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Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples

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Abstract

Three *Bacillus* strains isolated from honey samples and bee gut were pre-selected for their in vitro antimicrobial activity against *Paenibacillus larvae* and *Ascosphaera apis*, important honeybee pathogens. The analysis of their 16S rRNA sequences revealed that C4, M1 and G2III strains belong to the *subtilis* species. Surfactin synthesis was verified by IR spectroscopy and HPLC studies. Surfactin inhibited *P. larvae* but it failed to affect *A. apis*. Vegetative cells of *P. larvae* were affected as soon as they came in contact with the surfactin sample; two orders of magnitude less in log scale were recorded. Optimal surfactin production was observed in MEL medium, a broth with molasses as the only carbon source. *Bacillus subtilis* G2III strain exhibited the highest levels of surfactin synthesis in BHI and MEL broths: 1391 AU/ml and 2782 AU/ml, respectively. Since only *A. apis* inhibition was observed when cell suspensions were assayed, we suspect that there may be an antimycotic compound within cells. The co-production of surfactin and a fungicide by these strains might biologically control bee pathogens in apiculture.

Keywords: Honeybee pathogens; *Paenibacillus larvae*; Surfactin; Antimycotic compound

1. Introduction

The genus *Bacillus* species are ubiquitous, endospore-forming Gram-positive bacteria that are harmless to mammalians, with the exception of *Bacillus cereus* and *Bacillus anthracis*. Their primary habitat is the soil and, due to their extreme heat-resistant spores, they can colonize different environments such as honey, food, animals, insects, etc. In particular, different *Bacillus subtilis* strains are ingested in the diet as contaminants or as part of selected fermented foods like nato- or probiotic supplements, available not only for humans, but also for animals [8,20,40,45]. They are also biologically and commercially important as producers of a great variety of secondary metabolites such as antibiotics, bioinsecticides, enzymes and lipopeptides [11,22,29,36].

Paenibacillus larvae, a Gram-positive sporulated bacterium that causes the American foulbrood disease, is an extremely contagious and dangerous pathogen of honeybees [47]. It only takes 10 spores to make a 24-h-old larva ill [6], and economic loss in apiculture is substantial due to high mortality rates. Oxytetracycline has been used to control American foulbrood, but resistant strains have already been identified [1,14,32]. Chalkbrood is a fungal disease caused by the heterothalic ascomycete *Ascosphaera apis* that results in mummified bee larvae [23,47]. Although it is not as serious as American foulbrood, since the spread of the fungus within a colony is limited, pharmacological and chemical treatments have proved to be ineffective in controlling it because they fail to kill the spores in the beehives [17,23]. As the presence of these

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pathogens causes significant economic loss to apiculture, natural approaches to controlling and preventing these diseases must be found.

Therefore, the aim of this work was to phenotypically and genotypically isolate and characterize *Bacillus* strains associated with the bee intestine and/or honey and to determine their in vitro potential against *P. larvae* and *A. apis*, with the purpose of exploring feasible biological control.

2. Materials and methods

2.1. Honeybee intestinal tract and honey sampling, culture media and growth conditions for Bacillus strain isolation

Honey samples from Atamisqui, Morillos and Tartagal (northwestern Argentina towns) and independent pools of the whole intestinal tract, midgut and posterior intestine of different honeybees from El Galpón (Salta, Argentina) were homogenized in saline solution (0.85% w/v NaCl) [43]. The samples were carefully handled to avoid contamination either by environmental material or by the external surface of the bee body. Each honey sample was dissolved with sterile distilled water and warmed at 80 °C for 10 min to kill vegetative cells. All samples were spread on brain–heart infusion agar (BHI, Britania, Argentina) and on MPYGP solid medium (1.5% yeast extract, 1% Müeller–Hinton broth, 0.2% glucose, 0.3% K_2HPO_4, 0.1% sodium piruvate; 2% agar; pH 7) [12]. Plates were incubated at 37 °C for 24 h, and typical *Bacillus* colonies were transferred to BHI broth.

2.2. Identification of selected strains by pheno- and genotypic characterization

The following biochemical tests were performed to identify the strains: Gram and spore stains, light microscopy observation, mobility, Proskauer and catalase tests, nitrate reduction, growth at different incubation temperatures and NaCl concentrations, starch hydrolysis and use of glucose, xylose and manitol [16]. All strains isolated were stored at −20 °C in BHI broth with 20% v/v glycerol.

For genotypic characterization, isolates were genetically characterized according to the internal transcribed spacer (ITS) by analysis of the 16S subunit of rRNA, and sequencing was performed on both strands by the commercial services of Macrogen Inc. (Seoul, Korea). Briefly, the intergenic 16S–23S transcribed spacer PCR (ITS-PCR) was carried out using nucleotide single universal strand primers S-D-BACT-1494-A-S-20 (GTCGTAACAAGGTAGCCGTA) and L-D-BACT-0035-A-A-15 (CAAGGCATCCACCGT) [10,31]. The extracted genomic DNA was amplified in a 25 µl reaction mixture containing 0.2 µl Taq polymerase, 2.5 µl 10× buffer STR, 0.1 µl primer, 17.5 µl PCR water and 5 µl DNA. Amplification consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, and a final extension at 72 °C for 7 min [31]. Control reaction mixtures lacking template DNA were also included in each experiment. The PCR products were separated on 0.8% agarose gel electrophoresis running at 65 V for 50 min. Gel patterns were visualized by ethidium bromide staining and photographs taken under UV light.

2.3. Indicator microorganisms and growth conditions

P. larvae strains (Azul, I, II, III, IV, RaB 1, 8 and 10), kindly provided by Dr. Terzolo and Eng. Borracci (INTA-Balcarce, Argentina), were activated in MPYGP agar at 37 °C for 72 h in a microaerophilic atmosphere. *A. apis* strains (M, B, F and V), provided by Eng. Bailac (San Luis, Argentina), were propagated on MY20 medium (0.5% meat peptone, 0.3% yeast extract, 0.3% malt extract, 20% glucose and 2% agar) [44] at 30 °C for 5–7 days.

2.4. Antimicrobial activity assays

These tests were carried out by the well-diffusion assay [5]. Both cellular suspensions and cell-free supernatants (CFSs) corresponding to the different isolated bacilli were analyzed. CFSs were obtained from these bacteria cultured in BHI broth for 24 h at 37 °C without shaking. They were centrifuged (10,000 × *g* for 15 min at 4 °C), filter-sterilized (0.45 µm pore size cellulose acetate filter) and kept at 4 °C until use. For the agar diffusion technique, 10 ml of molten MPYGP 1.5% agar were inoculated with 300 µl of a *P. larvae* cell suspension obtained as follows: bacterial colonies grown in MPYGP agar were recovered using a sterile cotton swab soaked in buffered-peptone water, and they were resuspended in 2 ml of MPYGP broth (*ca.* 1×10^7 CFU/ml); then, they were poured into Petri plates. The suspension was left to cool and solidify, and wells were punched in it. Twenty-three microliters of CFS were placed in each hole made on *P. larvae* lawn (*ca.* 1×10^8 CFU/ml). After incubation at 37 °C for 48 h, plates were examined for the presence of inhibition halos. An *A. apis* spore suspension was extended in duplicate on MY20 agar. One was left as control of normal mold growth and 25 µl of the *Bacillus* cell suspensions or the CFSs were placed in the holes of the other plate. Plates were incubated at 30 °C for up to 7 days.

2.5. Hemolytic activity

To test hemolytic activity, fresh cultures of all *Bacillus* strains studied were streaked on Columbia agar plates containing 5% sheep blood (Laborit, Argentina) and incubated for 48–72 h at 37 °C. Clear zones around the colonies indicated β-hemolysin activity [49].

2.6. Screening for lipopeptide synthesis

Precipitates of different CFSs resulting from acidification with 55 µL of concentrated HCl were recovered by centrifugation (14,000 × *g* for 25 min at 4 °C). Lipopeptides were extracted with methanol [49]. The solvent was evaporated and the precipitate was dissolved in sterile distilled water at pH 8.

2.6.1. Saccharomyces cerevisiae lysis assay

Colonies of the different bacilli of around 5–10 mm in diameter were obtained on BHI agar plates after 24 h incubation at 37 °C. Plates were refrigerated for 24 h more and overlaid with Sabouraud agar (Britania, Argentina) seeded with *S. cerevisiae*. According to Feignier et al. [15], the iturin-producer bacilli gave a zone of yeast growth inhibition. The iturin non-producer strains were detected by the absence of such an inhibition zone.

2.6.2. HPLC analyses

Twenty-five microliters of solution were injected into a Spherisorb C18 column (5 µm, 250 × 4.6 mm) and monitored at 220 nm. Flow speed was 0.5 ml/min. The mobile phase was TFA 0.8 mM:acetonitrile 99.94%. No gradient was used to elute the column. A purified surfactin sample (Sigma) was used as control.

2.7. IR studies

IR absorption spectra were obtained with an IR-TF Bruker model IFS-88 spectrometer. IR spectra were recorded for 400–4000 wave numbers (cm^{-1}) with a resolution of 2 wave numbers per wave number. Samples were prepared by dispersing the solid uniformly in a matrix of potassium bromide.

2.8. Surfactin effect studies on P. larvae *viability*

Quantitative analysis was performed by direct contact between *P. larvae* and the CFS or the partially purified surfactin fraction. *P. larvae* Azul was used as the indicator strain because no significant differences were observed among pathogenic strains (Table 1). The *P. larvae* cell suspension was prepared as described before. Mixtures of equal volumes of the cell suspension and CFS, or the surfactin fraction, were placed in contact and incubated at 37 °C for 30 min, 1, 2 and 24 h under microaerophilic conditions. Peptone water dilutions were made and viable *P. larvae* cell number was determined by plate count.

2.9. Surfactin synthesis in different culture media

The selected *Bacillus* strains, M1, C4 and G2III, were inoculated in BHI broth, nutritive broth (NB: meat extract 3 g/l, meat peptone 5 g/l) and MEL medium ($(NH_4)_2SO_4$, 7.0 g/l; Na_2HPO_4 $12H_2O$, 3.8 g/l; $MgSO_4$ $7H_2O$, 0.7 g/l; yeast extract, 0.5 g/l and molasses 0.2 g/l) prepared in our laboratory [27]. Active cells from cultures in BHI broth were used to inoculate the other media (inoculum 1% v/v). They were then incubated at 37 °C for 96 h. Surfactin was recovered from the CFS at different times during the incubation period. Its antimicrobial activity was determined against *P. larvae* and *A. apis* by the well-diffusion assay. Surfactin production was determined by HPLC analyses, and its titer (AU/ml) was determined in the different fractions by the serial dilution assay [9] using *P. larvae* Azul as indicator strain. Briefly, 23 µl of each dilution were put in 5 mm holes made in MPYGP agar plate inoculated with 100 µl of an active culture of the pathogen. Plates were incubated at 37 °C for 24 h under microaerophilic conditions. The titer of the fraction, as AU/ml, is defined as the reciprocal of the largest dilution that still inhibits development of the sensitive strain.

2.10. Statistical analysis

Statistical analysis was carried out according to Tukey's test and considered significant at $P < 0.05$ level. Assays were performed in triplicate.

3. Results and discussion

Innocuous bacterial strains that may produce antimicrobial substances are of great interest to confine or inhibit different

Table 1
Identification and characterization of *Bacillus* sp. strains from honey and bee gut samples.

Isolate	Origin	Anaerobic growth	Growth 7% NaCl	Growth at 50 °C	Starch hydrolysis	Xylose	Mannitol	Glucose	Hemolysis	Lipopeptides[a]	Accession GenBank
B. subtilis G2III	Bee gut	–	+	+	+	+	+	+	β	+	EU195330
B. subtilis subsp. *niger* C4	Honey	–	+	+	+	+	+	+	β	+	EU195328
B. subtilis M1	Honey	–	+	+	+	+	+	+	β	+	EU557670
B. subtilis Mori 2	Honey	–	+	+	+	+	+	+	β	+	EU195329
Bacillus sp. G2B	Bee gut	–	+	+	+	+	+	+	ND	+	ND
Bacillus sp. Cachi	Honey	–	+	+	+	+	+	+	β	+	ND
Bacillus sp. ID2	Bee gut	–	–	+	+	–	+	+	β	+	ND
Bacillus sp. C3	Bee gut	–	+	+	+	–	+	+	β	+	ND
Bacillus sp. M3	Honey	–	+	+	–	–	–	–	β	+	ND
Bacillus sp. IG1	Bee gut	–	+	+	+	–	–	+	β	+	ND
Bacillus sp. IG	Bee gut	–	+	+	+	–	+	+	β	+	ND
Bacillus sp. IG3	Bee gut	–	+	+	+	–	–	–	β	+	ND
Bacillus sp. G3C	Bee gut	–	+	+	+	+	+	+	–	+	ND
Bacillus sp. G1	Honey	–	+	+	+	–	+	+	–	+	ND
Bacillus sp. M4	Honey	–	+	+	+	+	+	+	ND	+	ND

ND: not determined.
[a] Precipitated by concentrated HCl addition.

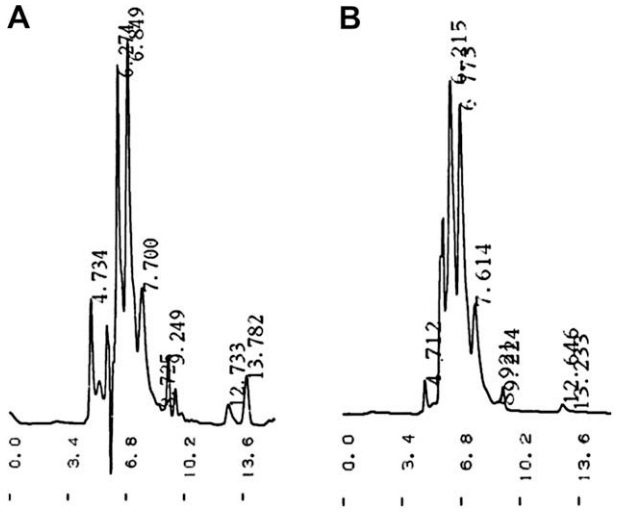

Fig. 1. Chromatographic profile by HPLC analyses of a crude mixture of surfactins produced by *B. subtilis* G2III in MEL medium. A, sigma surfactin standard; B, G2III strain fraction.

pathogens or spoilage microorganisms by natural means. With the exception of *B. anthracis* and many *B. cereus* toxin-producer strains, *Bacillus* strains are considered safe. The non-toxigenic species, as they do not harbor drug resistance genes and are non-pathogenic, are safe and could be used for agricultural, veterinary and human purposes [13,41,46]. Within this context, sixty mobile, spore-forming, catalase-positive and Gram-positive aerobic bacilli were isolated from different honey samples and bee gut. Fifteen were pre-selected for their antimicrobial properties. In all cases, the final pH after bacterial growth in BHI broth was similar to that of the non-inoculated medium (6.0–6.5). They grew in the presence of 7% NaCl, at 50 °C and the Voges Proskauer test was positive. They did not grow in anaerobiosis or in thioglycolate broth. They used maltose, glucose and xylose and hydrolyzed starch. Hence, according to these phenotypic properties, these bacteria were identified as *B. subtilis* [16]. Recent taxonomic studies have revealed that *B. subtilis* should be considered as a complex group of closely related species [33,38,39] and variation in DNA sequence readily distinguishes the *B. cereus* group of organisms from the closely related *B. subtilis* group, suggesting that both species may be identified without mistakes [3,4,7,19,21,25,30,33,34,48]. Hence, 16S rDNA sequence analysis of the selected bacilli was determined and it was observed that C4, M1 and G2lll strains exhibited 99, 99 and 98% DNA sequence identity to database entries associated with known *Bacillus* species, respectively. Then, both BLAST sequence similarity of their 16S rDNA sequence and biochemical tests indicated that these strains are closely related to *B. subtilis* (99%). The 16S rRNA nucleotide sequence data of *B. subtilis* C4, G2III and M1 were deposited in GenBank (accession numbers EU195328, EU195330 and EU557670, respectively) (http://www.ncbi.nlm.nih.gov). The C4 strain also showed a reddish film in the liquid–air interface of different media after 72 h of incubation at 37 °C. However, this pigment was not detected in the colonies of this bacterium when it grew on a solid surface. Many authors suggested that there are some pigmented variants of *B. subtilis* that can grow in a culture medium supplemented with tyrosine and these variants could be named subsp. *niger* [16,42]. These strains usually present this pigment, but not on all culture media; this property may occasionally be lost during serial transfer. In this case, since *B. subtilis* C4 always showed this property, it could be acknowledged as *B. subtilis* subsp. *niger* for this phenotypic characteristic and the aforementioned references. However, further in-depth assays must be carried out in order to confirm this hypothesis. These results are summarized in Table 1.

The three *Bacillus* strains selected, C4, M1 and G2lll, produced different lipopeptides which were detected by the presence of a flocculent precipitate when concentrated HCl was added to their CFS. These microorganisms presented β-hamolytic activity on sheep blood agar but they failed to lyse *S. cerevisiae* cells. These results suggest surfactin production [15,49]. Moreover, the IR spectrum in KBr showed characteristic peptide bands at 3305 cm^{-1} (NH stretching mode) and at 1645 cm^{-1} (stretching mode of the CO–N bond), bands at 2956–2924 cm^{-1}, 2869 cm^{-1}, 1463 cm^{-1} and 1377 cm^{-1} that indicate aliphatic chains ($-CH_2$, $-CH_3$) and a band observed at 1734 cm^{-1} that corresponds to a carbonyl group (data not shown). HPLC analyses also revealed that all these strains synthesize surfactin, since all of them presented similar chromatographic profiles which closely resembled those of the surfactin standard employed (Fig. 1A and B).

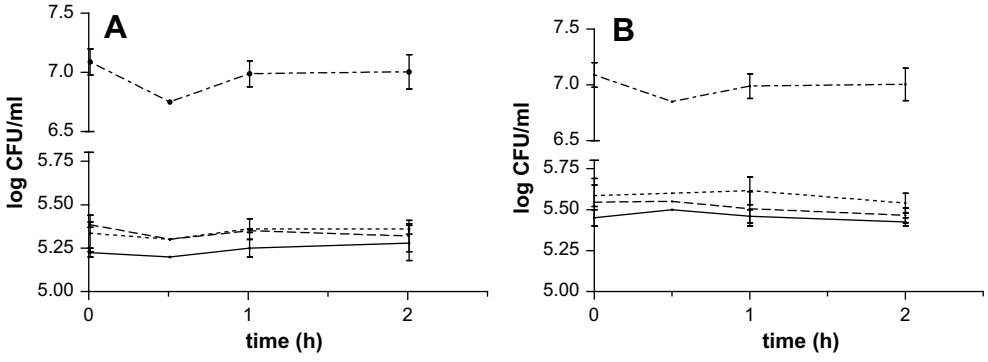

Fig. 2. *P. larvae* Azul inhibition by direct contact with: A, surfactin fraction; B, cell-free supernatant (CFS). - · - *P. larvae* Azul control; ··· *B. subtilis* subsp. *niger* C4; - - - *B. subtilis* M1; — *B. subtilis* G2III.

Table 2
B. subtilis surfactin synthesis in different culture media. In vitro *P. larvae* inhibition by the well-diffusion assay.

Surfactin fraction	Culture medium[a]	*P. larvae*[a] inhibition halo (mm)								
		I	II	III	IV	Azul	RaB	1	8	10
B. subtilis M1	BHI	10	9	10	10	13	10	12	11	10
	MEL	18	17	17	17	20	14	18	18	27
	CN	–	–	–	–	–	–	–	–	–
B. subtilis G2III	BHI	18	20	20	18	18	18	21	23	19
	MEL	22	24	24	22	24	20	23	22	24
	CN	–	–	–	–	–	–	–	–	–
B. subtilis subsp. *niger* C4	BHI	2	2	3	4	3	2	3	2	2
	MEL	5	7	6	6	6	8	5	4	4
	CN	–	–	–	–	–	–	–	–	–

No inhibition was detected.
[a] *P. larvae* lawn was prepared in MPYGP agar.

Hence, our results would agree with those obtained by other authors who used similar techniques [15,26,27,49].

Natural surfactin produced by *B. subtilis* is a mixture of isoforms with slightly different properties as a result of substitutions in amino acids and the aliphatic chain [18,24,28,35]. Surfactin is not only an excellent biosurfactant, but also exhibits several other important biological activities: it is an antibiotic with antitumoral and antiviral action [36,46]. The techniques employed here revealed an antagonistic action of both CFS and the fractions containing surfactin upon the different *P. larvae* strains. An immediate effect on *P. larvae* viability was detected: as soon as surfactin was placed in contact with the cells, the number of viable cells fell by almost 2 orders of magnitude (Fig. 2A). No statistically significant differences were found between the inhibitory effect of CFS and the surfactin fraction (Fig. 2A and B, respectively). A longer contact time between cells and surfactin did not enhance the inhibitory effect. This particularly interesting result reveals that the vegetative cells of this sporulated pathogen are very sensitive to the effect of surfactin, as they die after a very short period of contact. Furthermore, the similarity between the effect of CFS and the surfactin fraction would indicate that surfactin is responsible for the marked antagonistic effect. To our knowledge, this is the first report of *B. subtilis* strains that inhibit *P. larvae* by surfactin synthesis, although several *Bacillus* and *Paenibacillus* species have been reported to inhibit *P. larvae* [2]. Alippi and Reynaldi [2] observed that *P. larvae* inhibition could be related to bacteriocin-like compounds produced by *B. cereus* and *licheniformis*, or by enzymatic activity in *B. laterosporus* and *B. megaterium* strains, but their results were preliminary because they did not determine the chemical nature of the metabolites involved in the inhibitory activity.

Surfactin synthesis was evaluated in two conventional laboratory culture media: BHI (with peptones and glucose) and NB (no carbon source). MEL, a low-cost culture medium designed by Joshi et al. [26], was also assayed because the molasses that it contains as a carbon source is a waste product of the Argentinean northwestern sugar refineries. It would be a cheap alternative for obtaining biomass and biosurfactant on a large scale. HPLC analyses revealed that the three *Bacillus* strains synthesized surfactin in all media. However, synthesis reached an inhibitory concentration of *P. larvae* strains only in BHI and MEL media (Table 2). Each strain behaved differently, although they all synthesized more surfactin in the MEL medium. *B. subtilis* G2III synthesized the highest

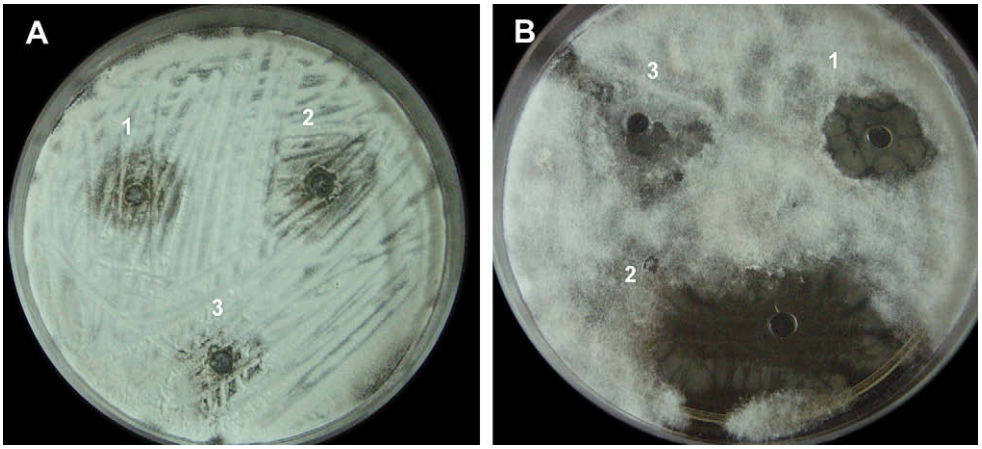

Fig. 3. Inhibition of A, *A. apis* M and B, *A. apis* B by cell suspensions of 1: *Bacillus subtilis* subsp. *niger* C4, 2: *B. subtilis* G2III and 3: *B. subtilis* M1.

concentration of surfactin after 72 h of incubation at 37 °C, and 1391 AU/ml and 2782 AU/ml were detected in BHI and MEL broth, respectively. *B. subtilis* C4 strain synthesized the least amount of the biosurfactant. The results of this analysis were consistent with previous studies showing that satisfactory production of biosurfactants is reached in media supplemented with varying concentrations of molasses as the carbon source [26,27].

These strains also exhibited an antifungal effect and growth inhibition was detected against *A. apis* (Fig. 3A and B). The antifungal effect was only detected with *Bacillus* cell suspensions. It has been reported in several articles that different *B. subtilis* strains that synthesize surfactin also produce iturin, and the synthesis of this biosurfactant would be accompanied by *Bacillus* cell sporulation [41]. Our selected *B. subtilis* cells and their lipopeptide fraction failed to produce inhibition of *S. cerevisiae*, a simple assay suggested by Feignier et al. [15] to test for iturin-producer *Bacillus* strains. These results, together with HPLC surfactin detection in the CFS, would allow us to infer that iturin was not the antifungal compound synthesized by these strains.

Only two articles have reported growth inhibition of *A. apis* by different *Bacillus* spp. strains [17,37]. A remarkable feature of strains isolated in this study is that the same *B. subtilis* strains (C4, M1 or G2III) that inhibit *P. larvae* by surfactin synthesis also affect *A. apis* via a fungicide that might be bonded to the cell surface.

Our results are promising because we isolated and selected three different *B. subtilis* strains that can inhibit two important honeybee pathogens by two different mechanisms: surfactin synthesis and fungicide or cell-to-cell interaction. Hence, the antimicrobial compounds of strains characterized in this work may have a valuable potential as biological control agents.

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References

[1] Alippi, A.M., López, A.C., Reynaldi, F.J., Grasso, D.H., Aguilar, O.M. (2007) Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees. Vet. Microbiol. 125, 290–303.

[2] Alippi, A.M., Reynaldi, F.J. (2006) Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources. J. Invertebr. Pathol. 91, 141–146.

[3] Ash, C., Collins, M.D. (1992) Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett. 94, 75–80.

[4] Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E., Collins, M.D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus* and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. 41, 343–346.

[5] Audisio, M.C., Terzolo, H.R., Apella, M.C. (2005) Bacteriocin from honeybee beebread *Enterococcus avium* active against *Listeria monocytogenes*. Appl. Environ. Microbiol. 71, 3373–3375.

[6] Bambrick, J.F., Rothenbuhler, W.C. (1961) Resistance to American fouldbrood in honey bees. IV. The relationship between larvae age at inoculation and mortality in a resistant and in a susceptible line. J. Insect Pathol. 3, 381–390.

[7] Bourque, S.N., Valero, J.R., Lavoie, M.C., Levesque, R.C. (1995) Comparative analysis of the 16 to 23S ribosomal intergenic spacer region sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. Appl. Environ. Microbiol. 61, 1623–1626.

[8] Cartman, S.T., La Ragione, R.M., Woodward, M.J. (2007) Bacterial spore formers as probiotics for poultry. Food Sci. Tech. Bull. Funct. Foods 4, 21–30.

[9] Daba, H., Pandian, S., Gosselin, J.F., Simard, R.E., Huang, J., Lacroix, C. (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. Appl. Environ. Microbiol. 57, 3450–3455.

[10] Daffonchio, D., Borin, S., Frova, G., Manachini, P., Sorlin, C. (1998) PCR fingerprinting of whole genomes: the spaces between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveals a different intraespecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int. J. Syst. Bacteriol. 48, 107–116.

[11] Desai, J.D., Banat, I.M. (1997) Microbial production of surfactant and their commercial potential. Microbiol. Mol. Biol. Rev. 61, 47–64.

[12] Dingman, D.W., Stahly, D.P. (1983) Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl. Environ. Microbiol. 46, 860–869.

[13] Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O., Cutting, S.M. (2004) Characterization of *Bacillus* probiotics available for human uses. Appl. Environ. Microbiol. 70, 2161–2171.

[14] Evans, J.D. (2003) Diverse origins of tetracycline resistance in the honeybee bacterial pathogen *Paenibacillus larvae*. J. Invertebr. Pathol. 83, 46–50.

[15] Feignier, C., Besson, F., Michel, G. (1995) Studies on lipopeptides biosynthesis by *Bacillus subtilis*: isolation and characterization of iturin-, surfactin-mutants. FEMS Microbiol. Lett. 127, 11–15.

[16] Gibson, T., Gordon, R.E. (1986) Bergey's Manual of Determinative Bacteriology. Baltimore: The Williams & Wilkins Co.

[17] Gilliam, M. (1993) Chalkbrood control. In L.J. Connor, T. Rinderer, H.A. Sylvester, & S. Wongsiri (Eds.), Asian Apiculture (pp. 589–595). Cheshire, CT 06410, USA: Wicwas Press.

[18] Grangemard, I., Peypoux, F., Wallach, J., Das, B.C., Labbe, H.C., Caille, A., Genest, M., Maget-Dana, R., Ptak, M., Bonmatin, J.M. (1997) Lipopeptides with improved properties: structure by NMR, purification by HPLC and structure–activity relationships of new isoleucyl-rich surfactins. J. Peptide Sci. 3, 145–154.

[19] Green, C.J., Stewart, G.C., Hollis, M.A., Vold, B.S., Bott, K.F. (1985) Nucleotide sequence of the *Bacillus subtilis* ribosomal RNA operon, rrnB. Gene 37, 261–266.

[20] Green, D.H., Wakeley, P.R., Page, A., Barnes, A., Baccigalupi, L., Ricca, E., Cutting, S.M. (1999) Characterization of two *Bacillus* probiotics. Appl. Environ. Microbiol. 65, 4288–4291.

[21] Harrel, L.J., Andersen, G.L., Wilson, K.H. (1995) Genetic variability of *Bacillus anthracis* and related organisms. J. Clin. Microbiol. 33, 1847–1850.

[22] Healy, M.G., Devine, C.M., Murphy, R. (1996) Microbial production of biosurfactants. Resour. Conserv. Recycl. 18, 41–57.

[23] Heath, L. (1982) Development of chalkbrood in a honeybee colony: a review. Bee World 63, 119–130.

[24] Hosono, K., Suzuki, H. (1983) Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. I. Purification, physicochemical properties and structures of fatty acid residues. J. Antibiot. 36, 667–673.

[25] Johnson, Y.A., Nagpal, M., Krahmer, M.T., Fox, K.F., Fox, A. (2000) Precise molecular weight determination of PCR products of the rRNA interspace spacer region using electrospray quadruple mass spectrometry for differentiation of *B. subtilis* and *B. atrophaeus*, closely related species of bacilli. J. Microbiol. Meth. 40, 241−254.

[26] Joshi, S., Bharucha, Ch, Desai, A.J. (2008) Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. Biores. Technol. 99, 4603−4608.

[27] Joshi, S., Bharucha, Ch., Jha, S., Yadav, S., Nerurkar, A., Desai, A.J. (2008) Biosurfactant production using molasses and whey under thermophilic conditions. Biores. Technol. 99, 195−199.

[28] Kanatomo, S., Nagai, S., Ohki, K., Yasuda, Y. (1995) Study on surfactin, a cyclic depsipeptide. I. Isolation and structure of eight surfuctin analogs produced by *Bacillus natto* KMD 2311. Yakugaku Zasshi. 115, 756−764.

[29] Katz, E., Demain, A.L. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. Bacteriol. Rev. 41, 449−474.

[30] Lechner, S., Mayr, R., Francis, K.P., Prüss, B.M., Kaplan, T., Wiessner-Gunkel, E., Gordon, S.A., Stewart, B., Scherer, S. (1998) *Bacillus weihenstaphensis* sp nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Bacteriol. 48, 1373−1382.

[31] Martínez, M.A., Siñeriz, F. (2004) PCR fingerprinting of rRNA intergenic spacer regions for molecular characterization of environmental bacteria isolates. In J.F.T.G. Spencer, & A.L. Ragout de Spencer (Eds.), Environmental Microbiology: Methods and Protocols. Book Series Methods in Biotechnology (pp. 159−166). Totowa, NJ: Humana Press.

[32] Miyagi, T., Peng, C.Y.S., Chuang, R.Y., Mussen, E.C., Spivak, M.S., Doi, R.H. (2000) Verification of oxytetracicline-resistant American foulbrood pathogen *Paenibacillus larvae* in the United States. J. Invertebr. Pathol. 75, 95−96.

[33] Nakamura, L.K. (1989) Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and proposal for *Bacillus atrophaeus* sp. nov. Int. J. Syst. Bacteriol. 39, 295−300.

[34] Nakamura, L.K., Roberts, M.S., Cohan, F.M. (1999) Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizeni* subsp. nov. Int. J. Syst. Evol. Microbiol. 49, 1211−1215.

[35] Peypoux, F., Bonmatin, J.M., Labbe, H., Grangemard, I., Das, B.C., Ptak, M., Wallach, J., Michel, G. (1994) [Ala4]Surfactin, a novel isoform from *Bacillus subtilis* studied by mass and NMR spectroscopies. Eur. J. Biochem. 224, 89−96.

[36] Phister, T.G., O'Sullivan, D.J., McKay, L.L. (2004) Identification of bacilysin, chlorotetaine, and iturin A produced by *Bacillus* sp. strain CS93 isolated from pozol, a Mexican fermented maize dough. Appl. Environ. Microbiol. 70, 631−634.

[37] Reynaldi, F.J., De Giusti, M., Alippi, A.M. (2004) Inhibition of the growth of *Ascosphaera apis* by *Bacillus* and *Paenibacillus* strains isolated from honey. Rev. Arg. Microbiol. 36, 52−55.

[38] Roberts, M.S., Nakamura, L.K., Cohan, F.M. (1994) *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. Int. J. Syst. Bacteriol. 44, 256−264.

[39] Roberts, M.S., Nakamura, L.K., Cohan, F.M. (1996) *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis*, isolated from soil in Death Valley, California. Int. J. Syst. Bacteriol. 46, 470−475.

[40] Sanders, M.E., Morelli, L., Tompkins, T.A. (2003) Sporeformers as human probiotics: *Bacillus, Sporolactobacillus*, and *Brevibacillus*. Comp. Rev. Food Sci. Food Saf. (CRFSFS) 2, 101−110.

[41] Sandrin, C., Peypoux, F., Michel, G. (1990) Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. Biotech. Appl. Biochem. 12, 370−375.

[42] Smith, N.R., Gordon, R.E., Clark, F.E. (1946) Aerobic Mesophilic Sporeforming BacteriaMiscellaneous publication no. 559. DC: U.S. Department of Agriculture.

[43] Snodgrass, R.E., Erickson, E.H. (1992) The Anatomy of the Honeybee, the Hive and the Honeybee. Hamilton, USA: Dadant and Sons. pp. 103−169.

[44] Takatori, K., Tanaka, I. (1982) *Ascosphaera apis* isolated from chalk brood in honey bees. Jap. J. Zootechnol. Sci. 2, 89−92.

[45] Urdaci, M.C., Bressollier, P., Pinchuk, I. (2004) *Bacillus clausii*. Antimicrobial and immunomodulatory activities. J. Clin. Gastroent. 38, S86−S90.

[46] Van Dyke, M.L., Lee, H., Trevors, J.T. (1991) Applications of microbial surfactants. Biotechnol. Adv. 9, 241−252.

[47] Williams, D.L. (2000) A veterinary approach to the European honey-bee (*Apis mellifera*. Rev. Vet. J. 160, 61−73.

[48] Wunschel, D., Fox, K.F., Black, G.E., Fox, A. (1994) Discrimination among the *Bacillus cereus* group, in comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. Syst. Appl. Microbiol. 17, 625−635.

[49] Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Knapp, R.M., McInerney, M.J. (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Meth. 56, 339−347.