by azocasein.

From the results obtained it is proposed that the EOs could act by inhibiting the production of proteases, inhibiting the transport, secretion thereof, inhibiting the quorum sensing or avoiding the export of proteases. All extracellular bacterial proteases are synthesized as inactive pre-proenzyme consisting of a signal peptide, a prosequence, and a mature sequence. The signal peptide acts as a signal for translocation of pre-proenzyme to membrane. The pre-proenzyme is processed into the proenzyme by the signal peptidase. The prosequence then acts as a molecular chaperone leading to an autocleavage of the peptide bond linking the pro and mature sequences (Inouye *et al.*, 2007). The EOs acted at some point of this regulatory mechanism.

The inhibition of proteases of *P. larvae* by EOs could be considered an attractive form of therapeutic intervention, since the blocking of bacterial virulence factors does not kill or inhibit the growth of pathogenic bacteria. This strategy is expected to generate low selection pressure on the bacteria and thus it might decrease the emergence of bacterial resistance and prevent disruption of beneficial microbiota on the hives. In further research, it will be interesting to isolate and biochemically characterize the potential autoinducers of *P. larvae* and to investigate their relationship with protease regulation.

The EOs studied in the present work have a potential to be used in the control of the damages caused by *P. larvae* in hives with symptoms of American Foulbrood.

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