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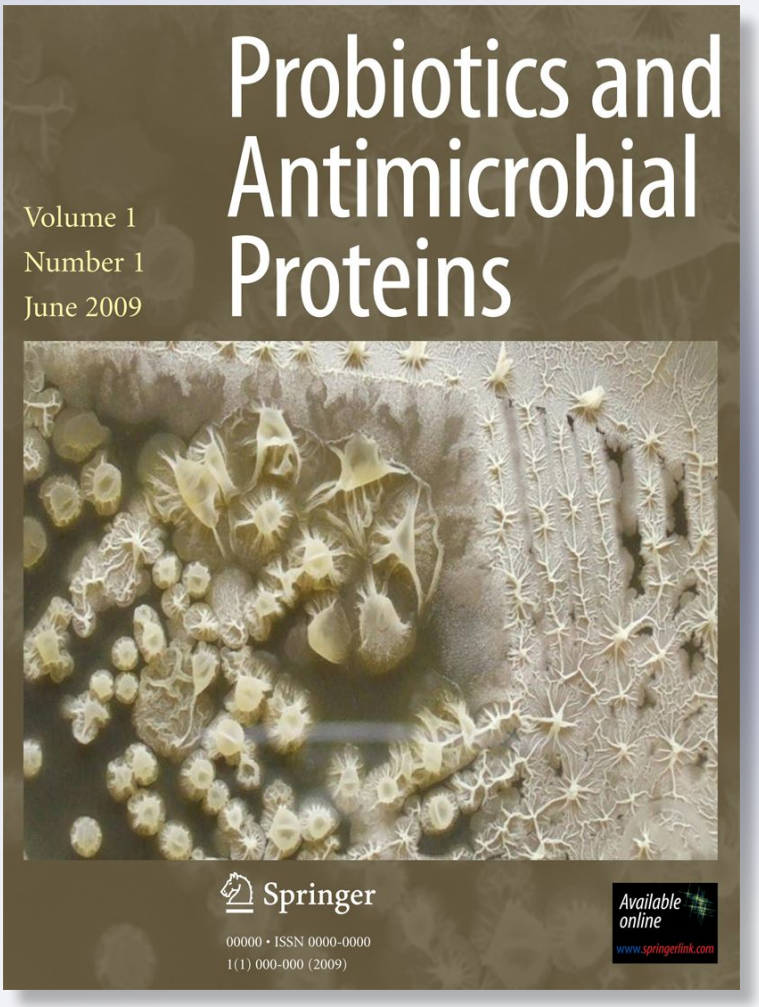

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Beneficial Effects of *Bacillus subtilis* subsp. *subtilis* Mori2, a Honey-Associated Strain, on Honeybee Colony Performance

D. C. Sabaté · M. S. Cruz · M. R. Benítez-Ahrendts · M. C. Audisio

Abstract A *Bacillus* spp. strain isolated from a honey sample in Morillos (Salta, Argentina) was phylogenetically characterized as *B. subtilis* subsp. *subtilis* Mori2. The strain was administered to bee colonies as a monoculture in one litre of sugarcane syrup (125 g/L) at a final concentration of 10^5 spores/mL to evaluate the bee colony performance. The treated colony was monitored, and any changes were compared with the control hives. All conditions were identical (weather, nourishment and supervision), except for the *Bacillus* spore supplement. The new nourishment, which was administered monthly from May to December 2010, was accepted by the bees and consumed within ca. 24–48 h. Photograph records and statistic analyses revealed significant differences in the open and operculated brood areas between the treated and control groups. The status of the colony improved after the second administration of the *Bacillus* spores until the end of the experiment. A higher number of bees were counted in the treated groups (26% more than the control) with respect to the initial number. Furthermore, at the time of harvest, honey storage in the treated hives was 17% higher than in the control hives. In addition, spore counts of both *Nosema* sp. and *Varroa* sp. foretica in treated hives were lower than in the control hives. These results with experimental hives would indicate that *B. subtilis* subsp. *subtilis* Mori2 favoured the performance of bees; firstly, because the micro-organism stimulated the queen's egg laying, translating into a higher number of bees and consequently more honey. Secondly, because it reduced the prevalence of two important bee diseases worldwide: nosemosis and varroosis.

Keywords Apis mellifera · *Bacillus subtilis* subsp. *subtilis* · Probiotic · Colony performance

Introduction

The Argentinean Republic is an important producer and exporter of honey [1]. Indiscriminate use of antibiotics has had a negative impact on the commercialization of apiculture products. In order to combat disease, beekeepers frequently use antibiotics and pesticides that, apart from the production of resistant pathogens [2–4], cause an imbalance of the normal bee microbiota [5]. This latter factor affects the bees' health and may alter its orientation and consequently may reduce the number of hive integrants [6]. Furthermore, the use of antibiotics or chemical products increases the risk of contamination of the hive products as they can remain in the honey and affect its quality, making it unsuitable for human consumption [7]. In numerous countries, antibiotics like chloramphenicol have been found in honey and other apiculture products [8, 9].

Natural and non-contaminant alternatives could be safer options to maintain a hive strong and healthy. An ideal solution should be innocuous to the bees (offspring and adults), not leaving any residue in the hive products,

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mainly honey, and easy to apply. The use of probiotics or competitive exclusion cultures has been used successfully in humans and animals such as poultry, pigs and fish [10–14].

Different *Bacillus* strains have been selected and studied as probiotic supplements and have been proven suitable not only for human but also for animal consumption. As these bacteria form spores, they present advantages over bacteria like *Lactobacillus* and *Bifidobacterium* traditionally used in probiotic formulae. They are able to withstand high temperatures and can be maintained viable without alteration and without refrigeration for long periods of time [15, 16]. They are also able to survive the low pH of the stomach [17]. The harmlessness of strains belonging to the *Bacillus* genus, except for *B. anthracis* and several *B. cereus* strains, has been thoroughly studied, and adverse effects have never been observed. In 2008, *B. coagulans* as GanedenBC^{30} was the first *Bacillus* strain that was defined as GRAS. To date, the use of *B. subtilis* as a food supplement has been approved, for example, in Italy and the United Kingdom [13].

The objective of the current study was to assess the effect of a *Bacillus* spp. strain, isolated from honey, as a probiotic supplement on the development of *Apis mellifera* beehives. It also evaluated the impact of this treatment in the normal index or prevalence of *Nosema* sp. and *Varroa* sp. in the beehive.

Materials and Methods

Bacterial Strain and Growth Conditions

Bacillus sp. Mori2 was isolated in the province of Salta from a honey sample of Morillos (Salta, Argentina) [18, 19]. This bacterium was characterized by classical biochemical tests for the *Bacillus* genus [20], and the typing of genus, species and subspecies was done by both the 16S ribosomal DNA sequence and the *B. subtilis gyrA* gene fragments analysis with PCR reactions as described as follows:

Phylogenetic Characterization of Bacillus sp.

DNA extraction was carried out with an active culture after incubation in 5 mL of brain heart infusion broth (BHI, Britania, Argentina) at 37 °C for 24 h according to Miller [21].

For the genotypic characterization, the isolate was genetically characterized by analysis of the subunit 16S of rRNA, and sequencing was performed on both strands by the commercial services of Macrogen Inc. (Seoul, Korea). Briefly, intergenic 16S–23S transcribed spacer PCR (ITS-PCR) was carried out using nucleotide single universal strand primers S-D-Bact-0008-a-S-20 (AGAGTT TGATCCTGGCTCAG) and S-D-Bact-1495-a-A-20 (CTA CGGCTACCTTGTTACGA) [22]. 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. 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 volts for 50 min. Gel patterns were visualized by ethidium bromide staining, and photographs taken under UV light. Online search for similarity was carried out at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

Phylogenetic Characterization at Subspecies Level

Bacillus subtilis gyrA gene fragments was amplified with PCR using *gyrA*-f (5-CAGTCAGGAAATGCGTACGTC CTT-3) and *gyrA*-r (5-CAAGGTAATGCTCCAGGCAT TGCT-3) primers [23]. Polymerase chain reaction (PCR) was carried out by amplification of DNA in 25-µL reaction mixture containing the same amounts of reagents listed in "Phylogenetic Characterization of *Bacillus* sp". Amplification consisted of an initial denaturation step at 94 °C for 2 min; 40 cycles of 94 °C for 30 s; 51 °C for 45 s and 72 °C for 60 s; and a final extension at 72 °C for 7 min [24]. The PCR products were analysed as it was explained above.

Bacillus Sporulation in Different Culture Media

Cell viability and sporulation of the *Bacillus* strain were assayed in BHI broth and a culture medium containing molasses as a carbon source. This latter medium contained 0.7% (w/v) $(NH_4)_2SO_4$, 0.38% (w/v) Na_2HPO_4 $12H_2O$, 0.07% (w/v) $MgSO_4$ $7H_2O$ and 0.05% (w/v) yeast extract [25] and was supplemented with a different concentration of molasses: 1, 2 and 3% (w/v), named MEL1, MEL2 and MEL3, respectively. The effect of agitation and the addition of 1 mg/mL manganese sulphate salts on the sporulation were also assayed.

An active 24-h culture of the strain grown in BHI broth and with an initial cell density of approximately 10^7 cfu/mL was used to inoculate the different media at 1% (v/v). The media were incubated at 37 °C for 1 week, and during this period, cell viability and sporulation were assayed by cell counts on agar plates. Sporulation was assayed by heating 2 mL of each growth culture at 96 °C for 10 min to

eliminate vegetative cells. BHI agar was used for quantification, and plates were incubated at 37 °C for 24 h.

Bees, Hive Location and Environmental Conditions

The research was carried out with africanized local *Apis mellifera* L. bees kept in standard Langstroth hives.

The bee colonies used in this assays were nuclei prepared with an open brood frame, an operculated brood frame plus the bees present in this frame and finally, an open frame of honey. After 48 h, a royal chamber with INTA-PROAPI (Instituto Nacional de Tecnología Agropecuaria-Proyecto Integrado de Desarrollo Apícola, Argentina) genetic was incorporated.

Bacterial Spore Administration

Spores delivery to the bees was carried out by a Doolittle-type feeder. The minimum sugar concentration, which was accepted by the bees, was previously determined in the laboratory equal to 125 g, in a final volume of 1 L of tap water [26]. Adequate decimal dilutions of the strain were spread on BHI agar and incubated at 37 °C for 24 h. Cell viability and sporulation were determined as described in "*Bacillus* Sporulation in Different Culture Media".

Dose

The experiment comprised ten hives, each consisting of 10 frames. Five hives were administered with *Bacillus* spores added to syrup, and the other 5 were used as controls and only administered syrup. One Litre of saccharose syrup (125 g/L), supplemented or not with the bacilli at a final concentration of 1×10^5 spores/mL, was administered to each hive once a month for 8 consecutive months (from May to December 2010).

Bee Colony Parameters Tested

The evolution of the hives treated with bacterial spores was monitored, and any change was compared with the untreated control hives. All other conditions (weather, nourishment and supervision) were identical.

The parameters considered to qualify the general state of the colonies during the evaluation were as follows: number of bees in each frame, open and operculated brood areas and quantity of honey. During each visit to the apiary, photographs were taken of each frame (control and treated hives) and later analysed in the laboratory with software designed by the group. This image-analysis technique was developed taking into account the methods described by other authors with many modifications [26, 27]. After being photographed, the bee colonies were then fed with the spores. The honey stored was quantified as follows: It was taken into account that on a certain surface of the honeycomb, there were 20 cells with 10 g of honey, on average. To obtain these values, immature and capped honey were considered.

Effect of the Bacterial Supplement on the Bee Health Status

The effect of administration of the probiotic bacterial strain on the incidence of *Nosema* and *Varroa* was determined every 30 days according to the following methodology:

Nosemosis

Abundance of *Nosema* sp. was determined on 60 bees once the units were installed, and from then, every 30 days according to a method by Cantwell [28]. The *Nosema* spore load per adult bee (spores/bee) was measured using a Neubauer chamber. The following scale was applied to measure the degree of infestation: weak (<500,000), medium (between 500,000 and 1,000,000) and strong (>1,000,000).

Varroosis

Infestation of *Varroa* (*destructor*) was assayed in adult bees, using the jar test, originally designed by de Jong et al. [29] and modified by SENASA [30]. It has to be mentioned that flumethrin, a broad spectrum acaricide used in beekeeping, was administered to both control and treated colonies in May 2010.

Statistics

The variance analysis was carried out using DBCA on 5 control colonies (i.e. without any treatment) and on 5 spores-treated colonies, on the date of observation and photographic results as a variable and a probability error of $P < 0.05$ and $P < 0.01$. Comparison of the average values was carried out using the Tukey test to a significant level of $P > 0.05$ and $P = 0.01$.

Results

Phylogenetic Characterization of *Bacillus* spp. Strain

The 16S rDNA sequence analysis of the selected bacilli was determined, and it was observed that the Mori2 strain exhibited 99% DNA sequence identity to database entries associated with known *B. subtilis* strains. The 16S rRNA nucleotide sequence data of *B. subtilis* Mori 2 has been

deposited in the GenBank (accession numbers EU195329) (http://www.ncbi.nlm.nih.gov). Aslo, the *gyrA* gene codes for the A subunit of DNA gyrase in *Bacillus* strain. When the *gyrA* gene sequencing was carried out, it was found that *B. subtilis* Mori 2 showed 99% homology with the DNA gyrase sequence of *B. subtilis* subsp. *subtilis* 168 from the GenBank database using the BLAST program. The *gyrA* nucleotide sequences of the strain were deposited into the GenBank with accession number HQ828989. The genetic similarity of the strain can be observed in the phylogenetic tree given in Fig. 1. The phylogenetic distance of these strains with human pathogens of the *Bacillus* genus, like *B. cereus* and *B. anthracis*, should be stressed.

Sporulation of *Bacillus* in the Different Culture Media

Bacillus subtilis subsp. *subtilis* Mori2 reached the stationary growth phase after 24 h of incubation in the four media assayed. The highest cell density was 8.81 ± 0.10 log cfu/mL in MEL2 after 144 h of incubation, compared to 7.6 ± 0.15 log cfu/mL in BHI (data no shown).

Sporulation was favoured in MEL3 with 7.5 ± 0.05 log cfu/mL after 144 h of incubation, followed by MEL1 and MEL2 with 6.3 ± 0.20 and 6.15 ± 0.15 log cfu/mL, respectively, whereas BHI showed the lowest number of spores: 5.75 ± 0.05 log cfu/mL (Fig. 2a).

Agitation did not alter spore formation in MEL3, medium with the highest number of spores, while addition of manganese salts enhanced sporulation from 7.5 ± 0.05 to 8.2 ± 0.30 log cfu/mL after 96 h of incubation (Fig. 2b).

Due to these results, *B. subtilis* subsp. *subtilis* Mori2 was precultured in MEL3 supplemented with Mn salts prior to administration of the spores to the hives.

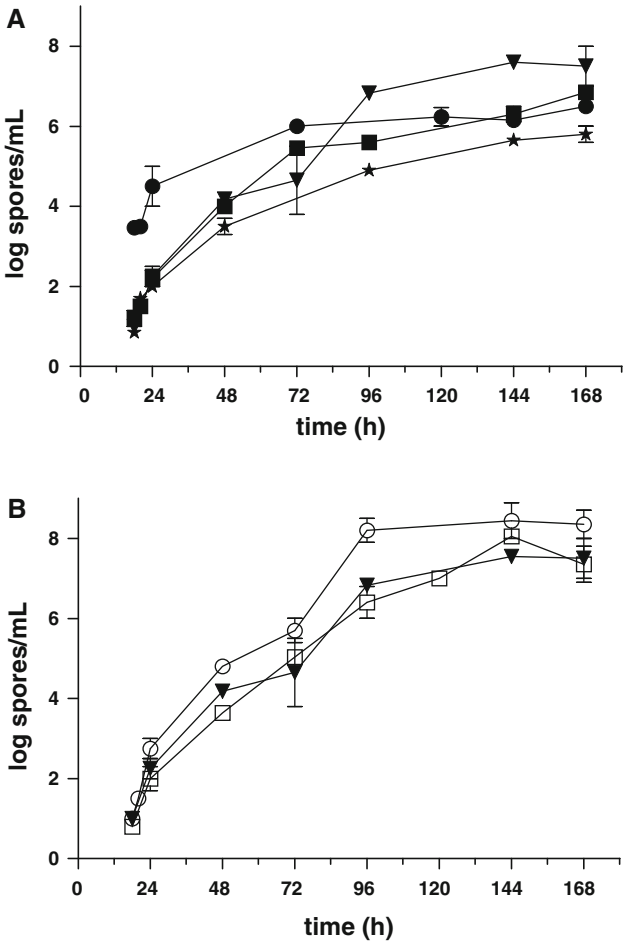

Fig. 2 Sporulation of *B. subtilis* subsp. *subtilis* Mori2 in the culture media assayed: (**a**) *asterisk* BHI; *filled square* MEL1; *filled circle* MEL2; *filled inverted triangle* MEL3, incubated at 37 °C without shaking; and (**b**) in MEL3 and incubated at 37 °C: *filled inverted triangle* without shaking; *open square* with shaking; *open circle* MEL3 supplemented with Mn salts. See text for medium conditions

Administration of Spores to Bees in Experimental Hives

It was estimated that bees in a hive consisting of 5 frames consumed 1 L of syrup with a concentration of 125 g/L of sugar and 10^5 spores of *B. subtilis* subsp. *subtilis* Mori2/mL between 24 and 48 h, as after this period the feeders, previously loaded with 1 L of syrup with bacteria, were empty.

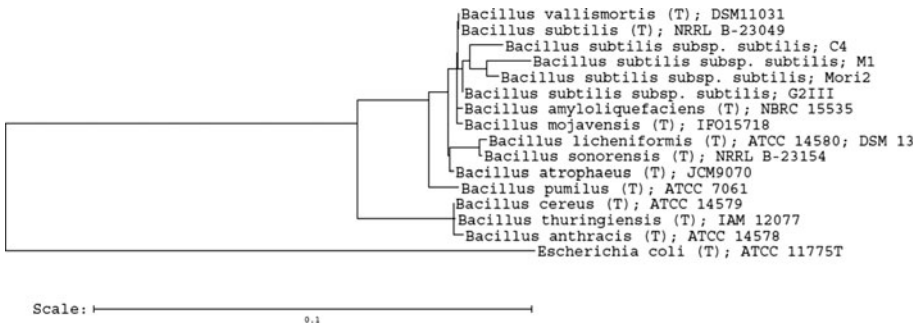

Fig. 1 Phylogenetic tree of the *B. subtilis* subsp. *subtilis* Mori2 strain was constructed using the tree builder program of the Ribosomal Data Base Project II (http://rdp.cme.msu.edu)

Effect of *B. subtilis* subsp. *subtilis* Mori2 on the Beehive Colonies

General Condition of the Colonies

When the bacterium was administered to the hives, the open brood area was larger after 2nd month onwards after application when compared with untreated hives: In June and July, values were 49 and 47% higher, respectively (Fig. 3a). At the same time, the operculated brood area was 25 and 31% larger in June and July, respectively, in hives treated with *B. subtilis* subsp. *subtilis* Mori2 compared to those without treatment (Fig. 3b).

Three months after administration of the bacterium, the area corresponding to treated bees was 26% larger than that of the untreated control bees. These figures maintained relatively stable during August and September with 23 and 20%, respectively. This would indicate an increase in the number of bees after the third month (Fig. 3c).

The stored honey, immature and capped, was expressed in grams of honey/cm^2, and was collected at the end of flowering season of the area in which the apiary is located (December–January). In the treated hives, a greater accumulation of honey of 20 and 35% was observed, compared to the control hives in the months of November and December, respectively (Fig. 3d).

Disease Incidence

Nosema Figure 4 shows a reduction in *Nosema* spore counts per bee, carried out monthly from May to December 2010, for treated compared to untreated hives.

Varroa The difference in percentage of infestation with foretic *Varroa* sp. between bees treated with *B. subtilis* subsp. *subtilis* Mori2 and control bees is shown in Fig. 5. Infestation was measured in open brood frames, using the jar test. During the 1st month after administration of the spore infestation with *Varroa* was reduced to zero, due to a previously administered sanitary treatment with Flumethrin, allowed into the apiculture. During all the assay, varroosis in the treated hives presented values close to zero, while the control hives were above the hives treated with spores since September.

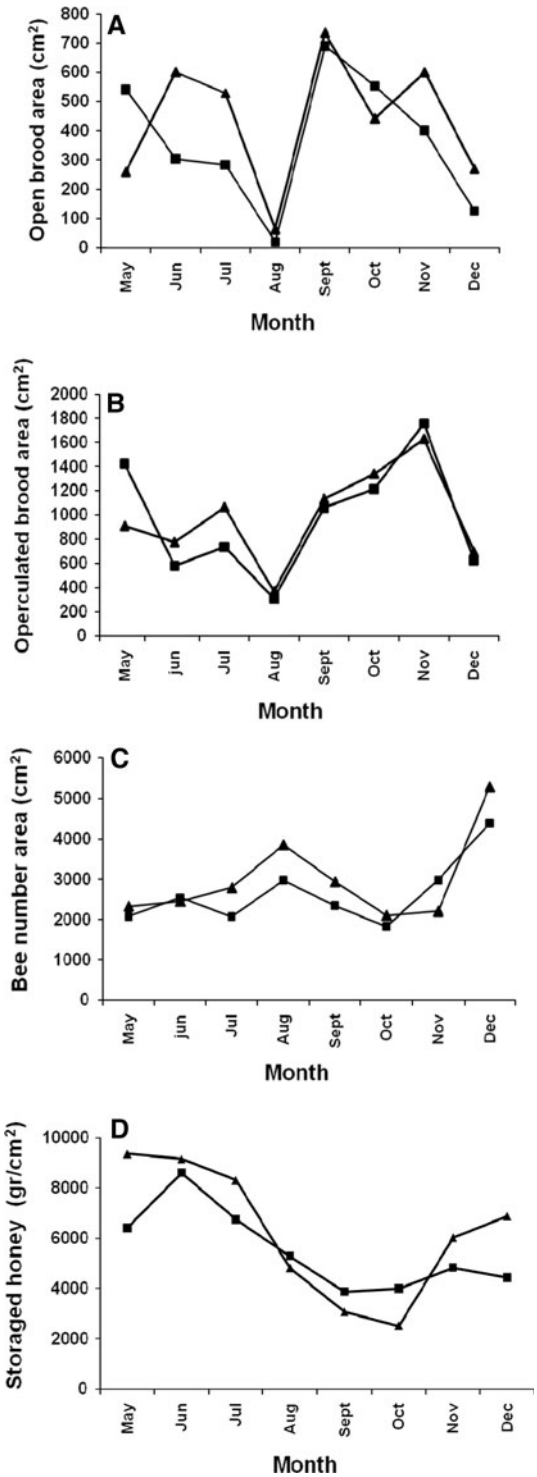

Fig. 3 Evolution of different beehive parameters during the trial using *B. subtilis* subsp. *subtilis* Mori2 (from May to December 2010: open and operculated brood areas (**a** and **b**, respectively), bee number area (**c**) and honey production (**d**) (*filled square* control, *filled triangle* treated)

Discussion

In order to use a micro-organism as a probiotic, it is important to determine with certainty its genus, species and subspecies, if applicable. In the current study, the *Bacillus* strain Mori2, isolated from honey, was selected for its ability to synthesize surfactin and its anti-*Paenibacillus larvae* activity [19]. The 16S sequence analysis showed 99% homology to *B. subtilis*. However, *B. subtilis* can be further classified into two subspecies: *B. subtilis* subsp. *subtilis* and

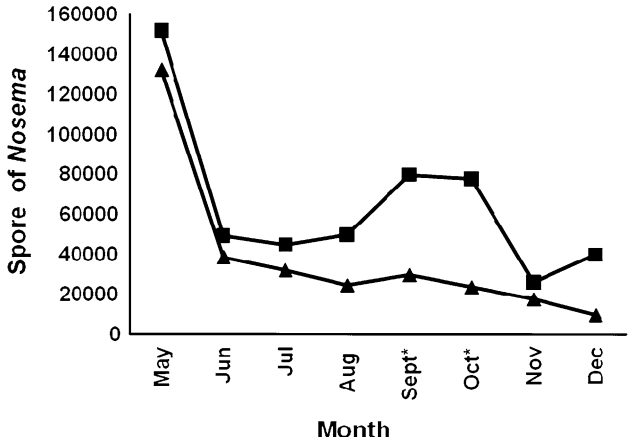

Fig. 4 Spore counts of *Nosema* sp. present in *Apis mellifera*. Samples were obtained from the gut of bees from hives treated with *B. subtilis* subsp. *subtilis* Mori2 (*filled triangle*) and control hives (*filled square*). (*Asterisk* months were differences in spores was significantly different.)

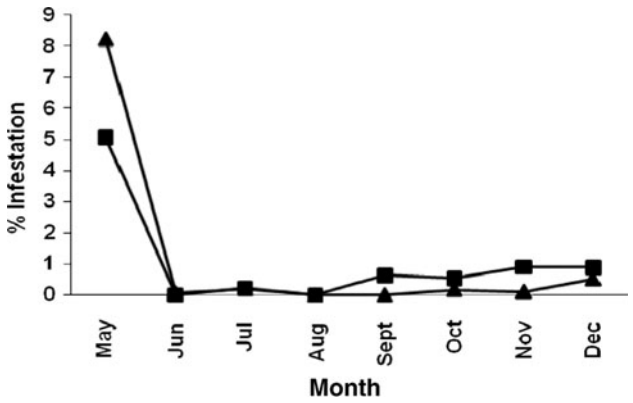

Fig. 5 Infestation (%) of foretic *Varroa* sp. present in *A. mellifera*, obtained through the flask technique using bees from hives treated with *B. subtilis* subsp. *subtilis* Mori2 (*filled triangle*) and control hives (*filled square*)

B. subtilis subsp. *spizizenii* [31]. Sequencing of the *gyrA* gene, which encodes the A subunit of DNA gyrase in *Bacillus*, is a useful tool to discriminate between species closely related to *B. subtilis* and between its subspecies [32]. The *gyrA* gene sequencing showed that *B. subtilis* Mori2 can be classified as *subtilis* subsp. *subtilis* Mori2 because it presented 99% homology to DNA gyrase from *subtilis* subsp. *subtilis* 168, phylogenetically characterized by Kunst et al. [23]. Iurlina and Fritz [33] determined the presence of bacilli in commercial honey and apiary samples in Argentina and found that 23% of the samples contained *Bacillus* spp., identified as *B. cereus*, *B. pumilus* and *B. laterosporus*. Therefore, the presence of *subtilis* subsp. *subtilis* in honey had not been reported until this paper.

Although different *Bacillus* species are used as biocontrol agents and probiotic supplements [16, 17], only a few studies exist on the production of vegetative cells for these purposes and the effect of nutrients in the culture medium to obtain a high spore density [34–36]. It is known, however, that transformation of vegetative cells into spores is induced by the exhaustion of nutrients such as carbon, nitrogen, phosphates or other essential micronutrients [37, 38]. Therefore, an adequate culture medium should be compatible with vegetative growth and spore formation [39]. Several authors have used molasses as a carbon source in a culture medium for growth and sporulation of *Bacillus* spp. strains [40–42]. As it was decided to administer spores from this bacterium to experimental hives, an economical culture medium was assayed in order to obtain a high and profitable number of spores. The low-cost culture medium described in the present study contains molasses as a carbon source, and growth and sporulation of the *Bacillus* strain was satisfactory. The number of spores in MEL1, MEL2 and MEL3 was higher than obtained in conventional BHI laboratory medium, and consequently, use of this inexpensive medium would be satisfactory for sporulation of this strain. The highest number of spores was 5×10^8/mL in a culture medium containing 3% (w/v) molasses and supplemented with manganese sulphate. This result agrees with other authors' whose have been informed that manganese sulphate favours sporulation [20]. Sella et al. [43] obtained about 10^9 cfu/g *Bacillus* spores when they used 2% (w/v) soy molasses in a solid medium and after 9 days of incubation. In the current study, the highest number of *B. subtilis* subsp. *subtilis* Mori2 spores was obtained after 96 h (4 days). Several authors have informed that sporulation in *Bacillus* strains should be carried out under agitation [44–46]; however, agitation did not favour sporulation in *B. subtilis* subsp. *subtilis* Mori2.

In apiculture, bees can be fed an artificial diet that can be either supportive or incentive [47]. In the first case, sugar cane is used to prepare a syrup with a water–sugar proportion of 1:1 (1 kg sugar cane/1 L of water). When saccharose syrup is used as an incentive diet in the north of Argentina, where this study was carried out, the proportion of water and sugar was 2:1 (2 kg sugar cane/1 L of water) or 1:1. It should be highlighted that the current study administered neither a supportive nor an incentive diet, as the syrup, administered once a month, contained 125 g/L of sugar, which is far below the normally used concentration. Acceptance of the bacteria administered in syrup by the bees was a key factor in the continuation of the different assays. The sugar concentration of the syrup (125 g/L) was chosen based on previous results in our laboratory with a *Lactobacillus johnsonii* strain, a micro-organism that is more demanding on nutrients than *Bacillus* [26].

In order to assess the different hive parameters, the technique of Calatayud and Verdú [48] was employed. The original method was designed to monitor the population of

Varroa jacobsoni Oud in beehives. Statistical analysis of the results revealed that hives treated with 1×10^5 *Bacillus* spores were significantly different from control hives. In vivo administration of *B. subtilis* subsp. *subtilis* Mori2 created a beneficial effect on the bee colonies. An increase in open and operculated brood could be observed after 2 and 3 months, translating into a higher number of bees. Also, at the end of the assays, it was observed in the treated hives a greater accumulation of honey compared to the control hives (November and December 2010).

A variety of bacteria has been used as probiotic supplements to hives. Máchová et al. [49] administered lactic acid bacteria (LAB) and *Bacillus* strains, isolated from bee gut, commercially available probiotics and dietary supplements to bees as a supplementary diet. The authors examined the bacterial colonization of the digestive tract, survival and general state of health of the bees and found that LAB more effective than the bacilli. Evans and López [50] studied the effect of commercially available probiotics containing *Lactobacillus* and *Bifidobacterium* on the immune response of bees. However, none of these authors determined the effect of the different probiotic supplements on the honey production.

It is interesting to mention that in the present study, *B. subtilis* subsp. *subtilis* Mori2 spores were administered to experimental hives with healthy colonies that were monitored according to sanitary regulations currently in force in Argentina. It was found that the administration of these bacteria positively affected the sanitary status of the hive. During the 4th month, the incidence of *Nosema* sp., an agent causing disease worldwide, was significantly reduced in hives treated with the selected *Bacillus* spores compared to untreated control hives. The technique used to quantify the number of *Nosema* spores did not allow differentiation between *Nosema apis* and *N. ceranae*. Treatment against *Nosema* is generally recommended when a hive presents an incidence of 500,000 spores/bee [51]. The values registered in the current study were well below this threshold, but coincidentally, the values decreased between May and June, coinciding with *Varroa* incidence/index values.

Varroosis is another worrying disease for beekeepers that is produced by the ectoparasite *Varroa destructor* and worldwide distributed [52]. The results obtained in this work showed that infestation with *Varroa* during the 1st month after administration of the probiotic bacilli was reduced to zero due to sanitary treatment with Flumethrin, a practice that is allowed in apiculture. Treatment with the acaricide was effective for 2 months (June–July), and then infestation with *Varroa* slowly increased until the end of the experiment (December 2010), when the first honey harvest of the 2010 season was gathered. Varroosis in the control hives was slightly higher than hives treated with *B. subtilis* subsp. *subtilis* Mori2. In the geological area where the apiary is located, this can be expected because between September and December (spring) egg laying by the queen bee increases and consequently the open and operculated brood area too. As a result, the foretic *Varroa* incidence increased because the brood areas grew.

These in vivo results indicate that *B. subtilis* subsp. *subtilis* Mori2 mainly favours open and operculated brood areas in beehives, demonstrating a strong stimulation of egg laying, higher stored honey, and reducing the *Varroa* and *Nosema* incident rates. Consequently, this probiotic culture might assist the beekeeper both in colony management and the creation of late nuclei and/or bee packages.

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