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ORIGINAL RESEARCH ARTICLE

Bacillus and *Brevibacillus* strains as potential antagonists of *Paenibacillus larvae* and *Ascosphaera apis*

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Species of *Bacillus* and *Brevibacillus* associated with honey bees are interesting sources of bioactive compounds with potential uses beyond the field of apiculture. Most *Bacillus* species and related genera produce a broad range of antimicrobial compounds, with activity against bacteria and fungi that include peptides, lipopeptides, bacteriocins, and bacteriocin-like inhibitory substances. By using biological tools, we evaluated the antagonistic activity of 34 bacterial strains against *Paenibacillus larvae* and *Ascosphaera apis*, the causal agents of American Foulbrood and Chalkbrood diseases of honey bee larvae, respectively. Data reveal that the antagonistic response was strain-specific, species-specific, and also medium-dependent. By using molecular tools, we investigated the distribution of antimicrobial peptide genes in the antagonist strains. The presence of homologous sequences to nine genes encoding for the synthesis of the antimicrobial peptides bacillomycin L (*bmyB*), fengycin (*fenD*), bacilysin (*bacA*), subtilin (*spaS*), iturin A (*ituD*, *lpa-14*; *ituC*), and surfactin (*sfp*; *srfAA*) was assayed by PCR. The distribution and frequency of these genes within the bacterial antagonists were also variable and strain-dependent, being the most common surfactins (*srfAA* = 44% and *lpa-14* = 38%), iturins (*ituD* = 47%), and bacilysin (*bacA* = 32%). Moreover, a positive correlation between presence of antimicrobial peptide genes and antagonism was found taking into account that 85% of the antagonists had at least one of the antimicrobial peptide genes. We also identified those antagonists active against different *P. larvae* genotypes. To our knowledge, this is the first study of the association between the presence of homologous sequences of antimicrobial peptide genes and antagonism against *P. larvae* and *A. apis* strains.

Cepas de *Bacillus* and *Brevibacillus* como antagonistas potenciales de *Paenibacillus larvae* y *Ascosphaera apis*.

Las especies de *Bacillus* y *Brevibacillus* asociadas con abejas melíferas son una fuente interesante de compuestos bioactivos con usos potenciales más allá del campo de la apicultura. La mayoría de las especies de *Bacillus* producen una amplia gama de compuestos antimicrobianos, con actividad contra bacterias y hongos que incluye péptidos, lipopéptidos, bacteriocinas y sustancias inhibidoras similares a bacteriocinas (BLIS). Con el objeto de buscar alternativas naturales para el control de loque americana y cría yesificada, se emplearon herramientas biológicas para evaluar la actividad antagónica de 34 cepas bacterianas contra cepas de *Paenibacillus larvae* y de *Ascosphaera apis,* agentes causales de estas enfermedades. Los resultados obtenidos mostraron que la respuesta antagónica fue medio-dependiente y cepa-dependiente. Se analizó por PCR la presencia de secuencias homólogas a 9 genes relacionados con la síntesis de péptidos antimicrobianos: bacilomicina L (*bmyB*), fengicina (*fenD*), bacilicina (*bacA*), subtilina (*spaS*), iturina A (*ituD*, *lpa-14*; *ituC*), y surfactina (*sfp*; *srfAA*). La distribución y frecuencia de estos genes en las cepas antagonistas también resultó variable y cepa-dependiente, siendo los más comunes surfactinas (*srfAA* = 44% y *lpa-14* = 38%), iturina A (*ituD* = 47%) y bacilicina (*bacA* = 32%). Se identificaron antagonistas bacterianos frente a distintos genotipos de *P. larvae* y se encontró una correlación positiva entre la presencia de genes vinculados con la producción de péptidos antimicrobianos y la inhibición del desarrollo de *P. larvae*. Este trabajo constituye el primer estudio de asociaciones entre presencia de estos genes y el antagonismo frente a *P. larvae* y *A. apis*.

Keywords: American Foulbrood; AFB; chalkbrood; antimicrobial peptides; biocontrol; brood bee diseases

Introduction

American Foulbrood of honey bees (AFB) is the most devastating bacterial disease affecting honey bee brood worldwide and is caused by the spore-forming Gram-positive bacterium *Paenibacillus larvae* (Genersch, 2010). AFB occurs in temperate or sub-tropical regions throughout the world and leads to losses not only in the apicultural economy but also in the pollination rates. In many countries, AFB-infected colonies are destroyed by burning the bees, brood combs, and all movable parts. However, in north and south American honey-producing countries, the antibiotic oxytetracycline

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Table 1. Source, culture conditions, and accession numbers of bacterial and fungal strains used in this study.

Species	Strain designation	Source and geographical origin	Medium and culture condition	Accession number
Indicator strains				
Ascosphaera apis	*Aa*37	Honey – Argentina	MY20 – 34 °C	N/A
Ascosphaera apis	*Aa*74	Larvae – Argentina	MY20 – 34 °C	N/A
Paenibacillus larvae ERIC I	ATCC 9545	ATCC	MYPGP – 37 °C	NR_118956.1
Paenibacillus larvae ERIC IV	ATCC 13537	ATCC	MYPGP – 37 °C	KT363749.1
Paenibacillus larvae ERIC I	PL38	Larvae – Argentina	MYPGP – 37 °C	N/A
Paenibacillus larvae ERIC I	PL45	Larvae – France	MYPGP – 37 °C	N/A
Paenibacillus larvae ERIC I	PL58	Larvae – Sweden	MYPGP – 37 °C	N/A
Paenibacillus larvae ERIC II	SAG 290	Honey – Unknown	MYPGP – 37 °C	N/A
Paenibacillus larvae ERIC II	SAG 10754	Honey – Unknown	MYPGP – 37 °C	N/A
Antagonists				
Culture collection strains				
Bacillus cereus	ATCC 11778	ATCC	BHI-T – 32 °C	JF749280.1
Bacillus licheniformis	NRRL B-1001	NRRL	BHI-T – 30 °C	N/A
Bacillus megaterium	NRRL B-939	NRRL	BHI-T – 30 °C	N/A
Bacillus pumilus	ATCC 7061	ATCC	BHI-T – 30 °C	AY876289.1
Bacillus subtilis	ATCC 10783	ATCC	BHI-T – 30 °C	N/A
Paenibacillus amylolyticus	NRRL B-14940	NRRL	MYPGP – 37 °C	NR_025882.1
Isolated from apiarian sources				
Bacillus cereus	m6c	Honey – Argentina	BHI-T – 32 °C	KP005456.1
Bacillus cereus	mv33	Honey – Argentina	BHI-T – 32 °C	KU230015.1
Bacillus cereus	mv50b	Honey – Argentina	BHI-T – 32 °C	KU230017.1
Bacillus cereus	m387	Honey – Argentina	BHI-T – 32 °C	KP005455.1
Bacillus cereus	m395	Honey – Argentina	BHI-T – 32 °C	KU230025.1
Bacillus cereus	m434	Honey – Argentina	BHI-T – 32 °C	KU230027.1
Bacillus cereus	LPcer1	Larvae – Argentina	BHI-T – 32 °C	KX431225.1
Bacillus cereus	MexB	Honey – Mexico	BHI-T – 32 °C	KU230012.1
Bacillus cereus	MexC	Honey – Mexico	BHI-T – 32 °C	KU230013.1
Bacillus clausii	Fr231	Honey – France	BHI-T – 30 °C	KU230014.1
Bacillus clausii	m448b	Honey – Brazil	BHI-T – 30 °C	KX685159.1
Bacillus licheniformis	mv55	Honey – Argentina	BHI-T – 30 °C	KU230018.1
Bacillus megaterium	m435	Honey – Mexico	BHI-T – 30 °C	KU230028.1
Bacillus pumilus	mv49b	Honey – Argentina	BHI-T – 30 °C	KU230016.1
Bacillus pumilus	mv81	Honey – Argentina	BHI-T – 30 °C	KU230019.1
Bacillus pumilus	m116	Honey – Argentina	BHI-T – 30 °C	KU230020.1
Bacillus pumilus	m350	Honey – Argentina	BHI-T – 30 °C	KU230023.1
Bacillus pumilus	m363	Honey – Argentina	BHI-T – 30 °C	KU230024.1
Bacillus pumilus	m414	Honey – Argentina	BHI-T – 30 °C	KU230026.1
Bacillus subtilis	xx	Larvae – Argentina	BHI-T –30 °C	KP177517.1
Bacillus subtilis	m329	Honey – Argentina	BHI-T – 30 °C	KU230021.1
Bacillus subtilis	m334	Honey – Argentina	BHI-T – 30 °C	KU230022.1
Bacillus subtilis	m347	Honey – Argentina	BHI-T – 30 °C	KP177515.1
Bacillus subtilis	m351	Honey – Argentina	BHI-T – 30 °C	KP177516.1
Brevibacillus borstelensis	RC	Honey – Argentina	MYPGP – 37 °C	KP177514.1
Brevibacillus laterosporus	BLAT169	Larvae – Argentina	MYPGP – 37 °C	KX102627.1
Brevibacillus laterosporus	BLAT170	Larvae – Argentina	MYPGP – 37 °C	KX431223.1
Brevibacillus laterosporus	BLAT171	Larvae – Argentina	MYPGP – 37 °C	KX431224.1

ATCC: American Type Culture Collection, USA; CCT: Colleçao de Culturas Tropical, Brazil; NRRL: Northern Utilization Research and Development Division, USA; SAG: Servicio Agrícola Ganadero, Chile.

(OTC) has been used by beekeepers to prevent and control AFB as an alternative to the burning of infected beehives. The widespread use of OTC has contributed to an increase in tetracycline resistance in the *P. larvae* populations by enhancing the interspecific transfer of tetracycline-resistant encoding plasmids-containing *tet*(L) genes (Alippi, León, & López, 2014; Murray & Aronstein, 2006).

PCR amplification of repetitive elements present in bacterial DNA (rep-PCR) is useful for genotyping, and ERIC-PCR amplification (rep-PCR by using enterobacterial repetitive intergenic consensus primers) has shown four *P. larvae* genotypes, named ERIC I, II, III, and IV (Genersch et al., 2006). This typing scheme correlates with phenotypic differences including spore surface configuration, colony morphology, and virulence (Djukic et al., 2014; Forsgren, Stevanovic, & Fries, 2008; Genersch, Ashiralieva, & Fries, 2005; Genersch et al.,2006; Neuendorf, Hedtke, Tangen, & Genersch, 2004). Laboratory infection assays revealed that ERIC I strains needs up to 12 days to kill the infected larvae, whereas ERIC II, III, and IV strains are fast killers and infected larvae die within 6–7 days (Ebeling, Knispel, Hertlein, Fünfhaus, & Genersch, 2016; Genersch, Ashiralieva, & Fries, 2005). Strains belonging to ERIC I and II have been isolated from AFB outbreaks over the

last decades, while ERIC III and IV are only represented by historical strains deposited in type culture collections (De Graaf et al., 2013).

Chalkbrood is an invasive mycosis caused by *Ascosphaera apis* that occurs throughout the world and exclusively affects bee brood. Although fatal to individual larvae, the disease does not usually destroy an entire bee colony. However, it can cause significant losses regarding both bee numbers and colony productivity, with reductions in the honey production of 5–37% (Heath, 1982). A broad range of chemotherapeutic compounds has been tested for their ability to control chalkbrood (reviewed by Hornitzky, 2001), some of them promising for controlling fungal growth either *in culture* or in honey bee colonies. Unfortunately, none of the tested compounds achieved the level of control required to fight the disease (Heath, 1982; Hornitzky, 2001). Considering that chalkbrood increased its incidence in recent years and, due to the lack of registered chemicals to fight it, the development of alternative control methods is of great interest (Aronstein & Murray, 2010).

For these reasons, alternative treatments for prevention and control of both diseases are necessary to ensure the sustainability of the beekeeping industry, including the use of biocontrol agents. Species of *Bacillus* and *Brevibacillus* that are associated with honey bees are interesting sources of bioactive compounds with potential uses beyond the field of apiculture. Most *Bacillus* species and related genera produce a broad range of antimicrobial compounds active against bacteria and fungi (Baruzzi, Quintieri, Morea, & Caputo, 2011). These compounds comprise peptides, lipopeptides, bacteriocins, and bacteriocins-like inhibitory substances (BLIS) that can be used as biocontrol agents and also as food preservatives (Abriouel, Franz, Omar, & Galvez, 2011; Baruzzi et al., 2011; Cochrane & Vederas, 2014, Mora, Cabrefiga & Montesinos, 2011, 2015; Sumi, Yang, Yeo, & Hamn, 2015). Based on their biosynthetic pathway, they can be separated into two different groups, i.e., ribosomally and non-ribosomally synthesized peptides. Abriouel et al. (2011) classified the ribosomally synthesized peptides into three classes containing several subclasses. Typical examples are subtilins, bacilysin, ericins, sublancin 168, subtilosins, and mersacidin produced by *Bacillus subtilis*; cereins and cereicidins produced by *Bacillus cereus,* megacins produced by *Bacillus megaterium* and also lichenin and lichenicidin produced by *Bacillus licheniformis.* Bacilysin and subtilins produced by strains of *B. subtilis* are active against a broad range of bacteria and some fungi like *Candida albicans* (Abriouel et al., 2011, Özcengiz & Öğülür, 2015).

On the other hand, antimicrobial lipopeptides produced by bacilli include non-ribosomally synthesized peptides that can be categorized, according to their chemical structure, as linear cationic, cyclic cationic or cyclic non-cationic lipopeptides. *Bacillus* cyclic non-cationic lipopeptides were the most studied and are classified into three main families: surfactin, iturin, and fengycin (Sumi et al., 2015). Within the surfactin family, surfactins show not only antimicrobial activity but also surface-active properties (Cochrane & Vederas, 2014). Within the iturin family, iturins and bacillomycins present considerable antifungal activity, as well as fengycins within the fengycin family (Cochrane & Vederas, 2014; Ongena & Jacques, 2007).

Species of *Bacillus* and *Brevibacillus* have been frequently isolated from apiarian sources including honey and honey bees (Alippi, Reynaldi, López, De Giusti, & Aguilar, 2004; Gilliam, 1979, 1997). We have previously shown that several species of *Bacillus* and related genera exhibited inhibitory effect against *P. larvae* and *A. apis in vitro* (Alippi & Reynaldi, 2006; Reynaldi, De Giusti, & Alippi, 2004). Also, we characterized two BLIS from *B. cereus* with specific activity against *P. larvae* (Minnaard & Alippi, 2016). The aim of the present study was to screen a collection of 34 bacterial antagonists for their *in vitro* antimicrobial activity against *P. larvae* and *A. apis* determining associations, if any, between the presence of several antimicrobial peptide genes and antagonism.

Materials and methods

Bacterial and fungal strains and media

A total of 41 bacterial strains and 2 fungal isolates were used and are listed in Table 1. Bacterial strains were divided into three groups. The first group included 28 antagonists of the genera *Bacillus* and *Brevibacillus* obtained from honey or honey bee larvae as described previously (Alippi & Reynaldi, 2006; Reynaldi et al., 2004). A second group included six reference strains of *Bacillus* and *Paenibacillus* species from Culture Collections used for comparisons. The third group consisted of seven strains of *P. larvae* belonging to different ERIC genotypes (Alippi et al., 2004; Genersch et al., 2006) that were used as bacterial indicator strains. *P. larvae* strains were isolated from AFB diseased larvae, honey samples from different geographical origins as previously described (Alippi & Aguilar, 1998; Alippi et al., 2004) or received as a culture. Also, two strains of the fungus *A. apis* isolated as previously described (Reynaldi et al., 2004) were used as fungal indicator strains.

Bacteria were maintained as stock cultures at −80 °C in the correspondent broth medium, MYPGP (Dingman & Stahly, 1983) or BHI (Gochnauer, 1973; Merck Química, Argentina) plus 20% glycerol (v/v; Table 1). For short-term storage, the strains were kept at 4 °C in the appropriate semi-solid medium. Fungal isolates were maintained in 20% glycerol at −80 °C; for short-term storage, the strains were kept at 4 °C on MY-20 medium (Reynaldi et al., 2004).

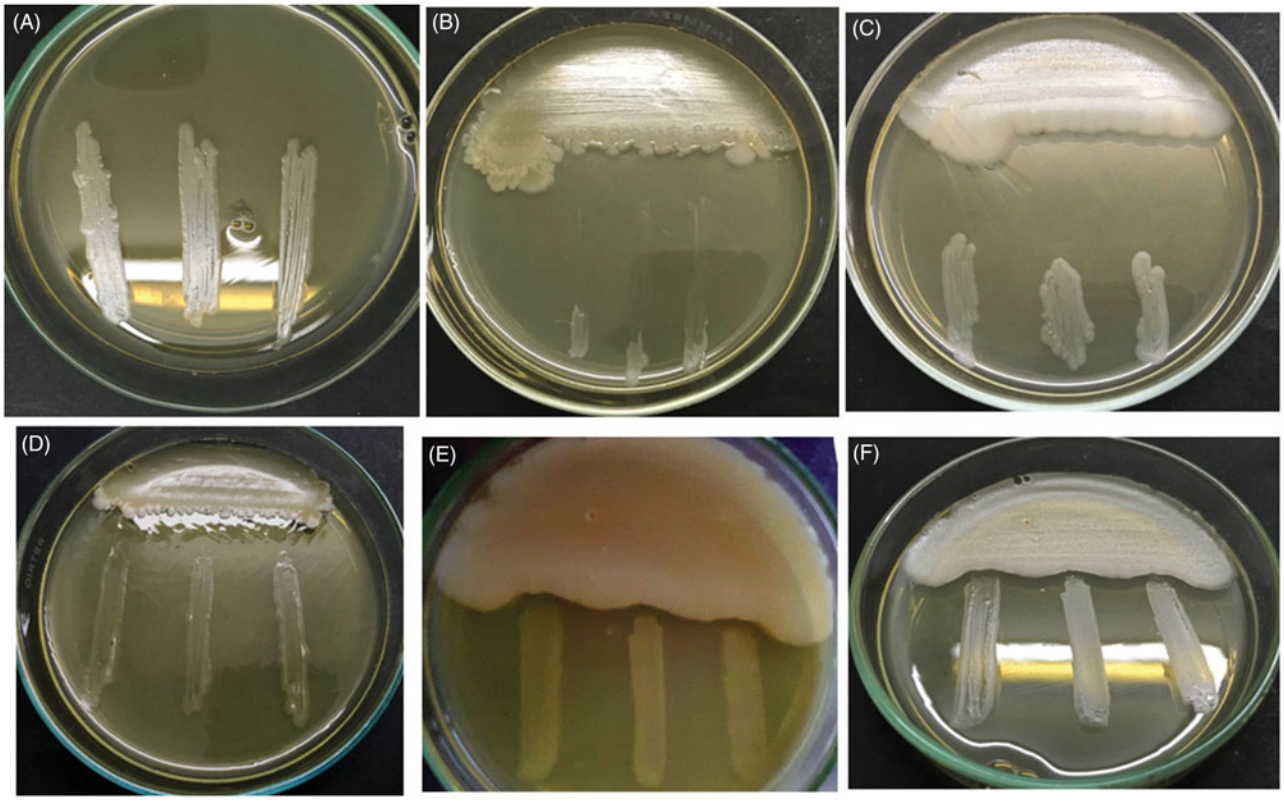

Figure 1. Patterns of inhibition observed in the perpendicular streak technique for *P. larvae* by testing diffusible compounds. (A) Control (*P. larvae* genotype ERIC I vs. sterile distilled water in MYPGP), (B) Excellent inhibition (R:4) *P. larvae* PL45 vs. *B. pumilus* mv81 in BHI-T, (C) Very good inhibition (R:3) *P. larvae* PL45 vs. *B. megaterium* m435 in MYPGP, (D) Good inhibition (R:2) *P. larvae* PL58 vs. *Br. laterosporus* BLAT169 in IST, (E) slight inhibition (R:1) *P. larvae* PL58 vs. *B. cereus* m395 in IST, and (F) Null inhibition (R:0) *P. larvae* PL38 vs. *B. cereus* mv33 in MYPGP.

Identification of bacterial antagonists

The identity of the 28 bacterial isolates of the first group was confirmed by using API 20E and API 50CH strips plus API 50CHB medium (bioMérieux, France) according to the manufacturer's instructions. The interpretation of the results was made by using the identification software api-*web* (bioMérieux, www.biomerieux.com). Strains were separated into one of the following three groups: (1) identification at the species level; (2) identification at the genus level; (3) no identification (low discrimination). Only results within group 1 were considered as valid and were subdivided into four subgroups: (1) excellent species identification with a percentage identification value (ID) of $\geq$99.9% and a *T* value $\geq$0.75; (2) very good identification (ID $\geq$99.0% and $T \geq 0.5$; (3) good identification (ID $\geq$90.0% and $T \geq 0.25$; and (4) acceptable identification (ID $\geq$80.0% and $T > 0$).

Also, colony morphology and microscopic examination of bacterial smears for the presence of unstained globules in the cytoplasm and size and location of spores were tested according to standard protocols (Gordon, Haynes, & Pang, 1973). Bacterial cultures were also tested by catalase reaction, anaerobic growth, production of lecithinase, decomposition of tyrosine, growth in different concentrations of NaCl, and starch hydrolysis (Gordon et al., 1973).

The strains were further identified by sequencing the 16S rDNA. Universal eubacterial primers used for 16S rDNA sequence analysis were 27f (5′-AGAGTTTGATC MTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGT TACGACTT-3′; Yu, Zhou, Yang, Liu, & Hu, 2013). The purified PCR products of approximately 1400 bp were sequenced by the dideoxy termination method by the commercial services of Macrogen Inc. (Seoul, Korea) or Unidad de Genómica, Instituto de Biotecnología, CICVYA-INTA (Hurlingham, Argentina). Sequence assembly and contig editing were performed by using CodonCode Aligner software (Codon Code Corporation, MA, USA). The partial sequences obtained were subjected to a Blast-N (http://www.ncbi.nlm.nih.gov) search to identify sequences with the highest similarity by comparison only with sequences obtained from cultures deposited in Culture Collections according to the criteria of Logan et al. (2009). Also, obtained sequences were analyzed by EZBioCloud (http://www.ezbiocloud.net) by comparison with those from type cultures (Yoon et al., 2017).

Screening of antagonistic potential

All *Bacillus*, *Brevibacillus*, and *Paenibacillus* strains (Table 1) were screened against *P. larvae* and *A. apis* to assess

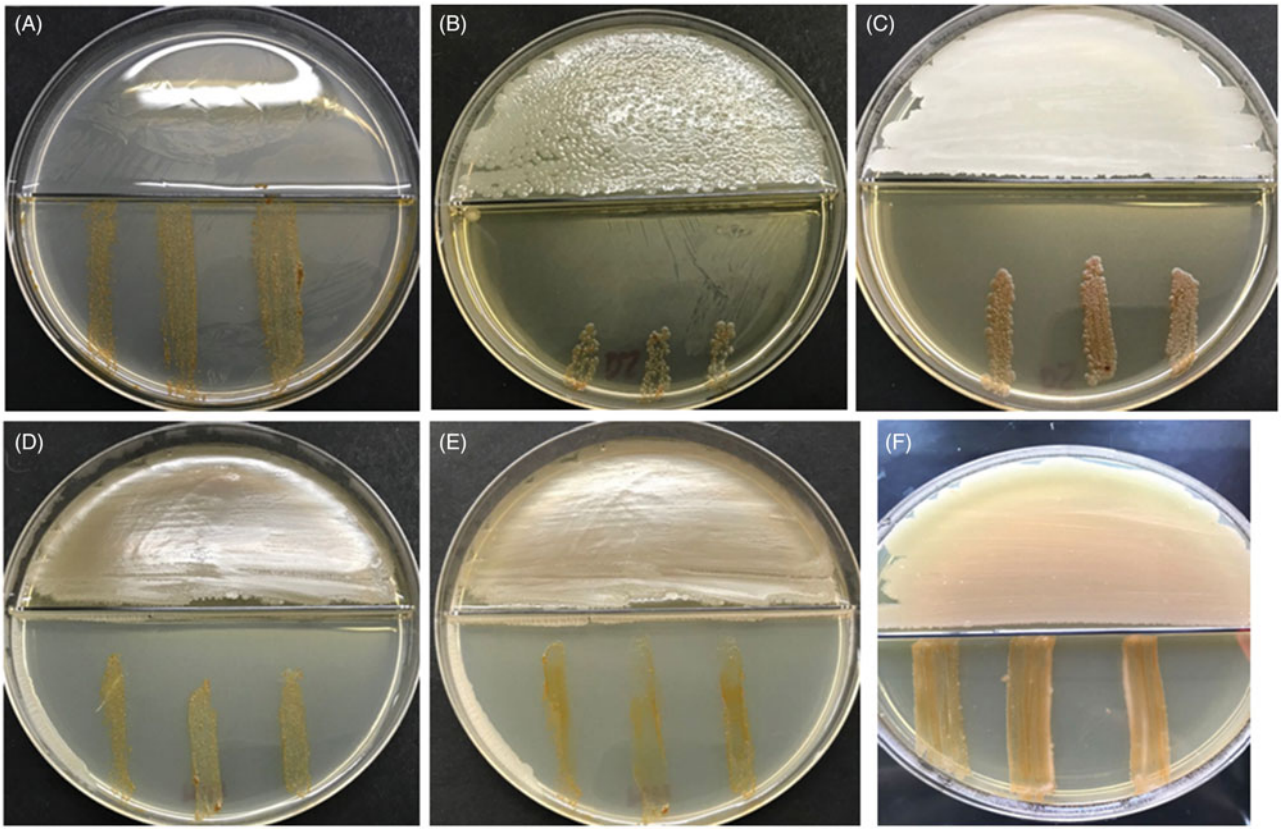

Figure 2. Patterns of inhibition observed by co-cultivation technique in two-compartment plates for *P. larvae* by testing volatile compounds (VOCs): (A) Control (*P. larvae* genotype II vs. sterile distilled water, (B) Excellent inhibition (R:4) *P. larvae* ATCC13537 vs. *B. pumilus* m116, (C) Very good inhibition (R:3) *P. larvae* SAG 290 vs. *B. subtilis* m334, (D) Good inhibition (R:2) *P. larvae* SAG 10754 vs.*B. pumilus* m363, (E) Slight inhibition (R:1) *P. larvae* SAG290 vs. *B. megaterium* m435, and (F) Null inhibition (R:0) *P. larvae* SAG290 vs. *Br. laterosporus* BLAT170.

their antagonistic potential due to the production of both diffusible and volatile organic compounds (VOCs).

To test inhibition due to diffusible compounds two methods were used, i.e., the perpendicular streak technique for *P. larvae* (Alippi & Reynaldi, 2006) and the central disk test assay for *A. apis* (Reynaldi et al., 2004). Each antagonist ($n = 34$) was tested against the 7 *P. larvae* indicator strains by using three different culture media prepared at a final concentration of 2.5% agar in order to avoid swarming. Media tested were MYPGP (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate agar), BHI-T (brain heart infusion agar fortified with thiamine) and IST (Iso-Sensi test agar, Oxoid™). The plates were incubated at the optimum temperature for each antagonist (Table 1) for 3 days to enable the production of diffusible compounds. Then, streaks from a 24 h active growing culture of each *P. larvae* indicator strain were made perpendicularly to the first streak, marking the plate at the edge of each one. Plates were prepared for each combination of antagonist-indicator strain in each media, including those for controls. In all cases, control plates were only streaked with each *P. larvae* indicator strain. All the plates were incubated at 37 °C for 24 and 48 h, recording any inhibition of *P. larvae* growth in comparison with controls. The scale of inhibition was registered as follows: null (0), slight (1), good (2), very good (3), and excellent (4).

Each bacterial antagonist ($n = 34$) was tested against *A. apis Aa*37 and *Aa*74 indicator strains by using MY-20 medium. For controls, only a central disk of fungal growth was used. Treated and control plates were incubated at 34 °C and the diameter of the fungal colony was recorded at 2 and 5 days. Percentages of mycelial growth inhibition (MGI) were calculated according to the formula proposed by Michereff, Silveira, Reis, and Mariano (1994) in comparison to controls.

To test inhibition due to VOCs, we employed the co-cultivation technique described by Zhang et al. (2013) with minor modifications, by using two-compartment polystyrene plates (Deltalab®, Spain). For testing *P. larvae* inhibition, one side of the plates was filled with BHI-T and inoculated with each bacterial antagonist by spreading a suspension adjusted to 0.5 Mc Farland, and the other side was filled with MYPGP. In the case of *Bacillus clausii* strains, due to the alkaliphilic nature of this species, the medium used was adjusted to pH 10. After incubation for 3 days at the corresponding temperature (Table 1), three streaks of each *P. larvae* indicator strain were made in the MYPGP side. The plates were sealed with Parafilm™ and cultured at 37 °C evaluating *P. larvae* inhibition at 24 h and 48 h, respectively.

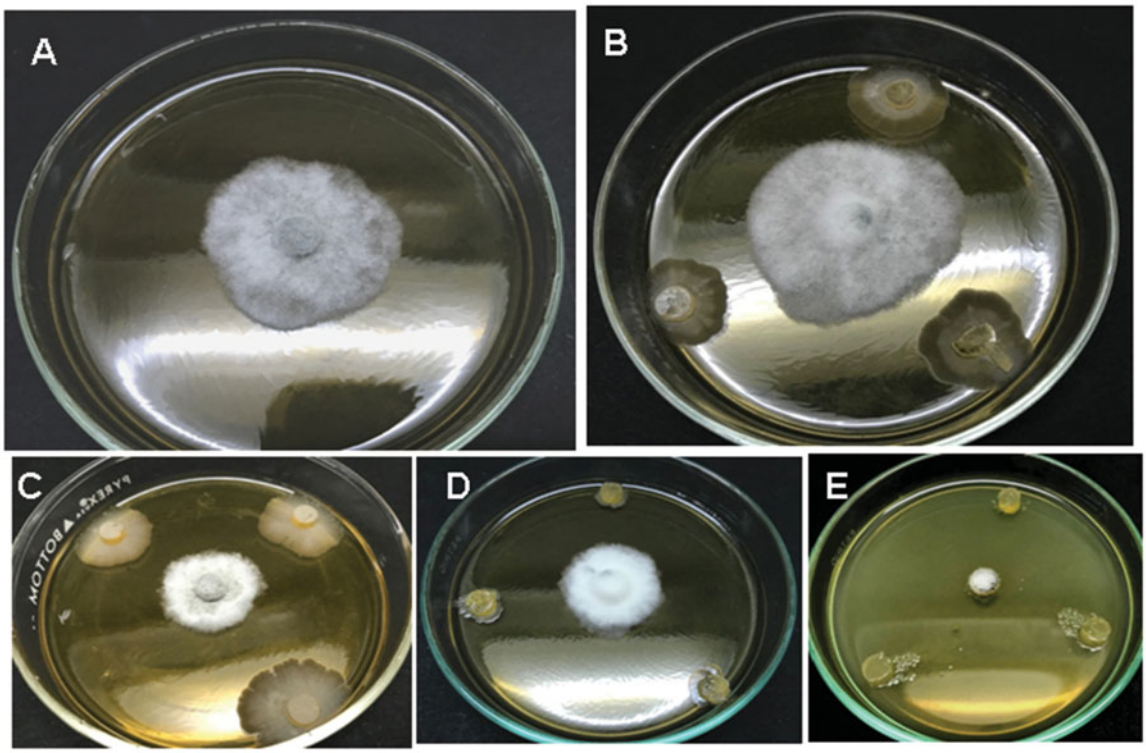

Figure 3. Patterns of inhibition of fungal growth of *A. apis* by testing diffusible compounds after 2 days of incubation. (A) Control *A. apis Aa*37, (B) *A. apis Aa*37 vs. *B. pumilus* m116 (11% MGI-R1), (C) *A. apis Aa*37 vs. *B. subtilis* m347 (21% MGI-R2), (D) *A. apis Aa*37 vs. *B. megaterium* m435 (44% MGI- R3), (E) *A. apis Aa*74 vs. *Br. borstelensis* (100% MGI-R5).

Control plates were only streaked with *P. larvae* indicator strains. The scale of inhibition was the same as mentioned before for testing diffusible compounds.

For testing *A. apis* inhibition, one side of the plate was filled with MY-20 and inoculated with an 8-mm disk of each fungal strain (Reynaldi et al., 2004), and the other side was filled with BHI-T pH10 in the case of *B. clausii* strains or with BHI-T for the rest of the antagonists. Control plates were the same but inoculated only with the fungal disk. Plates were sealed with Parafilm™ and cultured at 30 °C for 5 days. The diameters of the fungal colonies were recorded at 2 and 5 days. Percentages of MGI were calculated in comparison to controls as explained before.

Statistical analysis

Multiple correspondence analysis (MCA) was carried out by using Statistica 7 (Statsoft) to analyze the antagonistic potential of bacterial antagonists ($n = 34$) against *P. larvae* and *A. apis* for both, diffusible and VOCs. The antagonistic response variable against *P. larvae* ($n = 7$) was defined as R:0 (null); R:1 (slight); R:2 (good); R:3 (very good), and R:4 (excellent; Figures 1 and 2). In the case of *A. apis*, MGI values were transformed into the following scale of antagonistic response: R:1 (0–20%); R:2 (21–40%); R:3 (41–60%); R:4 (61–80%), and R:5 (more than 80%; Figures 3 and 4). In all cases, values measured after 2 days of incubation were used for the statistical analysis.

Surfactant capacity

Many *Bacillus* and *Paenibacillus* species produce lipopeptide biosurfactants with a broad spectrum of antimicrobial activities (Cochrane & Vederas, 2014). Besides their antimicrobial activity, these biosurfactants show surface-active property, i.e., formation of a superficial thick pellicle in broth cultures that are associated with biosurfactant production in most *B. subtilis* strains (Chollet-Imbert, Gancel, Slomianny, & Jacques, 2009).

With the aim of detecting biosurfactant activities, the drop-collapse method of Bodour and Miller-Maier (1998) was used. Polystyrene multiwell plates (96 well, Deltalab, Spain) were coated with 1.8 µl of 10W-40 mineral oil ELAION® per well and left at room temperature for 24 h to ensure a uniform coating. Antagonists have grown aerobically in an orbital shaker (100 rpm) at their optimum temperature (Table 1) in screw-capped tubes containing 5 ml of liquid medium. To assess the best condition for surfactant production, we tested three broth media, i.e., MYPGP, IST, and BHI-T. Each tube was centrifuged at 8500 × *g* for 5 min, and aliquots of 1 ml of supernatant were collected and replaced with 1 ml of fresh medium. A 5 µl aliquot of supernatant was delivered into the center of the well and drop collapse was determined visually after

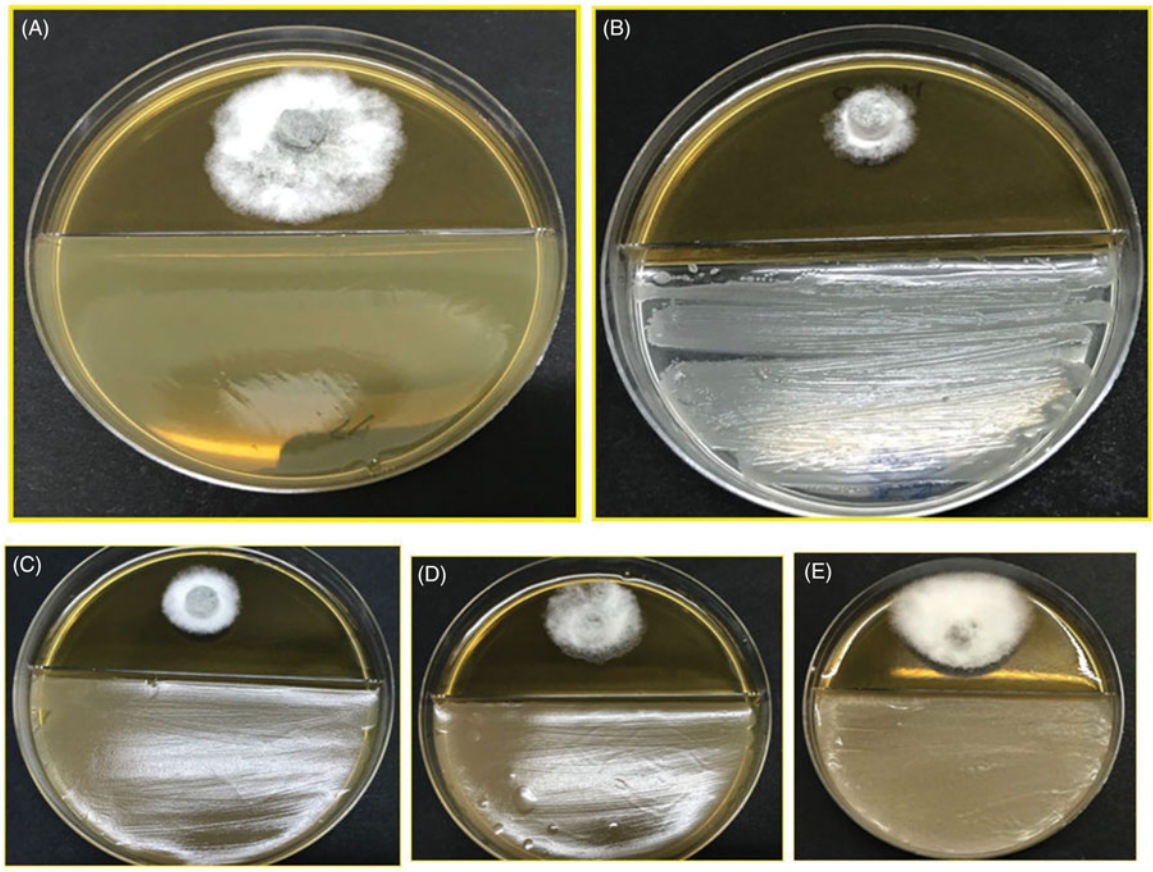

Figure 4. Patterns of inhibition of fungal growth of *A. apis* by testing VOCs in two-compartment plates after 2 days of incubation. (A) Control *A. apis Aa*74, (B) *A. apis Aa*74 vs. *Br. borstelensis* RC (67% MGI-R:4), (C) *A. apis Aa*74 vs. *B. licheniformis* NRRL B-1001 (44% MGI-R3), (D) *A. apis Aa*74 vs. *B. clausii* Fr231 (30% MGI-R2), and (E) *A. apis Aa*74 vs. *B. megaterium* m435 (15% MGI-R1).

1 min (Bodour & Miller-Maier, 1998). Sodium dodecyl sulfate (20% w/v) was used as a positive control. Samples were tested in triplicate and at intervals of 24 h for 7 days to assess the best incubation time for surfactant production.

The presence of lipopeptides was presumed if a precipitate was observed after adding HCl until pH 2 into the cell-free supernatants according to the technique described by Smyth, Perfumo, McClean, Marchant, and Banat (2010). Besides, shaken cultures were observed daily to detect superficial pellicle formation, thickness, and permanency (Chollet-Imbert et al., 2009). Hemolytic activity on blood agar plates according to standard protocols (Beecher & Wong, 1994) was also tested.

PCR assays

The presence of homologous sequences to nine genes encoding for the synthesis of the antimicrobial peptides Bacillomycin L (*bmyB*), Fengycin (*fenD*), Bacilysin (*bacA*), Subtilin (*spaS*), Iturin A (*ituD*, *lpa-14*; *ituC*), and Surfactin (*sfp*; *srfAA*) was assayed by PCR (Table 2). Total genomic DNA was isolated from bacterial strains as previously described (Alippi & Aguilar, 1998). Bacteria were grown in aerobic conditions at the appropriate temperature and culture media according to the species tested (Table 1). Oligonucleotide DNA primers used for PCR amplifications (Table 2) were custom synthesized by Fagos/Ruralex S.R.L. (Buenos Aires, Argentina).

PCR assays were carried out at least two times independently in two different thermocyclers (Eppendorf Mastercycler, Eppendorf AG, Hamburg, Germany and Primus 25 advanced, Erlangen, Germany). The amplification products were separated in 1.2% agarose gel in 0.5X TBE buffer, stained with ethidium bromide and visualized with a UV transilluminator (UVP, Upland, CA, USA). Gel images were digitalized by using a digital image capture gel documentation system (DigiDoc-It, UVP, v. 1.1.25, Upland, CA, USA).

Results

Identification of bacterial antagonists

The studied *Bacillus* isolates previously identified by classical microbiological methods (Alippi & Reynaldi, 2006; Reynaldi et al., 2004) as *B. cereus* (MexB, m6c, mv33, m387); *Bacillus megaterium* (m435); *Bacillus pumilus* (m350), *B. subtilis* (m329, m351), and *Brevibacillus laterosporus* (BLAT169, BLAT170, BLAT171) were confirmed both by api*web* software and 16S rDNA sequencing.

Table 2. Oligonucleotide primers used in this study.

Antimicrobial peptide gene	Primer name	Sequence (5′ → 3′)	Target	PCR Product size (bp)	Reference
Bacillomycin L	BMYBF	GAATCCCGTTGTTCTCCAAA	*bmyB*	370	Mora, Cabrefiga, and Montesinos (2011)
	BMYBR	GCGGGTATTGAATGCTTGTT			
Fengycin	FENDF	GGCCCGTTCTCTAAATCCAT	*fenD*	269	Mora, Cabrefiga, and Montesinos (2011)
	FENDR	GTCATGCTGACGAGAGCAAA			
Bacilysin	BACAF	CAGCTCATGGGAATGCTTTT	*bacA*	498	Mora, Cabrefiga, and Montesinos (2011)
	BACAR	CTCGGTCCTGAAGGGACAAG			
Subtilin	SPASF	GGTTTGTTGGATGGAGCTGT	*spaS*	375	Mora, Cabrefiga, and Montesinos (2011)
	SPASR	GCAAGGAGTCAGAGCAAGGT			
Iturin A	ITUCF	GGCTGCTGCAGATGCTTTAT	*ituC*	423	Mora, Cabrefiga, and Montesinos (2011)
	ITUCR	TCGCAGATAATCGCAGTGAG			
	ITUDF	ATGAACAATCTTGCCTTTTTA	*ituD*	1203	Hsieh, Lin, Meng, and Kao (2008)
	ITUDR	TTATTTTAAAATCCGCAATT			
	LPA-14F	ATGAAAATTTACGGAGTATA	*lpa*-14	675	Hsieh, Lin, Meng, and Kao (2008)
	LPA-14R	TTATAACAGCTCTTCATACG			
Surfactin	SRFAF	TCGGGACAGGAAGACATCAT	*srfAA*	201	Mora, Cabrefiga, and Montesinos (2011)
	SRFAR	CCACTCAAACGGATAATCCTGA			
	SFPF	ATGAAGATTTACGGAATTTA	*sfp*	675	Hsieh, Li, Lin, and Kao (2004)
	SFPR	TTATAAAAGCTCTTCGTACG			

On the other hand, *B. cereus* (mv81, m363), *Bacillus circulans* (RC, Fr231, m448b), *B. licheniformis* (m347), *B. megaterium* (m414), and *B. subtilis* (m116) were re-identified as *B. pumilus* mv81 (ID: 99.9%, $T = 0.89$), m116 (ID: 99.9%, $T = 0.92$), m363 (ID: 99.9%, $T = 0.91$) and m414 (ID: 99.9%, $T = 1.0$), *B. subtilis* m347 (ID: 99.9%, $T = 0.95$); *Brevibacillus borstelensis* (RC) and *B. clausii* (Fr231 and m448b), respectively. In the case of *Br. borstelensis* and *B. clausii*, these strains were identified by comparison with the description for both species according to Logan et al. (2002), Shida et al. (1995), Denizci, Kazan, Abeln, and Earslan, (2004), and Nielsen, Fritze, and Priest (1995), respectively because are not included in the api*web* database.

Finally, new isolated strains were characterized as *B. cereus* LPcer1 (ID: 94.0%, $T = 0.88$), MexC (ID: 92.3%, $T = 0.97$), mv50b (ID: 97.4%, $T = 0.92$), m395 (ID: 99.8%, $T = 0.83$), and m434 (ID: 99.9%, $T = 0.7$); *B. licheniformis* mv55 (ID: 99.9%, $T = 0.78$); *B. subtilis* xx (ID: 99.8%, $T = 0.88$), m334 (ID: 99.8%, $T = 0.99$), and *B. pumilus* mv49b (ID: 99.9%, $T = 0.89$).

Results obtained for colony morphology, size, shape, and location of spores, presence of unstained globules in cytoplasm, catalase reaction, anaerobic growth, production of lecithinase, decomposition of tyrosine, starch hydrolysis, and tolerance to NaCl were in agreement with those reported previously (Gordon *et al.*, 1973; Logan *et al.*, 2002; Shida *et* al., 1995) and those obtained when testing reference strains from culture collections.

The bacterial identity of the strains isolated from apiarian sources ($n = 28$) was confirmed by sequencing the 16S rDNA. Sequences were deposited at DDBJ/EMBL/Genbank under the accession numbers listed in Table 1.

Screening of antagonistic potential

A high variability was observed among bacterial antagonists against all *P. larvae* strains tested, considering both diffusible and VOCs (Table 3). The antagonist bacterial responses showed strain and species-specificity. MCA was used to analyze this variability, searching for associations, if any, between antagonists, indicator strains, and also antagonistic response. Figure 5(A–C) show the influence of the three culture media tested on the antagonistic potential due to the production of diffusible compounds against *P. larvae* indicator strains. Two main clusters were observed, i.e., cluster I that contains *P. larvae* strains belonging to genotype ERIC I and cluster II containing strains of ERIC II and IV (Figure 5A–C). On the three media tested, the most virulent genotypes ERIC II and IV were less antagonised with values of R:0 to R:2 than genotype ERIC I strains with values of R:3 and R:4 (Figure 5A–C). However, the antagonistic response was media-dependent, e.g., inhibition values obtained when tested BHI-T allowed a better differentiation of responses R:3 and R:4 (Figure 5C) compared to MYPGP (Figure 5A) and IST (Figure 5B), respectively.

Regarding species-specificity, most *B. pumilus*, *B. licheniformis*, *B. clausii*, and *Br. laterosporus* strains showed very good (R:3) and excellent (R:4) inhibition values against *P. larvae* genotypes ERIC I and IV and slight (R:1) to null (R:0) against genotype ERIC II (Table 3). Interestingly, strains of *Br. laterosporus* ($n = 3$) and *B. clausii* ($n = 2$) inhibited ERIC I but showed lack of inhibition to ERIC II (Table 3); although *B. subtilis* strains inhibited all *P. larvae* genotypes tested (Table 3).

Overall, the results obtained for VOCs (Table 3 and Figure 5D) were similar to those obtained for diffusible compounds; however, strains of *Br. laterosporus* ($n = 3$) and *B. clausii* ($n = 2$) were located as outliers (Figure 1D) with null response (R:0) against genotypes ERIC II and IV and slight to null response against ERIC I (Table 3). Also, *B. cereus* strains showed higher inhibition values for VOCs in comparison with those obtained by diffusion against all genotypes, particularly in the case of ERIC II

Table 3. Screening of antagonistic potential against different *Paenibacillus larvae* genotypes.

		P. larvae indicator strains																											
		ATCC9545				PL38				PL45				PL58				ATCC13537				SAG290				SAG10754			
		ERIC I				ERIC I				ERIC I				ERIC I				ERIC IV				ERIC II				ERIC II			
Antagonist		D	D	D	V	D	D	D	V	D	D	D	V	D	D	D	V	D	D	D	V	D	D	D	V	D	D	D	V
		MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T	MYPGP	IST	BHI-T	BHI-T
B. cereus	ATCC 11778	3	3	3	3	2	3	3	3	2	3	3	2	2	2	3	3	0	1	3	2	2	3	4	3	2	3	3	3
	m6c	3	3	2	2	2	2	3	3	2	3	3	3	1	2	3	2	0	1	1	2	0	0	0	1	0	2	0	1
	mv33	3	2	2	3	0	1	2	3	0	2	2	3	1	1	2	3	0	0	0	3	1	0	0	2	1	0	0	4
	mv50b	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	0	0	0	2	0	2	0	2	0	1	0	2
	m387	3	3	1	3	1	2	1	3	1	3	3	3	1	2	2	3	0	0	0	0	0	3	0	2	0	1	0	3
	m395	3	3	3	3	1	3	3	3	2	2	3	3	2	1	3	3	0	1	2	1	2	1	1	2	1	0	0	3
	m434	3	2	0	3	3	3	3	3	2	3	3	3	1	3	3	3	0	2	3	3	2	0	2	2	1	0	0	2
	LPcer1	1	2	2	3	1	2	3	4	2	3	2	2	3	3	2	3	1	1	1	3	2	0	0	1	2	0	0	3
	MexB	3	2	1	3	1	3	1	3	1	3	1	3	3	3	1	2	0	0	0	2	1	0	1	0	0	0	1	1
	MexC	2	1	1	3	2	2	1	2	2	1	2	2	2	2	1	3	0	0	0	0	2	1	3	1	1	0	0	2
B. clausii	Fr231	3	2	4	0	4	3	4	1	4	3	3	1	4	2	3	2	2	3	3	0	0	0	2	1	0	0	2	2
	m448b	2	1	4	0	3	1	4	0	4	1	4	0	4	1	4	0	2	0	3	0	0	0	0	0	0	0	0	0
B. licheniformis	NRRL B-1001	3	3	4	4	2	3	2	3	2	3	3	2	3	3	3	3	2	2	3	4	0	1	3	2	0	0	2	3
	mv55	2	3	3	3	3	3	3	3	2	2	3	3	3	2	3	3	3	2	2	3	0	1	2	1	0	0	1	3
B. megaterium	NRRL B-939	1	3	2	3	3	2	2	3	1	2	1	3	2	3	2	3	2	3	1	3	0	2	4	2	0	1	1	2
	m435	3	1	2	3	3	2	3	3	3	3	4	3	3	2	4	3	3	1	1	2	1	1	2	1	1	0	1	3
B. pumilus	ATCC 7061	3	2	3	3	3	3	2	3	3	3	3	3	3	3	3	3	1	0	3	3	1	0	3	0	2	0	3	3
	mv49b	2	1	2	2	2	2	2	3	3	3	2	3	3	2	2	2	0	2	1	3	0	2	0	3	0	2	1	3
	mv81	3	3	3	3	3	4	4	3	4	4	4	3	3	4	4	3	3	3	2	3	1	1	2	2	3	2	1	2
	m116	3	2	2	3	3	3	3	3	2	3	3	3	3	3	3	3	1	3	3	4	1	1	2	3	1	1	2	3
	m350	3	1	2	3	1	3	4	3	1	2	4	3	3	1	3	3	2	2	3	2	0	0	4	2	0	0	4	2
	m363	2	1	2	3	4	2	2	3	3	2	3	2	3	1	2	3	3	3	1	3	2	2	3	2	1	1	3	2
	m414	3	3	3	3	3	3	3	2	3	3	3	2	3	3	3	3	2	3	3	2	2	3	1	2	1	3	3	3
B. subtilis	ATCC 10783	3	2	3	4	3	3	3	4	4	2	2	3	3	2	3	2	1	1	1	2	0	4	4	2	0	3	2	2
	xx	2	2	3	3	2	3	3	3	1	2	3	3	1	2	3	2	2	3	3	1	2	4	4	2	2	3	3	1
	m329	1	2	1	1	2	3	3	3	1	3	3	3	1	3	4	3	1	2	3	0	1	4	4	2	1	2	4	2
	m334	2	3	1	3	3	3	4	4	3	3	1	3	2	3	3	3	2	3	1	1	2	3	3	3	2	0	0	2
	m347	3	3	3	2	3	3	4	3	3	3	3	3	3	2	3	3	0	2	2	3	1	4	2	2	2	3	0	1
	m351	3	2	3	2	3	3	3	3	2	3	3	3	3	3	3	3	1	3	3	2	2	4	3	2	1	2	0	3
Br. borstelensis	RC	3	3	3	2	3	3	3	2	3	3	3	4	3	3	3	3	1	1	2	2	0	2	3	2	0	1	1	3
Br. laterosporus	BLAT169	2	3	3	0	1	3	3	2	2	3	3	2	2	2	3	0	0	2	0	0	0	1	0	0	0	1	0	0
	BLAT170	3	4	3	1	3	4	3	2	3	4	3	2	3	4	4	0	3	4	3	0	0	1	0	0	0	0	0	0
	BLAT171	3	4	4	0	3	4	4	2	2	4	4	2	3	4	4	2	0	3	3	0	0	1	0	0	0	1	0	0
P. amylolyticus	NRRL B-14940	3	3	3	2	2	3	1	2	2	3	2	2	2	3	2	3	1	1	0	1	0	0	0	1	0	0	0	1

D: diffusible compounds; V: volatile compounds.
Scores were assigned based on the following scale of inhibition: 0: null, 1: slight, 2: good, 3: very good, and 4: excellent. Showed results correspond to 48 h.

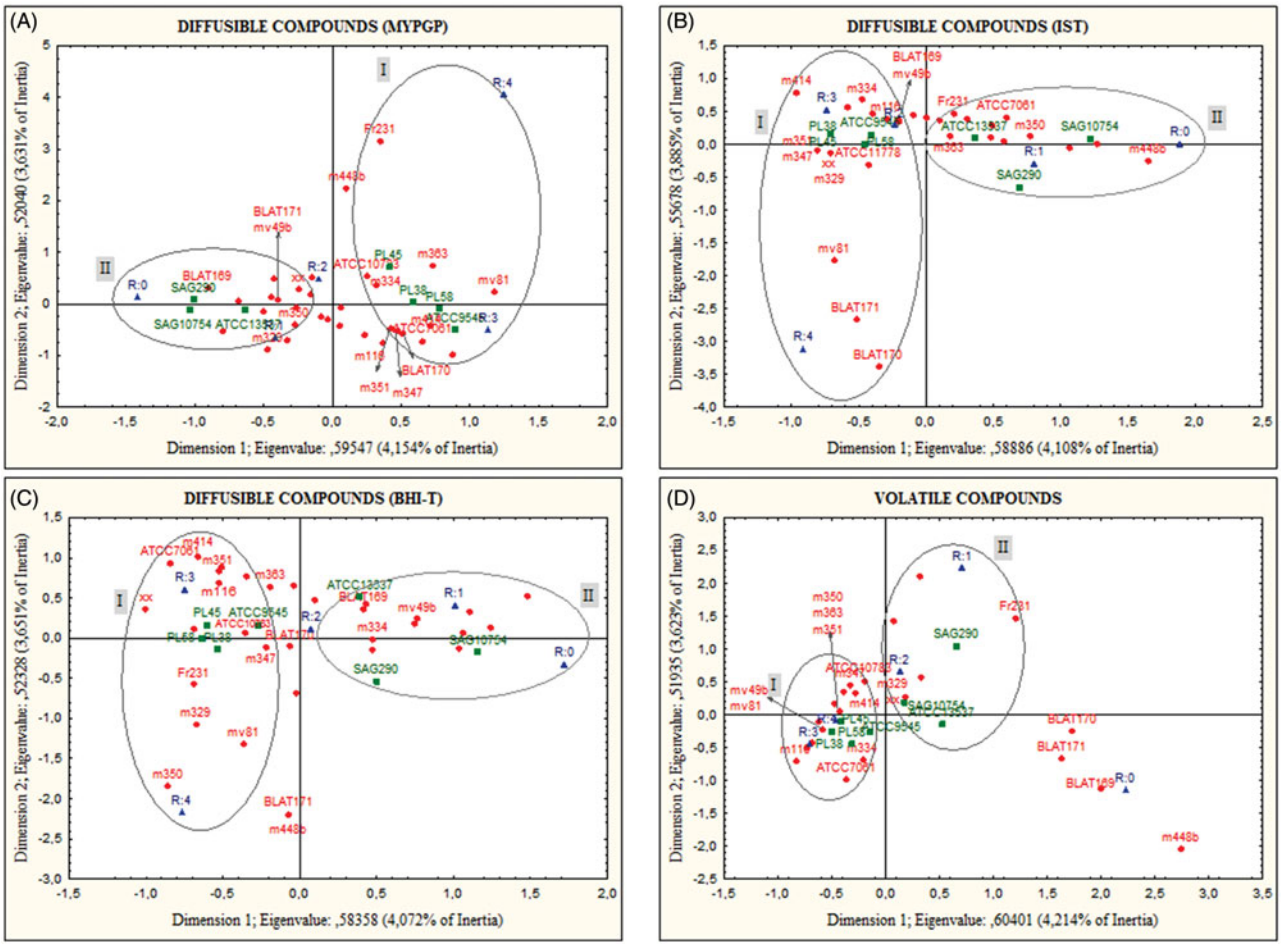

Figure 5. MCA of the antagonistic potential of bacterial antagonists against *P. larvae*. Analyzed variables: bacterial antagonists ($n = 34$), *P. larvae* indicator strains ($n = 7$), and antagonistic response (R: 0–4). Dimension 1 shows the intensity of R along the axis, while dimension 2 distinguishes the variability between bacterial antagonists. Ellipses are used to highlight the clusters (I and II) for grouping *P. larvae* indicator strains. Some strains are overlapped. (A, B, and C) Effect of diffusible compounds in MYPGP, IST, and BHI-T, respectively. (D) Effect of VOCs in BHI-T.

and IV (Table 3). Figure 6 shows the antagonistic potential against *A. apis* strains considering both diffusible (Figure 6A) and VOCs (Figure 6B). *Br. borstelensis* RC showed the best antagonistic response in both assays, particularly against *A. apis Aa*74 with values of R:5 and R:4, respectively (Figure 6A,B and Table 4) while variable results were obtained with the rest of bacterial antagonists (Table 4 and Figure 6A,B). For example, most of *B. subtilis* strains showed MGI values between 39% and 53% (R:3) against both *A. apis* strains when testing diffusible compounds. In the case of VOCs, the antagonistic response was strain-dependent; e.g., *Br. borstelensis* RC, *B. clausii* strains, *B. licheniformis* NRRL B-1001, *B. cereus* m6c and *B. pumilus* mv81 that showed higher antagonistic response values.

Surfactant capacity

Thirty-four antagonists showed highly variable results for parameters related to surfactant capacity depending on the species, strains, and media tested (Table 5). *B. subtilis* strains ($n = 6$) produced a drop-collapse in most media tested, while *B. cereus* strains ($n = 10$) yielded negative results. Also, all *B. subtilis* strains formed a thick superficial pellicle after short incubation periods (24–48 h) that remained during the rest of the experiment. Ninety-one percent of the antagonistic strains produced a precipitate after the acidification of the cell-free supernatants, obtaining the most consistent results when MYPGP broth was used. Also, with the exception of *B. subtilis* m329, all the antagonists showed hemolytic activity (Table 5).

PCR assays

The results on the distribution and frequency of the antimicrobial peptides genes were strain-dependant and highly variable (Figures 7 and 8). For instance, all *B. subtilis* strains ($n = 6$) were positive for genes related to the production of surfactin (*sfp* or *srfAA* or both; Figure 8) while bacilysin (*bacA*) was found in 5 out of 6 *B. subtilis* strains (Figure 7, lane 4 and Figure 8). Genes related to iturin production (*ituC*, *ituD* or *lpa-14*) were found in all *B. cereus* strains ($n = 10$; Figure 7, lane 9 and Figure

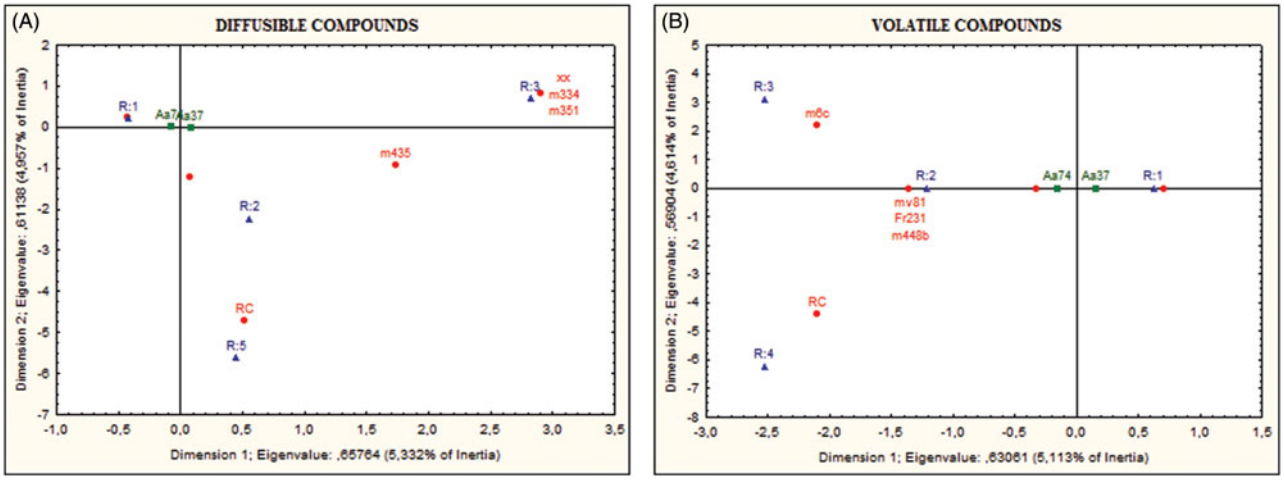

Figure 6. (A, B) MCA of the antagonistic potential of bacterial antagonists against *A. apis* considering diffusible and volatile compounds. Analyzed variables: bacterial antagonists ($n = 34$), *A. apis* indicator strains ($n = 2$), and antagonistic response (R: 1–5). Around R:1, most of the strains are overlapped.

8). Interestingly, surfactin (*sfp* and *srfAA*) and subtilin (*spaS*) genes were found in all *Br. laterosporus* strains tested ($n = 3$; Figure 7, lane 2 and Figure 8).

Despite this variability, the most common genes found within the bacterial antagonists were surfactins and iturins (*ituD* = 47%, *srfAA* = 44%, and *lpa-14* = 38%). Also, bacilysin (*bacA*) were found in 32% of bacterial antagonists. In contrast, bacillomycin (*bmyB*), *fengy*cin (*fenD*), and subtilin (*spaS*) were found only in less than 15% of the bacterial antagonists tested (Figures 7 and 8).

Discussion

A screening of bacterial antagonists of honey bee pathogens was achieved by using biological tools. Antagonistic activity of 34 *Bacillus*, *Brevibacillus*, and *Paenibacillus* strains mainly isolated from apiarian sources was evaluated against *P. larvae* and *A. apis*.

Our results showed a high variability among bacterial antagonists considering both diffusible and VOCs against *P. larvae*. The antagonistic response was strain-specific, species-specific, and also medium-dependent as observed by other authors with other antagonists (Alippi & Reynaldi, 2006; Benítez, Velho, de Souza da Motta, Segalin, & Brandelli, 2012; Forsgren, Olofsson, Vásquez, and Fries, 2010; Minnaard & Alippi, 2016). Differences in antagonism were observed between different *P. larvae* genotypes; for example, *Br. laterosporus* strains were excellent antagonists against genotypes ERIC I and IV but showed slight to null inhibition against ERIC II when testing diffusible compounds (Table 3 and Figure 5A–C); moreover, no inhibition against *P. larvae* ERIC II and IV was observed when testing VOCs (Table 3 and Figure 5D). *B. cereus* strains ($n = 10$) were very good antagonists against genotype ERIC I considering both diffusible and VOCs but showed lower inhibition values against ERIC II and IV when testing diffusible compounds compared to VOCs (Table 3). We also identified those antagonists active against different *P. larvae* genotypes, being the first report on the antagonism of *Bacillus* species against the most virulent genotype ERIC II, particularly in the case of some strains of *B. subtilis* and *B. pumilus*. Differences in genotype inhibition were also found by Forsgren et al. (2010) when testing lactic acid bacteria *in vitro* and in honey bee larvae infected with AFB.

Regarding *A. apis* inhibition, the antagonistic response was also strain-specific and species-specific. *Br. borstelensis* RC, *B. megaterium* m435, and *B. subtilis* xx, m334 and m351 were the best antagonists against *A. apis* when testing diffusible compounds (Figure 6A). Also, *Br. borstelensis* RC, *B. cereus* m6c, *B. licheniformis* ATCC7061, *B. pumilus* mv81, *B. clausii* Fr231 and m448b were very good antagonists when testing VOCs (Figure 6B).

The distribution and frequency of the homologous antimicrobial peptide genes within the bacterial antagonists were also strain-dependent and highly variable, being the most frequent surfactins (*srfAA* = 44% and *lpa-14* = 38%) and iturins (*ituD* = 47%). Also, bacilysin (*bacA*) was found in 32% of bacterial antagonists. Mora and colleagues (2011) working with a collection of 184 *Bacillus* isolates from plant environments reported that the most frequent antimicrobial peptide genes were *srfAA* (69%), *bacA* (61%), *bmyB* (55%), and *fenD* (40%), respectively. Our results are consistent with those obtained by Mora et al. (2011) regarding *srfAA* and *bacA* but not about *bmyB*, *ituD* or *fenD*. Moreover, a positive correlation between presence of antimicrobial peptide genes and antagonism were found, taking into account 85% of the antagonists had at least one of the antimicrobial peptide genes. To our knowledge, this is the first study of the association between the presence of homologous sequences of antimicrobial peptide genes and antagonism against *P. larvae* and *A. apis* strains.

Table 4. Percentage of inhibition of mycelial growth (MGI) on two *Ascosphaera apis* strains induced by different bacterial antagonists ($n = 34$).

		A. apis indicator strain			
		*Aa*37		*Aa*74	
Antagonist		D*	V*	D*	V*
B. cereus	ATCC 11778	0	14	0	0
	m6c	6	26	6	49
	mv33	0	13	2	22
	mv50b	12	0	0	6
	m387	3	19	0	0
	m395	15	25	2	0
	m434	9	13	0	0
	LPcer1	6	13	0	0
	MexB	0	21	0	11
	MexC	7	16	0	0
B. clausii	Fr231	8	30	8	30
	m448b	12	31	20	24
B. licheniformis	NRRL B-1001	6	31	6	44
	mv55	3	16	0	0
B. megaterium	NRRL B-939	17	21	12	21
	m435	44	0	39	15
B. pumilus	ATCC 7061	0	16	0	0
	mv49b	0	0	0	0
	mv81	6	29	6	31
	m116	11	14	0	0
	m350	0	0	9	0
	m363	6	7	17	35
	m414	34	7	0	0
B. subtilis	ATCC 10783	0	0	0	0
	xx	44	11	41	23
	m329	39	14	17	30
	m334	44	13	53	31
	m347	21	13	0	0
	m351	43	9	43	18
Br. borstelensis	RC	37	34	100	67
Br. laterosporus	BLAT169	0	0	14	0
	BLAT170	0	0	11	0
	BLAT171	18	0	9	0
P. amylolyticus	NRRL B-14940	17	15	18	15

*D: diffusible compounds, V: volatile compounds. Scores used in statistical analysis were assigned based on the following MGI percentage: Inhibition scale: R1: 0–20%, R2: 21–40%, R3: 41–60%, R4: 61–80%, R5: 81–100%. Showed results correspond to 48 h.

As far as we know, this is the first evidence of *Br. borstelensis* strain showing antagonism against *P. larvae* and *A. apis in vitro*. The only reference to biocontrol potential of *Br. borstelensis* is a BLIS of about 12 kDa active against *B. subtilis*, *Clostridium perfringens* and *Listeria monocytogenes* (Sharma, Gupta, & Gautan, 2014).

All *B. subtilis* strains tested here ($n = 6$) that showed high inhibition of *P. larvae* and *A. apis* contained at least three homologous genes for encoding production of bacillomycin L, fengycin, bacilysin, subtilin, iturin A, or surfactin (Figure 8). Our experiments are consistent with previous findings that reported *in vitro* activity against *P. larvae* or *A. apis* by *B. subtilis* (Alippi & Reynaldi, 2006; Omar et al., 2014; Reynaldi et al., 2004; Sabaté, Carrillo, & Audicio, 2009). Sabaté and co-workers (2009) concluded that *B. subtilis* strains inhibited *P. larvae* by surfactin production and, in the case of *A. apis* the inhibition was due to the production of an antimycotic compound. Other authors reported that *B. subtilis* and related species produced VOCs exhibiting antagonism against fungal plant pathogens, and that the antagonism was species-specific among different fungi and bacteria (Liu, Mu, Zhu, Du, & Liu, 2008). A synergistic activity of bacillomycin and fengycin against phytopathogenic fungi has been reported (Koumoutsi et al., 2004). Interestingly, we found the same combination of *fenD* and *bmyB* in two *B. subtilis* strains (xx and m351) that showed very good inhibition of *P. larvae* and *A. apis* strains when testing diffusible compounds (Figure 6A). Moreover, all *B. subtilis* strains tested showed surfactant capacity and formed a thick superficial pellicle with an intricate vein-like appearance similar to that reported by Branda, González-Pastor, Ben-Yehuda, Losick, and Kolter (2001). Furthermore, genes that mediate production of surfactin (*srfAA* and *sfp*) were shown to be required for the surfactant capacity and pellicle formation. Results presented here are not only in agreement with those reported by Branda et al. (2001) but also with the antagonistic response of these strains.

Despite the fact that *B. pumilus* strains studied here contained none or few antimicrobial peptides genes, our results, and also those obtained in previous studies (Alippi & Reynaldi, 2006), indicate that are potent antagonists against all *P. larvae* genotypes. Other authors reported bacitracin, pumicilin, and pumilacidin-producers *B. pumilus* strains with activity against Gram-positive bacteria (Abriouel et al., 2011; Sumi et al., 2015). Furthermore, the dipeptide antibiotic tetaine produced by *B. pumilus* B-180 was described as chemically and physically identical to bacilysin (Özcengiz & Öğülür, 2015) and *B. pumilus* strains mv81, m363, and m414 contained homologous gene of *bacA* (Figure 8).

B. clausii strains Fr231 and m448b showed strain-specific antagonism against *P. larvae* genotypes I and IV when testing diffusible compounds (Table 3). These results, together with those reported earlier (Alippi & Reynaldi, 2006), provide novel information about the antagonistic activity of *B. clausii* against *P. larvae*. We have to point out that *B. clausii* strain m448b contained *fen*D. Other authors reported a bacteriocin-producing strain of *B. clausii* active against *Agrobacterium tumefaciens* and *C. albicans* (Mouloud, Daoud, Bassem, Laribi Atef, & Hani, 2013) and also probiotic *B. clausii* strains with anti-staphylococcal but not anti-*Salmonella* activity *in vitro* (Urdaci, Bressollier, & Pinchuk, 2004).

Concerning *B. cereus*, all strains tested were good antagonists against genotype ERIC I considering both diffusible and VOCs. Also, showed higher inhibition values for VOCs in comparison with those obtained by diffusion against all genotypes, particularly in the case of ERIC II and IV (Table 3). *B. cereus* strains tested here contained homologous genes related to iturin A production (*ituC*, *ituD* or *lpa-14*; Figure 8). We believe that this is the first report of *B. cereus* strains active against

Table 5. Parameters related to surfactant capacity.

Bacterial antagonist	Media	Drop collapse[a]			Superficial pellicle[b]			Acidic precipitate[c]			Hemolytic activity[d]
		MYPGP	IST	BHI-T	MYPGP	IST	BHI-T	MYPGP	IST	BHI-T	Blood agar
B. cereus	ATCC11778	−	−	−	−	−	−	+	+	−	(+) β
	m6c	−	−	−	−	+	−	+	−	−	(+) β
	mv33	−	−	−	−	+	−	+	−	−	(+) β
	mv50b	−	−	−	+	+	+	+	+	−	(+) β
	m387	−	−	−	−	−	−	+	−	+	(+) β
	m395	−	−	−	−	+	−	+	+	−	(+) β
	m434	−	−	−	+	+	−	+	−	−	(+) β
	LPcer1	−	−	−	−	+	−	+	−	−	(+) βD
	MexB	−	−	−	−	+	+	+	+	−	(+) β
	MexC	−	−	−	−	+	−	+	−	+	(+) β
B. clausii	Fr231	−	+	−	−	++	++	+	+	−	(+) α
	m448b	−	+	−	−	−	+	+	−	+	(+) αβ
B. licheniformis	NRRL B-1001	−	−	+	++	++	++	+	+	+	(+) αβ
	mv55	−	−	−	−	+	−	+	+	+	(+) β
B. megaterium	NRRL B-939	−	−	+	−	−	−	+	+	−	(+) β
	m435	−	−	−	++	++	++	+	+	+	(+) β
B. pumilus	ATCC7061	−	−	+	−	−	−	+	+	+	(+) β
	mv49b	+	−	+	−	+	−	+	+	−	(+) β
	mv81	−	−	−	++	++	++	+	+	+	(+) αβ
	m116	−	−	−	+	+	+	+	+	+	(+) αβ
	m350	+	+	+	+	+	−	+	+	−	(+) β
	m363	−	−	−	+	+	−	+	+	+	(+) β
	m414	−	−	−	+	+	+	+	+	+	(+) β
B. subtilis	ATCC10783	+	+	+	++	++	++	+	+	+	(+) β
	xx	−	+	−	++	++	++	+	+	+	(+) β
	m329	+	+	+	++	++	++	+	+	+	(−)
	m334	+	+	+	++	++	++	+	+	+	(+) β
	m347	+	+	+	++	−	−	+	+	+	(+) αβ
	m351	+	+	+	++	+	++	−	+	−	(+) αβ
Br. borstelensis	RC	+	−	−	−	−	−	+	+	+	(+) αβ
Br. laterosporus	BLAT169	+	+	+	−	−	−	−	−	−	(+) αβ
	BLAT170	+	+	+	−	−	−	−	−	+	(+) αβ
	BLAT171	+	+	+	−	−	−	+	−	−	(+) β
P. amylolyticus	NRRL B-14940	+	+	+	−	−	−	−	−	−	(+) αβ

[a]Drop collapse: (−) negative response; (+) collapse of the drop. [b]Superficial pellicle formation (−) No pellicle formation; (+): thin pellicle observed and (++): thick pellicle observed. [c]Acidic precipitate: (−) Absence of precipitate, (+) precipitate formed. [d]Hemolytic activity: (−) negative, (+) separated in α: alpha hemolysis; β: beta hemolysis; α β: alpha-beta hemolysis and β D: beta discontinuous.

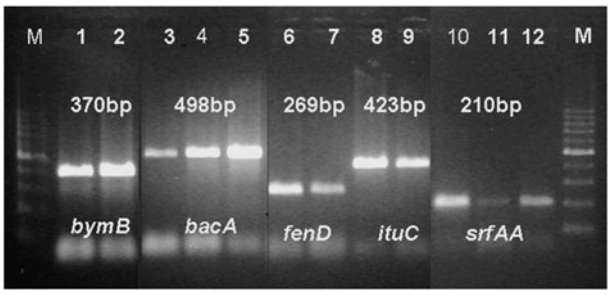

Figure 7. Composed gel showing PCR product profiles of representative samples tested. Lanes M: Molecular weight marker, CienMarker (Biodynamics, Argentina); lanes 1 and 2 *B. subtilis* strains m351 and xx, respectively containing *bymB*; lanes 3, 4 and 5: *B. pumilus* m414, *B. subtilis* m329 and *B. megaterium* m435 containing *bacA*; lanes 6 and 7: *B. cereus* m6c and *B. clausii* m448b containing *fenD*; Lanes 8 and 9: *B. pumilus* m350 and *B. cereus* mv33 containing *ituC*; lanes 10, 11 and 12: *B. licheniformis* mv55, *Br. laterosporus* BLAT170, and *Br. borstelensis* RC containing *srfAA*, respectively.

P. larvae in vitro that contains iturin A. Benítez and coworkers found an iturin-producing strain of *B. amyloliquefaciens* that showed *in vitro* activity against two strains of *P. larvae* (Benítez et al., 2012). They observed an extensive lysis and degradation of bacterial cells due to changes in the structure of cell walls and membranes by SEM. Furthermore, Hsieh, Lin, Meng, and Kao (2008) found that *B. subtilis*, *B. amyloliquefaciens* and *B. circulans* strains that contained homologous genes of *ituD* and *lpa-14* are iturin-A producing strains as confirmed by HPLC. Since they concluded that the PCR method was valuable for quick identification of iturin-A producing strains (Hsieh et al., 2008). In a previous study, Minnaard and Alippi (2016) characterized two BLIS obtained by *B. cereus* strains m6c and m387 of approximately 6.2 kDa and four bands between 6.2 and under 14.4 kDa, respectively. Both purified BLIS showed a narrow activity range mainly limited to *P. larvae* strains (10 from a total of 17 positives for BLISm6c and 12 from a total of 17 positives for BLISm387). Both BLIS were active against most *P. larvae* strains belonging to genotypes ERIC I (10 out of 11 tested) and ERIC II ($n = 2$), whereas strains belonging to ERIC IV ($n = 3$) were insensitive. In contrast, Gram-negative bacteria tested

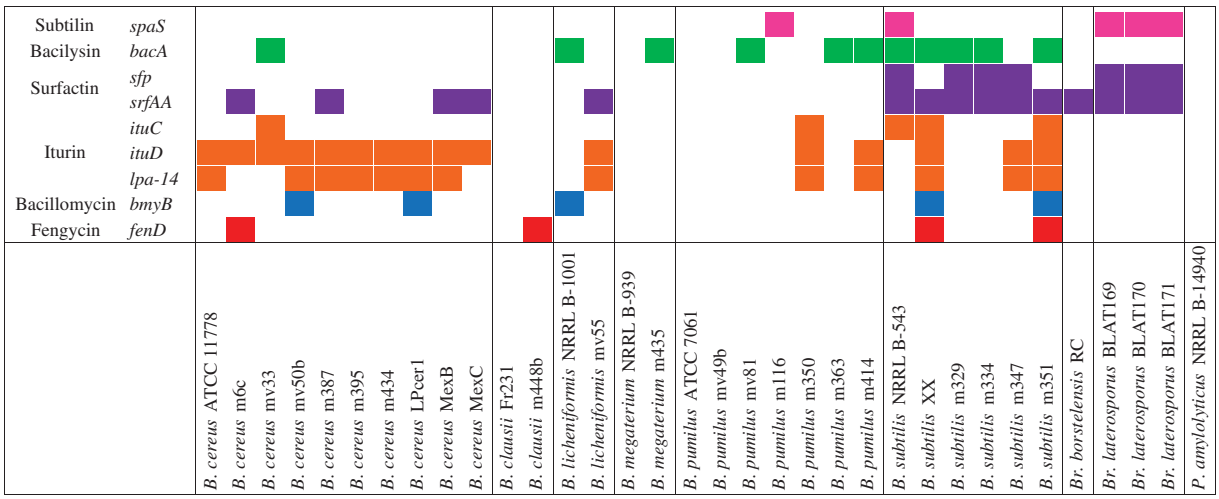

Figure 8. Distribution of antimicrobial peptide gene markers in 34 bacterial antagonists of different species of *Bacillus*, *Brevibacillus,* and *Paenibacillus*.

($n = 12$) were insensitive to both BLIS. Furthermore, *B. cereus* m6c and m387 were the best antagonists together with *B. cereus* mv33 and *B. subtilis* m387 against 17 *P. larvae* strains belonging to ERIC I and from different geographical areas (Alippi & Reynaldi, 2006). Additionally, strain m6c contained homologous genes of *srfAA*, *ituD*, and *fenD*, while strain m387 contained *srfAA*, *ituD*, and *lpa-14*. To our knowledge, this is the first report of the presence of a homologous gene of *fenD* in a *B. cereus* strain. The antimicrobial activity in *B. cereus* strains m6c and m387 could be related not only to the presence of antimicrobial peptide genes but also to the presence of different BLIS. Further studies are needed to clarify this hypothesis.

Br. laterosporus strains tested here ($n = 3$) contained the same combination of homologous genes of *srfAA* and *sfp* related to surfactin and also *spaS* related to subtilin production. Most *Br. laterosporus* strains showed excellent antagonism against genotypes ERIC I and IV but showed slight to null inhibition against ERIC II when testing diffusible compounds. However, slight to null inhibition was observed when testing VOCs against all genotypes (Table 3). Yang, Huang, Yuan, Zhang, and Yousef (2016) reported brevibacillin as a potent antimicrobial lipopeptide active against Gram-positive bacteria produced by *Br. laterosporus* strain OSY-II. Also, Jiang et al. (2015) reported a bogorol B producing *Br. laterosporus* strain JX-5 that exhibits activity against phytopathogenic fungi by permeating the fungal membrane, fracturing the nuclei, and damaging the cell walls. Other author reported strains of *Br. laterosporus* that inhibited several phytopathogenic fungi and also Gram-positive bacteria by the production of chitinases and antimicrobial peptides (Ruiu, 2013). Our strain *Br. laterosporus* BLAT170 inhibited the growth of several foliar fungal pathogens of wheat not only by inhibition against fungal growth and conidial germination *in vitro* but also by reducing disease incidence under greenhouse conditions (Alippi, Perelló, Sisterna, Greco, & Cordo, 2000). Even though previous studies reported antifungal activity by *Br. laterosporus* strains, including BLAT170, very low to null inhibition against *A. apis* was observed in the present study.

Despite the variability observed within the 34 *Bacillus*, *Brevibacillus*, and *Paenibacillus* strains, we can select the most potent strains against one or both pathogens, considering if diffusible or/and VOCs were involved. We can conclude that *B. pumilus* mv81, m350, and m414 showed very good inhibition against all *P. larvae* genotypes by diffusible and VOCs in most media tested; *B. clausii* Fr231 and m448b and *Br. laterosporus* BLAT169, BLAT170, and BLAT171 were excellent antagonists against genotypes ERIC I and IV by diffusible compounds. Furthermore, *B. subtilis* xx, m334, m351 and *Br. borstelensis* RC inhibited the growth of all *P. larvae* genotypes and also *A. apis* strains by diffusible compounds. Future work will be focused on the identification of the diverse bioactive compounds involved in the antagonism of these strains.

Disclosure statement

No potential conflict of interest was reported by the authors.

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