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ORIGINAL PAPER

Synergistic effect of surfactin from *Bacillus subtilis* C4 and *Achyrocline satureioides* extracts on the viability of *Paenibacillus larvae*

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Abstract The aim of this work was to determine the in vitro effect of the mixture between the lipopeptide surfactin, synthesized by *Bacillus subtilis* C4 (strain isolated from honey) and the most active vegetal extract from *Achyrocline satureioides*, a traditional medicinal plant, on local strains of *Paenibacillus larvae*, the agent of American Foulbrood in honeybees. Five *P. larvae* strains isolated in Córdoba, Argentina, were phenotypically characterized. These and 12 other *P. larvae* strains from different regions of Argentina were analysed. The antimicrobial activities of the essential oil, hexane (HE) and benzene extracts from *A. satureioides* were assessed against *P. larvae* and the HE showed the highest anti-*P. larvae* activity. A combination of the biosurfactant surfactin, produced by *B. subtilis* C4, and the HE of *A. satureioides* revealed a synergistic action on *P. larvae*. The effective surfactin concentration in the mixture decreased from 32 to 1 μg ml^{-1} and the HE concentration from 32 to 4 μg ml^{-1}, values similar or equal to minimal inhibitory concentrations observed for oxytetracycline. The fractional inhibitory concentration index confirmed synergism in 4 strains and partial synergism in one strain. The combination of surfactin synthesized by *B. subtilis* C4 and the HE from *A. satureioides* could be a natural alternative to help beekeepers to combat the American foulbrood agent *P. larvae.*

Keywords Surfactin · *Achyrocline satureioides* · *Paenibacillus larvae* · Synergism · Antimicrobial

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Introduction

American Foulbrood (AFB) is a bacterial disease that affects *Apis mellifera* L. (the honeybee) during the larval or pupal stage, and is caused by *P. larvae* (*P. larvae* White), a flagellated bacterium, whose main characteristic is the formation of highly resistant endospores (Williams 2000). The infectious power of *P. larvae* is serious because it requires only 10 spores to make ill a larva of less than 24 h old (Bambrick 1964). The major problem in prevention and control of this disease is the resistance of its endospores, which can survive for more than 35 years in honey and/or beekeeping material and resist high temperatures and the action of the most commonly used disinfectants (Bambrick and Rothenbuhler 1961).

One of the main control measures in New Zealand and the United Kingdom is burning infected beehives. However, in Argentina, the USA and Canada, one of the most frequent control measures is the use of antibiotics, especially oxytetracycline (OTC), although resistant strains have been reported (Alippi 1996; Miyagi et al. 2000; Evans 2003).

Non-toxic, natural compounds can be an alternative to the use of antibiotics to control AFB and considerably diminish the resistance of pathogens as well as the amount of antibiotic residues in beehive products. Several studies have reported on natural compounds with antagonistic properties against *P. larvae* such as propolis (Antúnez et al. 2008; Bastos et al. 2008), essential oils and other derivate compounds from different plant species (Albo et al. 2003; Gende et al. 2009; Flesar et al. 2010). Inhibition of *P. larvae* by bacteria isolated from bees has also been reported (Evans and Armstrong 2005; Yoshiyama and Kimura 2009).

Our research group determined that surfactin, a biosurfactant synthesized by different *B. subtilis* strains isolated from honey and bee-gut samples, not only showed an anti-*P. larvae* effect (Sabaté et al. 2009), but also revealed inhibitory properties against *Nosema ceranae*, another important bee pathogen (Porrini et al. 2010). Also, Audisio et al. (2011) isolated lactic acid bacteria from bee-gut with an anti-*P. larvae* effect. González and Marioli (2010) determined that the essential oil and water extracts from *A. satureioides* (marcela, a Brazilian medicinal plant) presented an antagonistic effect against *P. larvae* strains. As this study was carried out with a reduced number of *P. larvae* strains, it was decided to isolate new strains of this pathogen from different geographical regions in Argentina and include two strains from the CIDEFI (Phytopathology Research Center) culture collection (La Plata, Argentina). It was also decided to obtain new *A. satureioides* extracts (liquid–liquid extraction with different organic solvents) and analyse the effect in vitro of a combination of the most active extract of *A. satureioides* and surfactin, synthesized by *B. subtilis* C4, on the viability of the AFB agent.

Materials and methods

Culture media and growth conditions for isolation of *P. larvae*

Samples were obtained from apiaries in the Río Cuarto department in Córdoba, Argentina (González 2005), exhibiting symptoms of American Foulbrood. Flakes and viscous material were extracted from the hives and suspended in tubes containing 5 ml of MYPGP (1.5% yeast extract, 1% Mueller-Hinton broth (Britania), 0.2% glucose, 0.3% K_2HPO_4 and 0.1% sodium pyruvate; pH 7.0). Tubes were first heated in a water bath (85–90°C) for 20 min to make the spores germinate and were then incubated at 37°C for 24 h under microaerophilic conditions. After, the culture was spread on MYPGP agar plates to isolate *P. larvae* colonies. The identification of this bee-pathogen was achieved by biochemical assaying: Gram-staining, catalase and Voges-Proskauer tests and hydrolysis of starch, gelatin and casein (de Vos et al. 2009). *P. larvae* strains were stored at −20°C on MYPGP agar with 20% (v/v) glycerol until further use.

P. larvae strains

Paenibacillus larvae strains (Azul, I, II, III, IV, 2, 3, 4, 5 and 6) were kindly provided by Dr. Terzolo and Eng. Borracci from INTA Balcarce, Argentina, and strains 7A (Buenos Aires) and 35A (Río Negro) were purchased from CIDEFI. The latter two (35A and 7A) were used as positive controls for the phenotypical analyses mentioned above. All strains were activated on MYPGP agar at 37°C for 72 h under microaerophilic conditions.

Surfactin

Surfactin, synthesized by *B. subtilis* C4 was obtained according to Sabaté et al. (2009). Briefly, the cell-free supernatant (CFS) was obtained from *B. subtilis* C4 cultured in BHI broth for 24 h at 37°C without shaking. This was centrifuged (10,000×*g* for 15 min at 4°C), filter sterilized using a 0.45 μm pore size cellulose acetate filter, and kept at 4°C until use. Precipitate of CFS resulting from the acidification with 55 μl of concentrated HCl was recovered by centrifuge (14,000×*g* for 25 min at 4°C). Lipopeptides were extracted with methanol according to Youssef et al. (2004). The solvent was evaporated and the precipitate containing the metabolite was dissolved in sterile distilled water (pH 9) for MIC determinations and in dimethyl sulfoxide (DMSO) for synergism assaying.

Achyrocline satureioides compounds: isolation and preliminary characterization

Essential oils (EO) were extracted from *A. satureioides* according to González and Marioli (2010). Recollected plant samples were dried between graphite paper sheets at 25°C. The aerial parts of the plant were macerated in a water/ethanol (1:1) mixture. The ethanol was eliminated from the macerate in a rotatory evaporator, and the hexane (HE) and benzene (BE) extracts were successively obtained by liquid–liquid extraction from the remaining water extract. The following preliminary studies were performed on the HE for phytochemical screening:

(a) tannins, the HE was suspended in water and 0.5 ml were treated with five drops of ethanolic $FeCl_3$ (0.1% w/w). A dark blue precipitate indicated presence of tannins (Harbone 1984). (b) flavonoids, the suspension was treated with concentrated HCl, Mg(s) and *n*-butanol. A positive result was indicated by a reddish coloration of the *n*-butanol (Geissman 1962).

Antibacterial activity of the *A. satureioides* substances

The broth microdilution technique according to Mann and Markham (1998) was used to determine the inhibitory effect of EO, HE and BE fractions on *P. larvae*. A cell suspension of each *P. larvae* strain was prepared at a cell concentration of 0.5 on the MacFarland scale. Microplates were poured with 170 µl of the bacterial inoculum and supplemented with 20 µl of each of the different samples to be assayed for antimicrobial activity. The same inoculum (170 µl) supplemented with 20 µl of the solvent without any substance, was used as positive control and 170 µl of the culture medium without inoculum supplemented with 20 µl of the different dilutions was used as negative control. The microplates were incubated microaerophilically at 37°C for 14–15 h and afterwards 10 µl of 0.01% resazurin were added and the plates were incubated again at 37°C for 2 h. Positive controls presented a pink color (bacterial growth) and negative controls a blue color (bacterial inhibition).

Minimal inhibitory concentrations (MICs) of oxytetracycline (OTC), surfactin and HE from *A. satureioides* against *P. larvae*

Two different methods were used for MIC assaying: the agar dilution and broth microdilution methods.

Agar dilution method

MYPGP agar was used as basal medium according to Alippi et al. (2007). OTC and surfactin concentrations were obtained from a stock solution (200 µg ml^{-1}) in sterile distilled water with a pH close to 9 in the case of surfactin. Different dilutions were made in 20 ml MYPGP agar, which was left to solidify in Petri dishes. Dilutions of plant extracts were made in DMSO. *P. larvae* cell suspensions were prepared as mentioned before and 10 µl of each suspension were seeded onto MYPGP agar, supplemented with the different compound concentrations. Inoculated plates were examined for growth after 24–48 h of incubation at 37°C. MYPGP agar without any compounds was used as the *P. larvae* growth control. The DMSO antimicrobial effect was also analysed against *P. larvae*.

Broth microdilution method

Serial dilutions of HE were carried out in a 0.15% agar-agar solution. The holes of a 96-well microplate were poured with 170 µl of the microbial inoculum and supplemented with 20 µl of the HE dilutions. Afterwards, the proceedings were as described before. MIC was defined as the lowest antibiotic concentration that avoided growth.

Checkerboard analysis of surfactin and HE and FIC index analysis

Synergism was assayed with the Checkerboard test (White et al. 1996), using surfactin and HE concentrations above and below the MIC values obtained before: between 1 and 64 µg ml^{-1} for surfactin and between 4 and 256 µg ml^{-1} for HE. Dilutions of both substances were made in DMSO. Different combinations of both substances with the corresponding controls were made on a microtiter plate, the effect on *P. larvae* viability being assessed using both the broth microdilution method as described before and disk diffusion (Audisio et al. 2005). With the latter technique, 10 ml of molten 1.5% MYPGP agar were inoculated with 300 µl of a *P. larvae* cell suspension, the mixture was then poured onto Petri dishes. The agar suspension was left to cool and solidify and wells were punched in the *P. larvae* lawn (0.5 on the MacFarland scale). Twenty-three µl of each combination were poured into each hole and after incubation at 37°C for 48 h, the plates were examined for the presence of inhibition halos.

MIC values of each individual agent in the mixtures were obtained from both assays.

Synergy was determined by calculating the fractional inhibitory concentration (FIC) index (Eliopoulos and Moellering 1996) as follows:

$$\Sigma\text{FIC index} = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$$

where [A] is the lowest concentration of substance A in the mixture that inhibited the pathogen, MIC_A the Minimum Inhibitory Concentration for the organism of substance A alone and the FIC_A is the FIC of substance A. [B], MIC_B, and FIC_B are the lowest concentration, the MIC and FIC for substance B, respectively. The FIC index was interpreted according to Kumar et al. (2004) as follows: a FIC <0.5 was defined as synergy, from 0.5 to 0.75 partial synergy, from 0.76 to 1.0 additive effect, from 1.0 to 4.0 indifference and >4.0 antagonism. Chi-square analysis was performed to determine differing synergy rates among the natural compounds.

Isobolographic analysis of the interaction of both substances against *P. larvae*

Results of the Checkerboard assay are given in isobolograms (Hall et al. 1983; Krogstad and Moellering 1986). The

intervening points are formed by a pair of individual FIC values of the different combinations generated. If the two agents have additive antimicrobial activity, the line connecting the x- and y-intercepts and the intervening points (the isobol) will be straight. If the two agents have synergistic antimicrobial activity, the FIC values of each agent will be lower and the isobol will be concave. For factors that are antagonistic in combination, the isobol will be convex.

Results

Isolation of local *P. larvae* strains

Five strains were isolated from apiaries with AFB symptoms in Río Cuarto, Córdoba (Argentina). From the colonies isolated, those presenting typical *P. larvae* morphology, i.e. a whitish, somewhat transparent and brilliant appearance, were selected (Table 1). All selected isolates were Gram-positive and the Voges Proskauer and catalase assays gave negative results. The hydrolysis of starch was negative, but positive with gelatin and casein. The strains 7A (Buenos Aires) and 35A (Río Negro) from CIDEFI were used as positive control for the tests.

A. satureioides substances: preliminary characterization and antimicrobial effects

The preliminary analyses showed that flavonoids and tannins may be present in the HE of *A. satureioides*. Also, the total phenolic content of this extract was 47 mg gallic acid equivalent to 100 g of plant material. The analysis of antagonistic activity of EO, HE and BE fractions from *A. satureioides* against different *P. larvae* strains revealed that the lowest dose of BE that inhibited the pathogen (strains IV, 2 and 3) was 63 μg ml^{-1}, while the lowest EO concentration was 5,700 μg ml^{-1} with antagonistic activity against *P. larvae* IV, 2, 10 and 11. Inhibition by HE (*P. larvae* IV) was observed at a concentration of 16 μg ml^{-1}. Then, HE was selected for synergism analysis as inhibition occurred at a lower concentration than with BE and EO fractions assayed.

MIC values of the antibiotic OTC, surfactin and plant substances

Two different techniques were used to determine MIC values: the disk diffusion and the broth microdilution method. The latter was chosen, because it is useful when working with small volumes, as was the case with the EO, HE and BE fractions. However, no significant differences were observed between either techniques. Table 1 shows the effect of the different fractions against the *P. larvae* strains. OTC was assayed in order to determine sensibility or resistance of the strains to this antibiotic and to compare its inhibitory dose with the effective dose of the natural substances examined in this study. With OTC, 77% of the strains showed a MIC of 0.5 μg ml^{-1} and the remaining strains 1 μg ml^{-1}. MIC values for surfactin and HE were higher than that for OTC: MICs for surfactin against different *Paenibacillus* strains were 32 μg ml^{-1} and for HE the MIC varied from 16 to 125 μg ml^{-1}.

Table 1 MICs of different antimicrobial agents against strains of *P. larvae* by the agar dilution method

Strains	Origin	MIC of OTC (μg ml^{-1})	MIC of surfactin (μg ml^{-1})	MIC of HE (μg ml^{-1})
P. larvae 2	INTA	0.5	32	63
P. larvae 3	INTA	0.5	32	32
P. larvae 4	INTA	0.5	32	125
P. larvae 5	INTA	0.5	32	63
P. larvae 6	INTA	0.5	32	63
P. larvae 7	Río Cuarto-Córdoba	0.5	32	16
P. larvae 8	Río Cuarto-Córdoba	0.5	32	125
P. larvae 9	Río Cuarto-Córdoba	0.5	32	32
P. larvae 10	Río Cuarto-Córdoba	0.5	32	63
P. larvae 11	Río Cuarto-Córdoba	0.5	32	63
P. larvae I	INTA	1	32	16
P. larvae II	INTA	1	32	32
P. larvae III	INTA	1	32	32
P. larvae IV	INTA	1	32	16
P. larvae azul	INTA	0.5	32	32
P. larvae 7A	Buenos Aires	0.5	32	32
P. larvae 35A	Río Negro	0.5	32	32

Synergism analysis

The following *P. larvae* strains were chosen for the synergistic studies: 3, 7, III, 7A and 35A. This selection was done as all the strains showed similar results and consists of at least one strain from the different regions analysed. It is important to highlight that DMSO, which was used as the compound solvent, did not affect *P. larvae* viability *per se*. A combination of surfactin and HE revealed a decrease in the MIC from 32 to 1 μg ml^{-1} for 4 of the 5 strains assayed with HE concentrations varying between 16 and 4 μg ml^{-1} (Table 2). It is important to highlight that, due to the combination with the plant extract, the surfactin dose could be lowered close or equal to the MIC values observed with OTC to inhibit *P. larvae* growth. The HE concentration could also be reduced significantly by combining it with surfactin: the MIC became 4 μg ml^{-1} when combined with 1 or 2 μg ml^{-1} of surfactin, except for *P. larvae* III, which needed higher concentrations.

In order to analyse the synergistic effect of the two antimicrobial agents in the mixture it was necessary to calculate the FIC index: $\Sigma FIC\ index = FIC_A + FIC_B$. Calculating the FIC index determined that synergism was observed in 4 of the 5 strains assayed, with values of 0.16, 0.19, 0.28 and 0.37; partial synergism was found for the remaining strain with a FIC index of 0.56 (Table 3). These results were represented graphically by isobolograms (Fig. 1), which corresponded to the individual FIC of each agent in the mixture. A concave curve can be observed in all cases, and this proves a synergistic effect of the mixture of the different concentrations assayed. These results reveal the importance of combining different substances. As in this case, the combination of surfactin and a HE from *A. satureioides* allowed the inhibition of the pathogen at lower concentrations of the individual MIC of each agent and close or equal to conventional antibiotics.

Table 2 Individual and mixed MICs of the antimicrobials on *P. larvae*

Strain	Individual MIC (μg ml^{-1})		Mix MIC (μg ml^{-1})	
	Surfactin	HE	Surfactin[a]	HE[b]
P. larvae 3	32	32	1	4
P. larvae 7	32	16	1	4
P. larvae III	32	32	2	16
P. larvae 7A	32	32	1	4
P. larvae 35A	32	32	1	4

[a] MIC of surfactin combined with concentrations between 4 and 16 μg ml^{-1} of HE

[b] MIC of HE combined with concentrations between 1 and 2 μg ml^{-1} of surfactin

Table 3 FIC index

Strains	FIC index of[a] surfactin-HE
P larvae 3	0.19
P larvae 7	0.28
P larvae III	0.56
P larvae 7A	0.37
P larvae 35A	0.16

[a] FIC index < 0.5 synergism; 0.5–0.75 partial synergism; 0.76–1 additive; between 1 and 4 indifferents; >4 antagonism

Discussion

Actually, there are no standards in the NCCLS or CLSI guidelines to establish cut-off points to define antibiotic resistance of *P. larvae*, the American foulbrood agent. Alippi et al. (2007) suggested considering a *P. larvae* strain susceptible to tetracycline when MIC < 4 μg ml^{-1}, intermediate for MIC values between 4 and 8 μg ml^{-1} and resistant for MIC > 16 μg ml^{-1}, as a standard. This parameter could be extended to another related antibiotic such as oxytetracycline (OTC), which is used in Argentina to control AFB. Thus, taking into account that criteria for OTC, in this work, the susceptibility of different *P. larvae* strains was assayed and the results revealed sensitivity in all cases, with MICs ≤ 1 μg ml^{-1}.

It is interesting to say that, even though there are no Maximum Residue Limits for tetracyclines in honey according to regulations issued by the European Community (Mutinelli 2003), OTC-resistant *P. larvae* strains have been identified in Argentina (Alippi 1996; Reynaldi et al. 2008). The Argentine Republic is one of the world's most important honey producers and exporters (Finola et al. 2007; Vázquez et al. 2009) and the abuse of antibiotics by beekeepers has generated important commercial problems. This situation needs an alternative and effective control of the disease with therapeutics or prophylactic feed additives that do not contribute to the phenomenon of bacterial resistance.

Different natural extracts such as essential oils from medicinal plants, herbs and spices have beenshown to possess antimicrobial properties and could serve as a source for antimicrobial agents against different pathogens (Bagamboula et al. 2003; Dorman and Deans 2000; Zaika 1988). Particularly, essential oils and their components are known to be active against a wide variety of microorganisms, including Gram-negative bacteria (Helander et al. 1998; Sivropoulou et al. 1996). In this study, the effect of different concentrations of surfactin and hexane extract (HE) of *A. satureioides* on viability of *P. larvae* was analysed with both the agar dilution and broth microdilution methods and no significant differences were observed

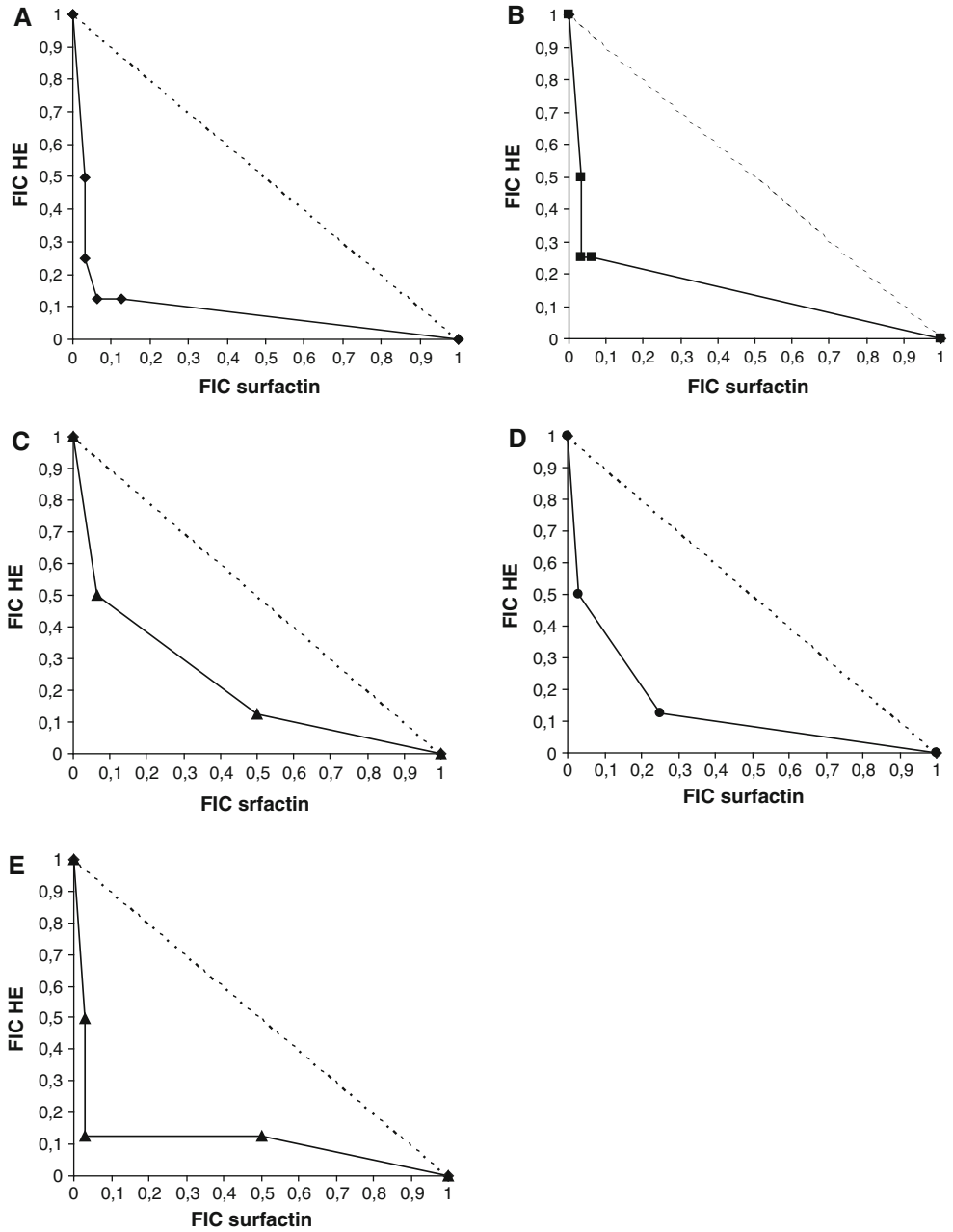

Fig. 1 Isobolograms showing additive interactions between surfactin and HE tested in pairs. Data are the fractional inhibitory concentrations (FICs) of the 2 factors in combination. **a** *P. larvae* 3; **b** *P. larvae* 7; **c** *P. larvae* III; **d** *P. larvae* 7A and **e** *P. larvae* 35A

between either techniques. The HE of *A. satureoides* was effective against *P. larvae* within 16 and 125 μg ml^{-1}. It is interesting to observe that Flesar et al. (2010) studied the effect of 19 crude extracts from different plant against *P. larvae*, and even though none were *A. satureoides*, similar effects were found with eight extracts. *Laurus nobilis* L., *Rosmarinus officinalis* L., *Capparis spinosa* L., *Catha edulis*, *Eucalyptus citriodora*, *Zingiber officinale*, *Bixa orellana* L. and *Curcuma longa* L. were analysed and only three were effective at lower doses (MIC 2–8 μg ml−1) and the rest had activity at higher concentrations (MIC 128–256 and >256 μg ml−1).

On the other hand, the effective dose of the surfactin to inhibit the pathogen was 32 μg ml^{-1}. To date, there have been no studies about an effective dose neither of surfactin nor *A. satureioides* extracts against this pathogen and hence, this study will be the first in which these parameters have been determined against the *P. larvae* strains assayed. It could be interesting to evaluate the response of OTC-resistant *P. larvae* strains to surfactin and to HE; however,

these strains were not available for this study, and we could only obtain and analyse antibiotic-sensitive strains.

It is already known that flavonoids such as quercetin and iso-quercetin, among others, are present in the *Achyrocline* genus (Broussalis et al. 1989; de Souza et al. 2002). Also, prenylated compounds with biological activity were isolated from *A. satureioides* extracts (Carney et al. 2002). In this paper, it was qualitatively determined that the presence of flavonoids and tannins in the hexane extracts (HE), compounds could be involved in the anti-*P. larvae* effect.

There is scientific information about synergistic mixtures of natural products and antibiotics with a strong inhibitory effect against different pathogens (Rodrigues et al. 2009; Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009). In order to reduce both environmental contamination and the use of antibiotics or pesticides, combinations of natural antimicrobials are highly desirable as these mixtures allow for the broadening of the antagonistic spectrum. Their effectiveness at low concentrations implies a reduction in toxicity and cost. Although several techniques exist to examine the synergistic effect of a combination of two or more compounds, the checkerboard test is one of the most commonly used techniques and is relatively easy to apply (White et al. 1996). For the current study, it was applied and synergism in *P. larvae* strains 7 from Río Cuarto-Córdoba, 3 from INTA-Balcarce, 7A from Buenos Aires and 35A from Río Negro; and partial synergism in *P. larvae* III from INTA-Balcarce was determined. To HE alone, the effective dose for these strains was between 16 and 32 μg ml^{-1} and this decreased when mixed with surfactin, of values between 4 and 16 μg ml^{-1}. To surfactin was 32 μg ml^{-1} and this concentration decreased when mixed with HE to values equal to MICs for OTC (1 μg ml^{-1}) of susceptible strains. Thus, this study has demonstrated a decrease in surfactin and HE concentrations of 32 and 4 times, respectively, when mixed together, whilst maintaining the antagonistic effect against *P. larvae*. In the case of *P. larvae* III, an FIC index of 0.56 was measured, as this value is close to the arbitrary value that defines synergism and partial synergism (0.5), it can be said that the mixture of surfactin and HE was highly effective at very low concentrations against all strains assayed. As, there is no background mode of action of a synergistic combination between surfactin, synthesized by bacteria of the genus *Bacillus*, and *A. satureioides* extracts, future trials are essential to determine the mechanism/s of how these compounds give rise to the *P. larvae* inhibition.

Microbial production of biosurfactants for commercial use as replacements for their chemical synthesis presents several advantages such as lower toxicity, higher biodegradability, and activity is maintained at extreme pH, salinity and temperature (Desai and Banat 1997). Previous studies assaying the harmlessness of surfactin used in this study to *Apis mellifera* bees showed that administration of the drug to worker bees for 30 days at concentrations close to 10 mg ml^{-1} did not produce any toxic effect (Porrini et al. 2010). Furthermore, surfactin synthesized by *B. subtilis* C4 will have a beneficial side effect, as besides inhibition of *P. larvae* it also inhibits *Nosema ceranae*. On the other hand, in Argentina, Uruguay, Brazil and Paraguay, *A. satureioides*, commonly known as "Marcela", has been used successfully in natural medicines (Filot Da Silva and Langeloh 1994) and the toxicity studies of its extracts revealed that oral administration did not create any toxic risk for humans (Rivera et al. 2004).

As far as we know this is the first study that demonstrates that a combination of surfactin, synthesized by *B. subtilis* C4, and a hexane extract from *A. satureioides* has a synergistic inhibitory effect on viability of *P. larvae*, an important pathogen of honeybee. The low dose of both compounds in the final mixture can be a natural, non-contaminating alternative to collaborate with beekeepers. In future trials, the in vivo effect of these compounds on hives will be assayed.

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