# Natural strategies for the control of Paenibacillus larvae, the causative agent of American foulbrood in honey bees: a review

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**Review article**

# Natural strategies for the control of *Paenibacillus larvae*, the causative agent of American foulbrood in honey bees: a review

Rosa Maria Alonso-Salces[1,3], Noelia Melina Cugnata[1,3], Elisa Guaspari[1,3], Maria Celeste Pellegrini[1,2], Inés Aubone[1], Fiorella Giselle De Piano[3,4], Karina Antunez[5], Sandra Rosa Fuselli[1,2]

[1]Grupo de Investigación Microbiología Aplicada, Centro de Investigación en Abejas Sociales, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Dean Funes, B7602AYL, 3350, Mar del Plata, Argentina
[2]Comisión de Investigaciones Científicas, La Plata, Argentina
[3]Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina
[4]Cátedra de Apicultura, Departamento de Producción Animal, Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina
[5]Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

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**Abstract –** American foulbrood (AFB) is a severe bacterial disease that affects larvae of honey bees (*Apis mellifera*). The causative agent of AFB is the spore-forming bacteria *Paenibacillus larvae.* The use of antibiotics for the control of AFB has led to the appearance of resistant bacterial strains and residues in beehive products. Nowadays, antibiotics are legally banned in several countries, and the affected colonies have to be destroyed by burning the hives. Therefore, the development of alternative methods for the control and prevention of AFB is necessary. In this context, different natural strategies based on the application of essential oils, plant extracts, propolis, royal jelly, nonconventional natural molecules, bacteria, and bacteriocines, have been studied in vitro and in vivo for the prevention and control of *P. larvae*. The experimental data achieved from these studies are reviewed and discussed in the present review, which intend to be a starting point for future research in the field.

***Paenibacillus larvae*** **/ American foulbrood /** ***Apis mellifera*** **/ natural products**

## 1. 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*, which is a flagellated gram-positive bacterium, whose main characteristic is the formation of highly resistant endospores. This pathogen affects the breeding during the larval or pupal stages (Genersch et al. 2006); its spores being the infectious form. Honey bee larvae are more susceptible to infection during the first 36 h after egg hatching (Ashiralieva and Genersch 2006), indeed only ten

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Corresponding author: S. Fuselli, sfuselli@gmail.com
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spores are required to make a larva of less than 24 h old ill (Bamrick 1967). However, at later larval developmental stages, spore doses needed to successfully infect a larva are too high to occur under natural conditions (Genersch et al. 2005). The infection takes place by the ingestion of the spores with the food provided by adult worker bees (nurses) to the larvae (Hansen and Brodsgaard 1999). Spores germinate in the larval midgut and massively proliferate for several days. At a later stage, *P. larvae* reaches the peritrophic matrix, penetrates into the epidermal cells, producing septicemia, and finally causing the death of the larva. In the last instance, the corpses are digested by vegetative bacterial cells and converted to dried scales containing millions of *P. larvae* spores (Hansen and Brodsgaard 1999; Cornman et al. 2013; Djukic et al. 2014). The most conspicuous symptoms in colonies suffering from clinical AFB disease are irregular brood capping, showing capped and uncapped cells scattered irregularly across the brood frames; dark, sunken, and often punctured caps emitting a "foul" odor; brown glue residues of the dead larvae forming a characteristic ropey thread when it is pulled out with a wooden stick or a match; and a hard scale of larval residues at the bottom of the cell. Traditional AFB diagnosis is based on the observation of these clinical symptoms in the beehive and on the microbial cultivation of material from infected colonies (De Graaf et al. 2006).

The resistance of *P. larvae* endospores is a main issue in the control and prevention of AFB because these endospores can survive for more than 35 years in honey and/or on the beekeeping material and resist high temperatures as well as the action of the most commonly used disinfectants (Bamrick 1967). Most treatments are based on the use of broad spectrum antibiotics, which, in most cases, have been used continuously and excessively. Indeed, different antibiotics, such as sulfathiazole and oxytetracycline hydrochloride (OTC), are able to inhibit the growth of *P. larvae*, but its use and abuse for decades led to the appearance of resistant strains and residues that contaminate beehive products. For these reasons, the use of antibiotics for AFB treatment and prevention is forbidden in several countries, and the affected colonies have to be destroyed by burning the hives (Mutinelli 2003). This action represents a drastic solution with a high cost for beekeepers, and furthermore, it cannot be used as a preventive method to combat AFB (Kochansky et al. 2001). In this context, the development of alternative and effective methods for the control and prevention of AFB disease is crucial. These methods should consider the evidence of bacterial-resistant phenomenon and comply with strict EU policies as well as with current trends of green consumerism (Lewis and Ausubel 2006). In the present review, the research studies focused on the development of natural strategies for the control and prevention of AFB carried out so far are summarized, including the use of essential oils, plant extracts, propolis, royal jelly, nonconventional natural molecules, bacteria, and bacteriocines for the aimed purpose.

## 2. NATURAL PRODUCTS FOR THE PREVENTION AND CONTROL OF AMERICAN FOULBROOD

### 2.1. Essential oils

Essential oils (EOs) are natural volatile and complex products obtained as secondary metabolites of aromatic plants, having different biological effects (anticancer, anti-inflammatory, insect repellent, antimicrobial, antiviral, and antioxidant). Since EOs affect many targets at the same time, no cases of resistance or adaptation have been detected (Adorjan and Buchbauer 2010). Several EOs have been evaluated for the in vitro and in vivo control of *P. larvae*, as well as their acute oral toxicity to *Apis mellifera* (Tables I, II, and III in the Electronic supplementary material).

#### *2.1.1. In vitro assays to control P. larvae*

EOs from *Achyrocline satureioides*, *Carum carvi*, *Cinamomum* sp., *Cinnamomum zeylanicum*, *Citrus paradise*, *Cuminum cyminum*, *Cymbopogon citratus*, *Eucalyptus cinerea*, *Melaleuca alternifolia*, *Mentha*

*piperita*, *Minthostachys verticillata*, *Origanum majorana*, *Origanum vulgare*, *Polygonum bistorta*, *Salvia officinalis* and *Salvia sclarea*, *Syzygium aromaticum*, *Tagetes minuta*, *Thymus vulgaris*, *Verbena*, *Pimenta dioica* (L.) Merr., *Litsea cubeba* Pers., *Trachyspermum ammi* L., *Mentha arvensis* L., *Mentha spicata* L., *Illicium verum* Hook.f, *Myristica fragrans* Gronov., *Cinnamomum camphora* (L.) J. Presl., *Ocimum tenuiflorum* L., *Daucus carota* L., *Zingiber officinale* Rosc., and *Pelargonium graveolens* L., were able to inhibit the growth of *P. larvae*, as evinced by the agar diffusion technique (Floris et al. 1996; González and Marioli 2010; Roussenova 2011; Cecotti et al. 2012; Kuzyšinová et al. 2014; Ansari et al. 2015). EOs from *Cimbopogon citratus*, *Cinnamomum aromaticum*, *Citrus reticulate* var. *madurensis*, *Citrus paradisi*, *Heterothalamus alienus*, *M. alternifolia*, *M. piperita*, *O. majorana*, *O. vulgare*, *S. sclarea*, *S. aromaticum*, *T. minuta T. vulgaris*, as well as the mixtures of *C. citratus* and *T. vulgaris* EOs (20:80, *v*/*v*), and *C. citratus*, *T. vulgaris*, *Satureja hortensis*, *O. vulgare*, and *Ocimum basilicum* EOs (5:11:21:26:37, *v*/*v*/*v*/*v*/*v*) also showed antibacterial activity against *P. larvae* by the agar dilution technique (Alippi 1996; Alippi et al. 2001; Roussenova 2011). EOs from *Citrus sinensis*, *Cinamomum* spp., *C. cyminum*, *Eugenia* spp., *T. vulgaris*, *Verberna* spp., *Acantholippia seriphioides*, *C. zeylanicum*, *H. alienus* Spreng., *Pimpinella anisum*, *Foeniculum vulgare*, and *Eucalyptus globulosus*, and the mixture of *T. vulgaris* EO, thymol and *Cinamomum zeylanicum* EO (62.5:25:12.5, *v*/*v*/*v*) exhibited antibacterial activity against *P. larvae* by the broth macrodilution technique (Floris et al. 1996; Fuselli et al. 2005, 2006a, b; Ruffinengo et al. 2006; Gende et al. 2008a, b, 2009b, 2010b). *Cinamomum* spp. EO also showed high sporicidal activity (Floris et al. 1996). The mixture of *T. vulgaris* EO, thymol, and *Cinnamomum zeylanicum* EO (62.5:25:12.5, *v*/*v*/*v*) presented lower minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) than the pure EOs (Fuselli et al. 2006a). Gende et al. (2010b) attributed *E. globulosus* EO antimicrobial activity to limonene, one of its constituents (Gende et al. 2010b). EOs from *Artemisia absinthium*, *Artemisia annua*, *Lepechinia floribunda*, *C. paradisi*, *Cymbopogon nardus*, *Melaleuca viridiflora*, *C. zeylanicum*, *Rosmarinus officinalis*, *Carapa guianensis*, *Copaifera officinalis*, *M. alternifolia*, *Mentha* aff. *arvensis*, *Mentha* aff. *rotundifolia*, *P. dioica* (L.) Merr., *L. cubeba* Pers., *T. ammi* L., *M. arvensis* L., *M. spicata* L., *I. verum* Hook.f, *M. fragrans* Gronov., *C. camphora* (L.) J. Presl., *O. tenuiflorum* L., *D. carota* L., *Z. officinale* Rosc., and *P. graveolens* L. showed antibacterial activity against *P. larvae* by the broth microdilution technique (Fuselli et al. 2007, 2008a, b, 2009, 2010; Gende et al. 2010a, 2014; Maggi et al. 2011; Santos et al. 2012, 2014; Ansari et al. 2015; de Almeida Vaucher et al. 2015). The antimicrobial activities against *P. larvae* of the EOs of *M.* aff. *arvensis* and *M.* aff. *rotundifolia* from Argentina were assigned to some of their constituents, such as menthol, menthone, menthofuran, and piperitone oxide (Gende et al. 2014).

#### *2.1.2. Toxicity assays on A. mellifera*

*C. sinensis*, *Cinamomum* spp., *C. zeylanicum*, *C. cyminum*, *Eugenia* spp., *T. vulgaris*, and *Verbena* EOs were nontoxic for adult honey bees when they were fed with candy and the EO at different concentrations by systemic administration (Floris et al. 1996; Gende et al. 2009b). *C. citratus*, *T. vulgaris* and *O. basilicum* EOs, as well as *C. citratus* and *T. vulgaris* EO mixture (50:50, *v*/*v*) were moderately toxic to adult honey bees. However, the *C. citratus*, *T. vulgaris*, and *Coriandrum sativum* EO mixture (33.3:33.3:33.3, *v*/*v*/*v*) presented negative mortality curves, meaning that there was less mortality at high doses. This fact disclosed that bees did not consume candy with high quantities of *C. sativum* EO (Albo et al. 2008). When a solution containing a certain amount of EO was sprayed over a group of honey bees, *T. minuta*, *C. guianensis*, and *C. officinalis* EOs resulted to be nontoxic for adult bees (Eguaras et al. 2005; Santos et al. 2012); whereas *M. alternifolia* EO caused the death of the bees after 7 days of treatment. Nevertheless, the use of nanoparticles of

*M. alternifolia* EO did not produce any toxic effect on honey bees (Santos et al. 2014). *Eucalyptus globosus* and *R. officinalis* EOs and the nanoemulsion of *Carapa officinalis* EO were not toxic for adult worker honey bees when they were completely exposed to the EO, i.e., bees were in contact with the EO and ingested the EO (Gende et al. 2010a; Maggi et al. 2011; de Almeida Vaucher et al. 2015). The nanoemulsion of *C. guianensis* EO exhibited a toxic effect for larvae and adult honey bees, whereas the nanoemulsion of *C. officinalis* EO, a low toxic effect on larvae (de Almeida Vaucher et al. 2015).

#### 2.1.3. In vivo assays to control American foulbrood

The treatment of artificially infected nuclei of honey bee colonies with *C. zeylanicum* EO by systemic administration prevented and controlled AFB (Floris et al. 1996; Gende et al. 2009a). *T. vulgaris*, *C. citratus*, *O. vulgare*, and *S. hortensis* EOs were tested individually and as mixtures on artificially infected nuclei, even though AFB was neither controlled nor prevented by these treatments (Albo et al. 2001, 2003).

### 2.2. Plant extracts

Several plant extracts have been evaluated for the in vitro control of *P. larvae* and their acute oral toxicity to honey bees (Tables IV and V in the Electronic supplementary material).

#### 2.2.1. In vitro assays to control P. larvae

The antimicrobial activity of different extracts of *Flourensia riparia*, *Flourensia fiebrigii*, and *Flourensia tortuosa* against *P. larvae* were studied by the agar diffusion technique. The chloroform, ethyl ether, and hexane extracts at different concentrations (100 to 50000 μg $mL^{-1}$) showed inhibitory effect by this technique (Reyes et al. 2013). Dichloromethane and methanol extracts from the inflorescences of various species of *Hypericum* genus (100 μg of dry extracts) also presented antibacterial activity against *P. larvae*. When applying as little as 25 μg of these extracts, the inhibition halo diameters were comparable with those achieved when applying 1000 μg of lactic acid (positive control) (Hernández-López et al. 2014). The antimicrobial activities of *Azadirachta indica* (Neem) and *Vitex trifolia* (Barbaka) crude aqueous extracts (20, 40, and 60 mg $mL^{-1}$ $disk^{-1}$) against *P. larvae* resulted to be dose dependent, with the larger inhibition zones corresponding to Barbaka plant (Anjum et al. 2015). *F. riparia* ethyl ether and chloroform extracts as well as the *F. fiebrigii* ethyl ether extract inhibited *P. larvae* by the agar dilution technique (Reyes et al. 2013). Water extracts of *A. satureioides*, *Chenopodium ambrosioide*, *E. cinerea*, *Gnaphalium gaudichaudianum*, *Lippia turbinata*, *Marrubium vulgare*, *M. verticillata*, and *T. minuta* also inhibited the growth of *P. larvae* by this technique (González and Marioli 2010). The MIC values of the hexane extract of *A. satureioides* obtained by the broth microdilution and the agar dilution techniques varied from 16 to 125 μg $mL^{-1}$ (Sabaté et al. 2012). In another study, the hexane, benzene, ethy ether, and ethyl acetate extracts of *A. satureioides* yielded MIC values of 60, 131, 773, and 6545 μg $mL^{-1}$, respectively, by the broth microdilution method (González et al. 2015). The MIC of the *Melia azedarach* ethanolic extract determined by the broth macrodilution technique was 5000 μg $mL^{-1}$ (Gende et al. 2008b). The dichloromethane, ethyl acetate, *n*-butanol fractions, and the crude extract obtained with ethanol-water (7:3, *v*/*v*) of *Scutia buxifolia* (Boligon et al. 2013); the methanol-dichloromethane (1:1, *v*/*v*) extracts of *Humulus lupulus* and *Myrtus communi* (Flesar et al. 2010); the *Calendula officinalis* and *Nasturtium officinale* crude extracts obtained with ethanol-water (7:3, *v*/*v*); the ethanol-water (8:2, *v*/*v*) extract of *Laurus nobilis* (Damiani et al. 2014); and the *Cariniana domestica* *n*-butanol and ethyl acetate fractions (Piana et al. 2015) caused the inhibition of *P. larvae* growth by the agar dilution method.

#### 2.2.2. Toxicity assays on A. mellifera

*H. lupulus* and *M. communis* methanol-dichloromethane extracts, evaporated to dryness and solubilized in DMSO 5% (*v*/*v*), were not

toxic at concentrations as high as 100 μg bee$^{-1}$ by systemic administration (Flesar et al. 2010). *S. buxifolia* crude extract obtained with ethanol-water (7:3, *v*/*v*) and their *n*-butanol, ethyl acetate, and dichloromethane fractions, re-dissolved in DMSO to reach final concentrations of 50, 25, 6.25, and 1.56 mg mL$^{-1}$, respectively, were tested by spraying honey bees. No toxic effects for the bees were detected after 15 days of observation (Boligon et al. 2013). Similarly, no toxicity was observed for *N. officinale* crude extract and *C. domestica n*-butanol fraction re-dissolved in DMSO during the 15 days of treatment (Piana et al. 2015). However, some deaths of honey bees occurred during the first 3 days of treatment with *C. officinalis* and *C. domestica* ethyl acetate fractions. Complete exposure was implemented to analyze the effect of the chloroform and ethyl ether extracts from *F. riparia* and the ethyl ether extracts from *F. fiebrigii* on adult honey bees. These extracts, re-dissolved in ethanol at 96% (*v*/*v*), did not show lethal effects on exposed bees during in vitro conditions even at the highest concentration assayed (125 mg mL$^{-1}$) (Reyes et al. 2013).

## 2.3. Propolis

Propolis is a natural product derived from plant resins, produced by honey bees to seal the walls and entrance of the hive, and contributes to protect the colony against different pathogens. It is made of a complex mixture of different chemical compounds (from 80 to 300); the main components having been identified as polyphenols (flavonoids and phenolic acids), terpenoids, steroids, and aminoacids, whose concentrations vary according to the geographical and botanical origin of the propolis (Burdock 1998). Several biological properties such as antioxidant (da Silva et al. 2006), antimicrobial (Boonsai et al. 2014), antifungal (Kujumgiev et al. 1999), antiviral (Manolova et al. 1985), hepatoprotective, immunomodulatory, and anti-inflammatory (Burdock 1998) have been ascribed to propolis. In this sense, several papers account for the use of propolis from different geographical origins as antimicrobials against *P. larvae* to control AFB on *A. mellifera* (Table VI, VII, and VIII in the Electronic supplementary material).

### 2.3.1. *In vitro assays to control P. larvae*

Mlagan and Sulimanovic (1982) tested propolis from Yugoslavia and found that 5% (*v*/*v*) and 10% (*v*/*v*) propolis extracts inhibited the growth of *P. larvae* by the agar diffusion technique. Ethanolic extracts of propolis from Uruguayan apiaries also showed inhibition by this technique (Antúnez et al. 2008). Brazilian propolis from *Baccharis dracunculifolia* and *Vernonia polyanthes*, and North American propolis from *Populus* spp. presented activity as well. These propolis had similar effects to vancomycin, but a much lower amount of antibiotic (30 μg) than of propolis (9 mg on average) was required (Bastos et al. 2008). The ethanolic extracts of propolis from Romania (at 10 mg mL$^{-1}$) also inhibited the growth of *P. larvae* by this technique; this activity was attributed to flavones, flavonols, flavanones, and dihydroflavonols (Mihai et al. 2012). Bíliková et al. (2013) studied Bulgarian poplar propolis and their crude ethanolic extract as well as their petrol ether fraction and ethyl acetate fraction by the agar diffusion and the broth microdilution techniques. In this case, 100 μg of propolis extract was necessary to cause the same effect as 10 mg of OTC. Moreover, five individual components and a mixture of caffeates isolated from that propolis also showed antimicrobial activity, with pinocembrin and pinobanksin-3-*O*-acetate being the components with the highest antimicrobial effect (Bíliková et al. 2013). Boonsai et al. (2014) studied the methanol, dichloromethane, and hexane extracts of propolis from Thailand by the same techniques; only the methanolic extracts (100 mg mL$^{-1}$) exhibited antimicrobial activity, which was attributed to cardanol (a phenolic compound of the Anacardiaceae family). Lindenfelser (1968) examined the activity against *P. larvae* of 15 propolis samples from the USA and determined their MICs by the broth macrodilution technique. All samples showed similar levels of bactericidal activity at concentrations smaller than 10 μg mL$^{-1}$. As well, MICs of Uruguayan propolis were estimated by this technique, ranging between 0.32%

(*v*/*v*) and 0.64% (*v*/*v*) (Antúnez et al. 2008). Most ethanolic extracts of propolis samples collected in apiaries from different USA regions were found to inhibit *P. larvae* growth in a dose-dependent manner by the broth microdilution technique (Wilson et al. 2015).

#### 2.3.2. Toxicity assays on A. mellifera

The acute oral toxicity of several propolis on adult honey bees has been studied by the systemic administration technique (Table VII in the Electronic supplementary material). The ethanolic extract of propolis from Uruguay was not toxic for adult honey bees at least at 50% (*w*/*v*) (Antúnez et al. 2008). In contrast, the ethanolic extract of propolis from Egyptian old wax comb ($LC_{50}$ = 1.404) were found to be more toxic than Egyptian ($LC_{50}$ = 8.223) and Chinese propolis extracts ($LC_{50}$ = 15.047) (Kamel et al. 2013).

#### 2.3.3. In vivo assays to control American foulbrood

Field studies have been conducted using the systemic administration and the spraying procedure (Table VIII in the Electronic supplementary material). Lindenfelser (1968) showed that, despite their proven in vitro antimicrobial activities against *P. larvae*, North American propolis were not effective on naturally infected colonies. When bees were fed with propolis diluted in honey at a concentration of 500 μg $mL^{-1}$ (systemic administration), new infection was not detected while the treatment was still in progress; however, when treatment was discontinued, the disease reappeared. No toxic effects were noted on the adult bees of this colony, even though many deformed young bees emerged (Lindenfelser 1968). The efficiency of Egyptian, Chinese, and old wax comb Egyptian propolis extracts to control AFB on artificially infected bee colonies was evaluated using hybrid carniolan race colonies. The addition of 0.05% (*w*/*v*) of Egyptian propolis to the feed had a highly significant positive influence on controlling the growth of *P. larvae,* resulting in 100% bacterial reduction, as it happened when tylosin was used (200 mg). The activity of Chinese and old wax comb Egyptian propolis extracts at 0.1, 0.05, and 0.025% (*w*/*v*) were significantly different when compared with untreated colonies (Kamel et al. 2013). In another case, colonies from an apiary in Uruguay that had presented AFB on previous years (but with no clinical symptoms) were treated by aspersion and by feeding with propolis ethanolic extracts (6%, *v*/*v*). After 21 and 42 days of the application of the treatments, the number of *P. larvae* spores per gram of honey was significantly lower in the colonies treated with propolis ethanolic extracts than in the untreated colonies (Antúnez et al. 2008).

### 2.4. Royal jelly

Royal jelly (RJ) or larval jelly (LJ) is the larval diet of queen larvae and worker larvae secreted by the hypopharyngeal and mandibular glands of worker honey bees. The main components of jellies are carbohydrates, peptides, proteins, fat, and low molecular weight compounds (Bogdanov 2011). Low molecular weight proteins and peptides of RJ seem to play a host-defense role against honey bee pathogens (Bíliková et al. 2001). The first antibiotic compound identified in RJ was the fatty acid 10-hydroxi-Δ2-decenoic acid. Afterwards, a potent antibacterial protein, named royalisin (currently called defensin1), found in RJ was also described. Royalisin has specific activity against various gram-positive bacteria at low concentrations but not against gram-negative bacteria (Fujiwara et al. 1990). Antibacterial (Nascimento et al. 2015), immunoregulation (Sugiyama et al. 2012), antitumoral (Kimura 2008), anti-inflamatory (Kohno et al. 2004), reproprotective action (Jalali et al. 2015), antiviral (Hashemipour et al. 2014), and antioxidant (Pavel et al. 2014) activities have been attributed to RJ. In particular, the in vitro antimicrobial activity of RJ from different geographical origins against *P. larvae* has been also reported in literature (Table IX in the Electronic supplementary material). The use of RJ to control *P. larvae* growth was first observed by Hornitzky et al. (1998). Later, it was reported that purified royalisin also had inhibitory effect against the pathogen by the agar diffusion technique, even at the lowest tested concentration (5.4 μg $mL^{-1}$) (Bíliková et al. 2001). Other proteins found in RJ

were identified as apalbumins. The most abundant ones, apalbumin-2 and apalbumin-2a, were purified, and their activity against *P. larvae* was evaluated in liquid cultures. Apalbumin-2a 18.6 μM inhibited its growth at a similar rate than OTC, while apalbumin-2 did not present antibacterial activity at all (Bíliková et al. 2009). The analyses of RJs collected from individual colonies at two apiaries, one of which having shown incidence of AFB, revealed differences in the content of the antibacterial peptide. Most colonies from the apiary with AFB produced RJs with higher amounts of royalisin than the colonies from the healthy apiary. These results suggested that the differences in the peptide contents are related to genetic variability among colonies. However, it is also possible that the presence of *P. larvae* could have affected the content of royalisin in the RJs (Bachanová et al. 2002).

### 2.5. Nonconventional natural molecules

This heterogeneous group includes a number of compounds which share one common characteristic: they are pure natural substances, either commercial, such as fatty acids or that have 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 chalkbrood mummies (*A. mellifera* larvae infected by *Ascosphaera apis* fungus). This fact led scientists to seek the identity of the active compound. Several studies on the evaluation of nonconventional natural molecules used for the in vitro control of *P. larvae* and their toxicity on honey bees have been published (Tables X and XI in the Electronic supplementary material).

#### *2.5.1. In vitro assays to control P. larvae*

Lokvam and Braddock (1999) performed agar diffusion bioassays to determine the antimicrobial activity of resin from both sexes of *Clusia grandiflora*, a native species from South America. These resins showed pronounced inhibitory activity against *P. larvae*. The female plant resin inhibition zones were higher than those produced by the male plant resin. Chamone I (benzophenone isolated from the trunk latex of *C. grandiflora* Splitg. (Clusiaceae)) and nemorosone II (benzophenone isolated from the pollinator reward resin of the female flowers of the same plant) demonstrated to have a strong inhibitory effect on *P. larvae* (Lokvam et al. 2000). Linoleic acid (isolated from mycelia and spores of *A. apis*) was shown to be active against *P. larvae* at concentrations down to 2.5 μg disk$^{-1}$ (Feldlaufer et al. 1993a). Lauric and tridecanoic acids were found to be the most active saturated fatty acids against *P. larvae*, while palmitoleic and linoleic acids were the most active among the unsaturated ones. The introduction of a double bond or multiple double bonds seems to be necessary to maintain the antibiotic activity once the chain length of the fatty acid exceeds 14 carbons (Feldlaufer et al. 1993b). Fifteen fatty acids were proved to have antibacterial activity against *P. larvae*. The majority of the tested acids exhibited activity at the highest quantity tested (250 μg), except for myristoleic and lauric acids, which also showed reduced activity with diffusion disks containing 25 μg of the molecule (Hornitzky 2003). L-tenuazonic acid (isolated from *Alternaria raphani* and *Alternaria brassicicola* cultures) was proved to be a specific antibiotic against *P. larvae* (MIC of 1–5 μg disk$^{-1}$) by the agar diffusion technique, even though the MIC of OTC and gentamicine were lower (0.5 μg disk$^{-1}$) (Gallardo et al. 2004). By the agar dilution technique, L-tenuazonic acid yielded a MIC value of 32 μg mL$^{-1}$, similar to OTC (Gallardo et al. 2004). Exiguaflavanone K and 8-prenyldihydroisorhamnetin isolated from *F. riparia* showed antimicrobial activity against *P. larvae*, as well as 8-prenyleryodictiol and (2S)-8-(3″-methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone isolated from *F. fibrigii* (Reyes et al. 2013). The antimicrobial activity of six pure compounds isolated from species of the *Hypericum* genus: *Hypericum canariense*, *Hypericum drummondii*, *Hypericum mutilum*, and *Hypericum perforatum* on *P. larvae* vegetative cells were evaluated by the broth macrodilution technique (Hernández-López et al. 2014). Uliginosin B isolated from *H. mutilum*, 7-epiclusianone from *H. canariense*, and hyperforin from the medicinal species *H. perforatum* presented antimicrobial activity against *P. larvae*. Hyperforin was also tested on *P. larvae* spores. When using 10 μg of such compound, the CFU count of *P. larvae*

(4.40 CFU $mL^{-1}$) was lower than the negative control (114.6 CFU $mL^{-1}$) (Hernández-López et al. 2014). The antimicrobial activity against *P. larvae* of glycerol monolaurate and glycerol monolaurate nanocapsules were found to be significantly different, with MIC values being 62.2 and 142.8 μg $mL^{-1}$, respectively (Lopes et al. 2016). Commercial compounds of plant origin, such as sanguinarine, thymoquinone, capsaicin, nordihydroguaiaretic acid, and trans-2-hexenal, showed antimicrobial activity against *P. larvae* by the broth microdilution technique (Flesar et al. 2010).

#### 2.5.2. Toxicity assays on A. mellifera

The acute oral toxicity of capsaicin, nordihydroguaiaretic acid, sanguinarine, thymoquinone, and trans-2-hexenal was assessed on *A. mellifera* subsp. *carnica* by systemic administration. $LD_{50}$ values for capsaicin and thymoquinone were higher than 100 μg $bee^{-1}$, and for nordihydroguaiaretic acid and trans-2-hexenal, higher than 200 μg $bee^{-1}$. Therefore, these compounds were considered to be "virtually nontoxic." $LD_{50}$ value for sanguinarine was 153 μg $bee^{-1}$, which is above the potential therapeutic dose; hence, it poses no danger to honey bees (Flesar et al. 2010). Glycerol monolaurate resulted to be highly toxic on adult worker bees when tested at one- and twofold of the MIC. In contrast, glycerol monolaurate nanocapsules were proved to be virtually nontoxic when using the spraying procedure (Lopes et al. 2016).

### 2.6. Bacteria and bacteriocins

Probiotics are living microorganisms which, when administered in adequate amounts, confer a health benefit on the host. These microorganisms should fulfill a group of requirements, among others: ability to adhere to cells; exclude or reduce pathogenic adherence; persist and multiply; and produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth. Moreover, it is important that the microorganisms are safe, and therefore, noninvasive, noncarcinogenic, and nonpathogenic. A recommended alternative is to isolate microorganisms from the same host were they are applied (FAO/WHO 2006). Honey bees support a diverse microbial biome (Engel et al. 2012; Moran et al. 2012; Powell et al. 2014), which can be an important source for probiotics. Lactic acid bacteria (LAB) are usually found within bee guts and are known to protect their hosts via antimicrobial metabolites such as organic acids, hydrogen peroxide, and antimicrobial peptides, as well as modulation of host immune response (Vásquez et al. 2012). Many of these beneficial bacteria have been isolated from honey bee adults and larvae, and beehive products such as honey and pollen (Evans and Armstrong 2005, 2006; Yoshiyama et al. 2013; Jaouani et al. 2014). Bacteriocins are bacterial ribosomally synthesized, extracellular peptides, or proteins with an antibacterial activity usually against bacteria closely related to the producer (De Vuyst and Vandamme 1994; Riley and Wertz 2002). Bacteria and bacteriocins have also been studied for the control of *P. larvae* (Table XII in the Electronic supplementary material).

#### 2.6.1. In vitro assays to control P. larvae

Bacteria isolates, obtained from honey bee larvae or the gut of adult *A. mellifera*, belonging to the genera *Brevibacillus*, *Stenotrophomonas*, *Bacillus*, *Acinetobacter*, *Lactobacillus*, and *Bifidobacterium* showed antimicrobial activity against *P. larvae* by the agar diffusion technique (Evans and Armstrong 2006) (Forsgren et al. 2010). Many of these isolates were proved to produce surfactin, an antibiotic with antitumoral and antiviral action (Sabaté et al. 2009). Microorganisms (*Escherichia coli*, *Providencia alcalifaciens*, *Splingomonas melonis*, *Bacillus subtilis* and *Bacillus cereus, Enterococcus, Lactobacillus*, and *Weissella*) isolated from the midgut of *Apis cerana japonica* also exhibited inhibitory effects on *P. larvae* (Yoshiyama and Kimura 2009; Yoshiyama et al. 2013). Isolates of different species of aerobic spore-forming bacteria (ASFB) isolated from honey samples and brood combs presented antagonism with *P. larvae*. Ten of these ASFB strains were identified as *B. subtilis*, *Bacillus umilus*, *Bacillus licheniformis*, *Bacillus megaterium*, *B. cereus*, and *Brevibacillus laterosporus* (Alippi and Reynaldi 2006). Besides the activity of bacterial cells, cell-free supernatant of *Lactobacillus acidophilus*, *Lactobacillus*

*crispatas*, and *Lactobacillus jonsonii* obtained from the gut of worker honey bees also showed antimicrobial activity against *P. larvae* (Audisio et al. 2011). The antibacterial activity against *P. larvae* of both, the cell-free supernatant of *Bacillus thuringiensis* subsp. *entomocidus* and of entomocin 110 (bacteriocin isolated from the same species of *Bacillus*), was evaluated by the agar diffusion technique, showing inhibition of all 17 strains of *P. larvae* tested (Cherif et al. 2008). Regarding the cell-free supernatants extracts of 36 *Enterococcus* isolates, 20 of them showed strong inhibition, and the remaining 16 isolates caused a mild inhibition of *P. larvae* (Jaouani et al. 2014). Soil bacteria are also sources of microorganisms with antibacterial activity against *P. larvae*. Nguyen and Kim (2015a, b) proved the existence of a strong antimicrobial activity of *Bacillus polymachus* T515$^T$ and *Streptomyces polymachus* T258$^T$ on *P. larvae*. As well, an antimicrobial substance produced by *Bacillus amyloliquefaciens* (soil bacteria) also presented activity on vegetative cells and spores of *P. larvae* (Benitez et al. 2012).

#### 2.6.2. *Toxicity assays on A. mellifera and exposure bioassays*

Bacteria and bacteriocins toxicity on *A. mellifera* has been determined by the systemic administration method. Larval food was supplemented with crude and purified fractions of *B. amyloliquefaciens* antimicrobial factor (800 AU mL$^{-1}$). Honey bee larvae showed a mortality of 25.5%, while the mortality in the control group was 15% (Benitez et al. 2012). In the exposure bioassays, honey bee larvae were fed with a mixture of *Lactobacillus kunkeii*, *Lactobacillus* sp., *Bifidobacterium asteroids*, and *Bifidobacterium coryneforme*. The overall effect of adding the LAB mixture to the larval feed was a significant reduction in the number of infected larvae, irrespectively of the infective dose (Forsgren et al. 2010).

## 3. DISCUSSION

Taking into account the negative effects of *P. larvae* on honey bee colonies and the drawbacks of traditional methods to deal with AFB disease, the development of alternative and effective methods for its control and prevention has become mandatory. Among the natural strategies that have been developed during the last years, the use of EOs is a promising therapy. *Cinamomum zeylanicum* EO presented the best characteristics as natural product to control AFB, showing the highest antimicrobial activity, and being effective on AFB control on beehives and nontoxic for adult honey bees, even though toxicity on honey bee larvae has still to be tested. The nanoemulsion of *C. officinalis* EO also showed high antimicrobial activity against *P. larvae* and presented a low toxic effect on larvae; hence, it has been suggested as an alternative method to fight AFB. Besides, nanoemulsions can potentiate the antibacterial effect of EOs and protect against volatility. Enhancement of the treatment effectiveness may also be possible if EOs are used together with other active ingredients, such as individual natural compounds, plant extracts, other EOs or their main constituents. As well, propolis and RJ and its proteins, royalisin and apalbumin-2a, presented in vitro inhibitory effect against *P. larvae*; thus, future research should focus on the role of these substances in protecting honey bee larvae against *P. larvae* infection. Moreover, a large number of diverse nonconventional natural molecules have been reported to present significant growth-inhibitory action on *P. larvae* and low toxicity to bees. Among them, lauric acid and tridecanoic acid are potentially useful in suppressing AFB, being already commercially available for different purposes. Probiotics can also improve honey bee health, indeed several bacterial isolates obtained from honey bees or beehive products were able to inhibit in vitro the growth of *P. larvae*, and the oral administration of a mixture of lactic acid bacteria to the larval feed led to a significant reduction in the number of infected larvae.

Despite all this progress in alternative natural strategies to fight AFB disease, the fact that *P. larvae* is a spore-forming bacterium is the main limitation to any of the treatments proposed to the present against this bacterium due to the well-known resistance of their endospores. Some of the natural products studied for their antimicrobial activity against *P. larvae* have also shown sporicidal activity, such as *Cinamomum* spp. EO, some propolis ethanolic extracts, hyperforin and a

substance produced by *B. amyloliquefaciens*; however, none of them have achieved the eradication of AFB in honey bee colonies yet.

## 4. CONCLUSIONS

The research performed presently to study the in vitro and in vivo antimicrobial activity of natural products against *P. larvae*, as well as the toxicity of these natural products on adult honey bees, has been thoroughly reviewed throughout this paper. Regarding that honey bee larvae are the target of AFB disease, future research should focus on studying the effect of the natural compounds that are effective antimicrobials in vitro and nontoxic to adult honey bees on the honey bee larvae. Moreover, further studies on the distribution and effects of these natural products on beehive, adult honey bees, larvae, honey, RJ, and other beehive products are still necessary in order to understand pharmacokinetics and pharmacodynamics inside the beehive. As well, research on the effectiveness of these natural antimicrobials at field conditions is imperative. Moreover, further studies should be carried out on the sporicidal properties of these natural substances to destroy *P. larvae* spores for the prevention of AFB disease. And last but not least, the development of proper delivery modes of the natural products inside the beehives for the in vivo treatment and prevention of the illness is another important issue that requires further research, in order to put these natural strategies in practice under real beehive conditions.

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**Stratégies naturelles pour lutter contre *Paenibacillus larvae*, l'agent responsable de la loque américaine chez les abeilles : une synthèse**

***Apis mellifera* / agent pathogène / loque américaine / produit de lutte naturel**

**Natürliche Strategien zur Kontrolle von *Paenibacillus larvae*, dem Erreger der Amerikanischen Faulbrut bei Honigbienen. Ein Review.**

***Paenibacillus larvae* / Amerikanische Faulbrut / *Apis mellifera* / Naturprodukte**

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