Honey yield of different commercial apiaries treated with *Lactobacillus salivarius* A3iob, a new bee-probiotic strain

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Abstract

The main objective of this study was to determine the impact of *Lactobacillus salivarius* A3iob, a honey bee gut-associated strain (GenBank code access KX198010), on honey yield. Independent assays were conducted from May to September 2014 and 2015, in three commercial apiaries: Tilquiza, El Carmen and Yala, all located in north-western Argentina. Local *Apis mellifera* L. bees were kept in standard Langstroth hives; treated hives were fed once a month with 1×10^5 cfu/ml viable *Lactobacillus* cells, administered to the bees through a Doolittle-type feeder in 125 g/l sucrose syrup. Control hives were only given the syrup mixed with MRS sterile broth. The main honey harvest was done in December in all groups and we found that there was an overall increase in honey yield from the treated hives. In 2014, all treated hives produced between 2.3 to 6.5 times more honey than the controls. However, in 2015, higher honey average yields in the treated hives at El Carmen and Yala were obtained, yet not at Tilquiza, because of a slight mishap. They experienced the swarming of several bee colonies due to a higher number of bees without appropriate management, which caused the control group to yield more honey compared to the hives fed with *Lactobacillus*. Interestingly, at El Carmen, two honey harvests were recorded: one in winter and another in summer (July and December 2015, respectively). This unexpected result arose from the particular flora of the region, mainly *Tithonia tubaeformis*, which blooms in winter. *L. salivarius* A3iob cells prove to be a natural alternative that will positively impact the beekeepers' economy by providing a higher honey yield.

Keywords: honey bee, probiotics, honey yield, *Lactobacillus*

1. Introduction

Lactobacillus is a heterogeneous genus containing, at present, over one hundred species that vary significantly in phenotypic, genotypic, biochemical and physiological characteristics (Hammes and Hertel, 2009). Several of its species are found in the normal microbiota of the gastrointestinal and genitourinary tracts of animals and humans (Ahrné *et al.*, 1998; Boris *et al.*, 1997; Hammes and Hertel, 2009). They have also been found in insects such as termites (Bauer *et al.*, 2000), bumblebees (Martinson *et al.*, 2011) and honey bees (Audisio *et al.* 2011; Endo and Salminen, 2013; Gilliam, 1997; Olofsson *et al.*, 2014; Rada *et al.*, 1997). This group of bacteria has been used for centuries in fermented foods and in the food industry, and they have been certified as 'generally recognised as safe' (GRAS) microorganisms. These properties have led to different *Lactobacillus* species being used in probiotic supplements (Fernández *et al.*, 2002; Klein, 1998). The international and scientifically accepted definition of a probiotic is 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2006). It is proven that these viable microorganisms employ several mechanisms that promote probiotic effects, such as competitive exclusion, alteration of the intestinal microbial communities, enhancement of host defence barriers, and modification of host signalling (Collado *et al.*, 2010; Lee and Salminen, 2009; Ouwehand *et al.*, 1999).

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Argentina is an important international honey producer (ranked 4th largest, after Turkey and Ukraine) and exporter (ranked 2nd, after China) (Vázquez *et al.*, 2009). For this reason, health and performance of hives are key for a great number of beekeepers, and is therefore the driving force behind finding natural and safe alternatives to improve beehive health and strength, and controlling honey bee diseases. Thus, different lactic acid bacteria belonging mainly to the *Lactobacillus* genus were isolated from the bee gut in order to study their potential as a probiotic (Audisio *et al.*, 2011). In particular, *Lactobacillus johnsonii* CRL1647 was selected due to its beneficial effects on *Apis mellifera* L. colonies, such as queen egg-laying stimulation, an increase in bees per beehive and higher honey yield (Audisio *et al.*, 2015). In this study, we evaluated a new strain, isolated from bee-gut by our group and preselected due to its *in vitro* inhibition against *Paenibacillus larvae*. Its effect on honey production was determined after being administered to commercial apiaries located in different micro-phytogeographic regions of Jujuy, a province in north-western Argentina.

2. Materials and methods

Microorganisms, culture media and growth conditions

Lactobacillus spp. A3iob was isolated from bee gut, as was described by Audisio *et al.* (2011). It was cultured in Man-Rogose-Sharpe broth (MRS, Biokar, Beauvais, France) at 37 °C for 18 h under microaerophilic conditions. It was preserved as frozen stock in MRS broth plus 20% (v/v) glycerol at -20 °C.

Phylogenetic characterisation of the *Lactobacillus* strain

DNA was extracted from lactobacilli cells according to Pospiech and Neumann (1995). The isolated DNA was genetically characterised by 16S rRNA amplification and sequencing. The nucleotide single universal strand primers f-s-D-BACT 008 (AGAGTTGATCCTGGCTCAG); r-s-D-BACT-1495 (CTACGGCTACCTTGTTGTTACGA) from Invitrogen service were used (De Silva *et al.*, 1998). The PCR was carried out in a reaction volume of 50 µl, which contained 5.0 µl of 10× STR reaction buffer (Promega, USA), 20 ng of total DNA, each oligonucleotide in 0.1 mM concentration, and 1 U of Taq polymerase (Invitrogen, São Paulo, Brazil). Amplification consisted of an initial denaturation step of 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min. The final extension step was performed at 72 °C for 7 min (Martínez and Siñeriz, 2004). The extractions were performed twice, on separate occasions. Finally, the amplicons were purified and sequenced by two different services: by the sequencing service from CERELA (Tucumán, Argentina), and, in parallel, at the Unit of Genomics/Node National Genomics Platform, CATG-INTA-Castelar (Buenos Aires, Argentina). An online search for similarity was carried out at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov). The phylogenetic analysis of the *Lactobacillus* strain sequence was conducted with MEGA 5.1 β4 software.

Trials with commercial apiaries

The experiments were carried out for two consecutive years, 2014 and 2015, as part of a research project between CONICET and beekeepers from Jujuy. Each beekeeper normally handled each colony at the apiary, and when lactobacilli had to be administered, one person from our group went to the apiary and administered the lactobacilli-cell suspension. The growth of the colonies was monitored and any change was compared with control hives that were not fed *Lactobacillus* cells. All other conditions (weather, geographical location, feeding and supervision) were identical between control and treated hives in each apiary.

Apiaries location and environmental conditions

The assays were carried out at three commercial apiaries located on the narrow strip of the Andean Yungas rainforest that cross the valleys of Jujuy. The Yungas rainforest runs along the eastern slope of the Andes mountains with a height range of 400-3,000 meters above sea level. The vegetation of the Yungas is composed of floors, or strips, of vegetation. The location and environmental conditions (i.e. flora, weather, etc.) are briefly described in Table 1.

Honey bee

Local *Apis mellifera* L. bee colonies were kept in standard Langstroth hives. Each year after summer ended a new set of colonies was selected for the study in each of the three apiaries. At the beginning of the assay, each hive was made with an open brood frame, an operculated brood frame (both frames covered with bees), two honey frames and five new stamped wax frames. Queen cells, taken from selected colonies in the apiary, were inserted in the new hive. First, the opening of the hive was reduced by a frame feeder. As the queen, fertilised naturally, began to breed and lay eggs, the frame feeder was removed to make room for the growing population. Once the new bee colonies were obtained, they were uniformed; thereby, the same initial size of all the hives was ensured (Ahmed, 2008). Finally, colonies were randomly assigned to treatment or control group.

Bacterial dose and administration

The *Lactobacillus* cell applications were done once a month, from May to September in both years. It has been determined and standardised that transferring 200 µl of *L. salivarius* A3iob fresh monoculture into 5 ml of sterile MRS broth allowed for a growth of 1×10^8 cfu/ml after 24 h of incubation (37 °C and microaerophilic conditions).

Table 1. General climatic characteristics of the regions of Jujuy province, where apiaries are located.

	Valley	Yungas
Strip of vegetation	Selva Montana	Bosque Montano
Meters above sea level	700-1,500	1,500-3,000
Annual rainfall (mm)	1,500-2,000	≤1000
Dominant tree species[1]	Maroma (*Ficus maroma)*	Pino del cerro (*Podocarpus parlatorei*)
	Laureles (*Cinnamomum porphyrium, Nectandra pichurim, Ocoteapuberula*)	Yoruma colorada (*Roupala meisneri*)
	Pocoy (*Inga edulis, I. semialata, I. saltensis*)	Aliso del cerro (*Alnus acuminata*)
	Tipa blanca (*Tipuana tipu*)	Nogal (*Juglans australis*)
	Horco molle *(Blepharocalix salicifolius)*	Arbolillo (*Viburnum seemenii*)
		Molulo (*Sambucus peruviana*)
		Paloyerba (*Ilex argentinum*)
Apiary	El Carmen	Tilquiza
		Yala
Coordinates[2]	Lat: 24°23'15'S Long: 65°15'33'W	Lat: 23°56'38'S Long: 65°13'55'0
		Lat: 24°07'00'S Long: 065°22'59'W

[1] Tree species are named as common name (scientific name).
[2] Lat: Latitude; Long: Longitude; S: South; W: West.

Next, 5 ml of viable *L. salivarius* A3iob cells (taken directly from the overnight broth culture) were transferred and resuspended in one litre of 125 g/l sucrose syrup in order to achieve a final concentration of 1×10^5 cfu/ml (Audisio and Benitez-Ahrendts, 2011), and then immediately administered to the colony by a Doolittle feeder.

In the first year of the assay (2014), five hives at Tilquiza, four hives at El Carmen and nine at Yala were administered with *L. salivarius* A3iob (final concentration of 1×10^5 cfu/ml). For controls, five other hives at Tilquiza, four other hives at El Carmen and nine other hives at Yala were administered with syrup (125 g/l) plus 5 ml sterile MRS culture broth. In the second year (2015), the number of control hives was retained (5 at Tilquiza and 9 at Yala) and 3 hives were used at El Carmen. As for *Lactobacillus*-treated hives, seven hives at Tilquiza, six at El Carmen and nine at Yala were included in the study. Due to the increased honey yield obtained in 2014, the beekeepers decided to evaluate a larger number of treated hives in 2015.

It is important to mention that before administration, the number of viable *L. salivarius* A3iob cells was determined by a plate count on MRS agar. The plates were incubated at 37 °C for 48-72 h under microaerophilic conditions, as explained above.

Honey production and yield determination

Honey yield was used as a key parameter to describe the general condition of the colonies during the assays. The honey was harvested and registered using honey supers. All the tested apiaries were harvested in December 2014 and 2015. The exception was El Carmen which had an additional atypical harvest in July 2015.

Statistical analysis

The results of honey yield were expressed as mean ± standard deviation of the groups. A simple imputation was made for the data obtained in the apiary of El Carmen (Badler *et al.,* 2004). The assumptions of the variance analysis were verified. ANOVA was carried out using INFOSTAT software.

3. Results

Phylogenetic characterisation of the *Lactobacillus* strain

The 16 rRNA gene analyses were carried out twice and at different research centres. Both centres provided the same results: A3iob had a 99% homology, according to the BLAST analysis, with known *L. salivarius* strains, such as *L. salivarius* strain L6 or *L. salivarius* strain CICC 23174 from GenBank sequence library. The nucleotide sequence was published and deposited at GenBank with the following accession number: KX198010. The genetic homology of *L. salivarius* A3iob with related *Lactobacillus* species was

analysed and can be observed in the phylogenetic tree built using the neighbour-joining method (Figure 1).

Trials at commercial apiaries: honey yield

Honey harvested in summer 2014

In this year, the recorded honey yields were significantly higher in the hives that received *Lactobacillus* cells than in the control hives (Table 2). At El Carmen, 22.24±7.12 kg was harvested on average, around 130% more than the honey obtained from control hives (9.70±8.51 kg; P=0.0354). A similar result was recorded for Tilquiza, with an average honey yield of 23.30±0.57 kg compared to the control hives (3.57±4.90 kg; P<0.001), and Yala, which yielded an average of 23.49±8.99 kg for the treated hives compared to the control hives (3.76±7.45 kg, P<0.001).

Honey harvested in summer 2015

In this year, a different general situation was observed. There were apiaries where the administered lactobacilli increased honey yields and others where the bacteria did not have a positive effect, mainly due to beekeeping management. At Tilquiza, *Lactobacillus*-treated hives yielded 9.29±11.61 kg of honey, around 50% less than the control group (18.20±4.71 kg, P=0.1393). This difference can be easily explained. *L. salivarius* A3iob stimulated egg-laying by the queen and produced a larger number of bees in all the apiaries tested; thus, more honey was produced (i.e. at El Carmen, Yala and Tilquiza). However, each beekeeper handled this 'bee explosion' (as they referred to it) differently. Unfortunately, at Tilquiza several hives swarmed and the beekeepers lost them; consequently, less honey was obtained.

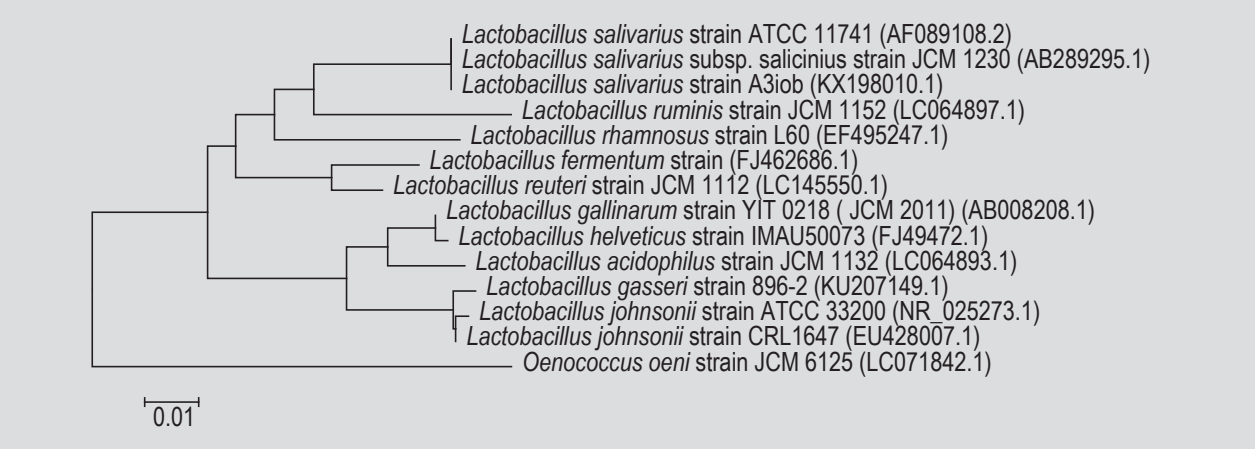

Figure 1. Phylogenetic tree of the *Lactobacillus salivarius* A3iob strain, constructed using the MEGA 5.1 β4 software. In brackets, accession number to the NCBI data base.

Table 2. Honey yield (kg) per colony of the three apiaries from Jujuy province, Argentina, during 2014.

Hive	Tilquiza		El Carmen		Yala	
	Control	A3iob	Control	A3iob	Control	A3iob
1	8.4	23.5	18.8	10.0	16.5	20.7
2	9.5	22.5	17.5	26.5	17.3	30.7
3	0.0	24.0	2.5	25.0	0.0	21.1
4	0.0	23.5	0.0	27.5	0.0	22.9
5	0.0	23.0	9.7[a]	22.2[a]	0.0	39.5
6					0.0	22.5
7					0.0	7.5
8					0.0	17.5
9					0.0	29.1
Mean weight ± standard deviation	3.58±4.90	23.30±0.57	9.70±8.51	22.24±7.12	3.76±7.45	23.50±8.99
Total weight	17.9	116.5	48.5	111.2	33.8	211.4

[a] Data imputed by average.

At Yala, however, we recorded a similar increase in honey yield as a result of *Lactobacillus* application. Harvested honey was significantly higher (P=0.001) in treated hives than in the controls: 11.82±1.10 kg and 8.73±1.44 kg of honey, respectively (Table 3). The hives at El Carmen recorded the best results. The annual average honey yield of this apiary for the hives that received *L. salivarius* A3iob was higher than that of Yala and Tilquiza, with 24.48±15.81 kg. However, this difference was not significant compared to the honey yield from El Carmen control hives (17.47±7.80 kg, P=0.3918).

Extra honey harvest in winter 2015

Two honey harvests were recorded at El Carmen in 2015. The second, in December, is the normal harvest time in the province of Jujuy. However, the first honey harvest occurred in July and constituted an atypical 'winter honey harvest' for this region of Argentina. This winter harvest made up 52.5% of the El Carmen annual honey yield. The hives that were given *L. salivarius* A3iob yielded an average of 11.97±9.50 kg of honey in July and 12.52±7.70 kg in December; the performance of the control group was 9.64±7.23 kg of honey in July, and 7.82±4.99 kg in December (P=0.5583). This 'extra' honey harvest could almost completely be attributed to the particular flora of the region, primarily *Tithonia tubaeformis* (known as 'pasto cubano' in Spanish), which blooms in winter (Figure 2D).

4. Discussion

Honey bees have mainly been studied due to their hive products, such as honey, pollen and propolis, and their role as commercial crop pollinators. Other research has also focused on the relevance of the honey bee microbiota composition on its physiology and health (Alberoni *et al.*, 2016; Audisio, 2017; Kwong and Moran, 2016; Powel *et al.*, 2014; Schwarz *et al.*, 2016). In particular, Budge *et al.* (2016) reported that *Lactobacillus* and *Leuconostoc* spp. strains were associated with healthier and bigger honey bee colonies in the UK. Similarly, Horton *et al.* (2015) found that productive colonies trended towards increased diversity and prevalence of *Lactobacillus* species.

However, very little scientific research exists about this social insect as a super-individual and the impact of a defined treatment on its honey yield (Audisio *et al.*, 2015; Horton *et al.*, 2015; Sabaté *et al.*, 2012). Argentina is the 4th largest producer and the 2nd exporter of honey worldwide, and bee-pollination services are not among the first priorities of Argentinean beekeeping, even though some scientific initiatives are trying to redirect their attention towards this ecological issue.

In this study, a monoculture of *L. salivarius* A3iob was administered to hives belonging to three commercial apiaries located in north-western Argentina, and the honey yield of each one was recorded throughout 2014 and 2015. Interestingly, a difference was observed between years. In 2014, the three studied apiaries rendered a significant difference in honey yield between the treated hives and the controls (more than 50% in all cases), and only a summer honey harvest was obtained. During this trial, no significant differences were detected among the apiaries, one of them located in the Valley region (El Carmen) and two in the Yungas (Tilquiza and Yala). We can gather from these results that neither the weather nor the flora nor the beekeeping practices at each apiary had an impact on honey harvest.

Unexpectedly, in 2015, a different result was observed. Each apiary behaved completely different. El Carmen

Table 3. Honey yield (kg) per colony of the three apiaries from Jujuy province, Argentina, during 2015.

Hive	Tilquiza		El Carmen		Yala	
	Control	A3iob	Control	A3iob	Control	A3iob
1	10.0	0.0	30.5	40.3	7.5	12.5
2	19.0	0.0	14.0	39.5	7.7	11.7
3	22.0	22.0	12.0	28.0	10.0	11.2
4	20.0	23.0	12.0[a]	27.1	11.3	12.0
5	20.0	20.0	18.8[b]	12.0	7.5	14.0
6		0.0		0.0	8.3	12.0
7		0.0			9.1	10.0
8					10.0	11.0
9					7.2	12.0
Mean weight ± standard deviation	18.20±4.71	9.29±11.61	17.47±7.80	24.48±15.81	8.73±1.44	11.82±1.10

[a,b] Data imputed by average and randomised, respectively.

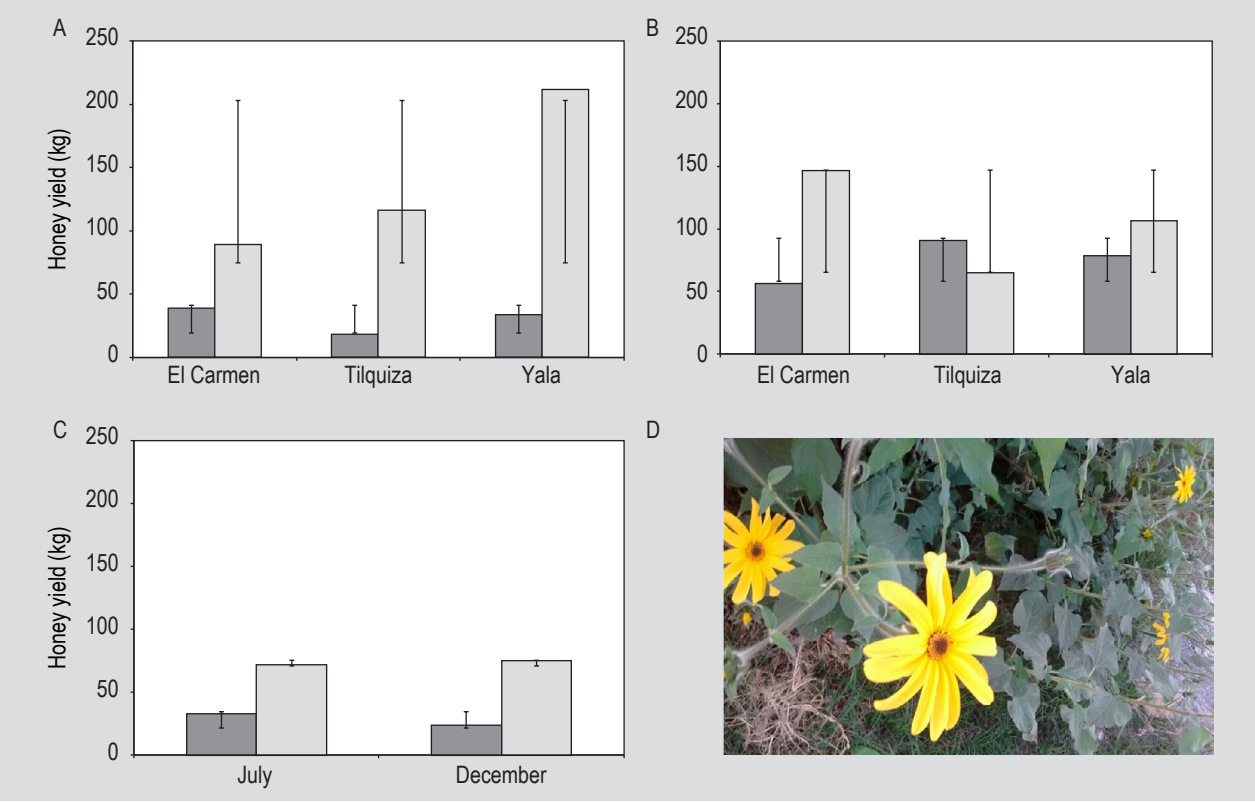

Figure 2. Honey yield from the three apiaries of Jujuy province, Argentina. (A) Total yield of the three apiaries in 2014. (B) Total yield of the three apiaries in 2015. (C) Performance of the apiary *El Carmen* throughout 2015. (D) *Tithonia tubaeformis*, widely distributed in the El Carmen locality. Dark grey bar indicates performance of *control* hives. Light grey bar indicates performance of *Lactobacillus salivarius* A3iob hives.

had the best average honey harvest; in contrast, Tilquiza recorded a negative impact on its honey yield compared to the control colonies. We have concluded this must be explained by human causes rather than the effect of *L. salivarius* A3iob cells because in all three treated apiaries a higher number of bees were observed in all hives that received the *Lactobacillus* cells. The only difference between apiaries was the management of the hives by the beekeepers. At Tilquiza, improper handling of such a high number of bees led to the swarming of several honeybee colonies. Thus, during the summer honey harvest no honey was stored. Surprisingly, El Carmen reported an additional winter honey harvest, due mainly to an unusual bloom of a typical plant of this region, 'pasto cubano', which did not occur in the areas surrounding the other apiaries. In spite of the differences in honey yield recorded during the trials over both years, no hive was lost and the general health of the hives was good. Overall, *L. salivarius* A3iob had a positive impact on the bee colonies, the only exception being a low honey harvest at Tilquiza in 2015, due to the reasons reported above.

The most important finding of this study was that in real situations, in terms of real beekeepers with their normal practices in real locations (i.e. with wild flora, weather, etc.), the administration of *L. salivarius* A3iob produced mainly positive results with respect to hive life and a higher honey yield; thus, it can be considered a potential bee-probiotic strain. At this point it is worth addressing other scientific articles which are reluctant to bee probiotic bacteria. Horton *et al.* (2015) states that 'All in all, our results do not appear to indicate that the feeding of certain probiotic bacteria to honey bee foragers is likely to result in increased honey production by honey bee colonies'. Similarly, Schmidt and Engel (2016) reported 'moreover, arbitrary probiotic treatments may not have beneficial effects on the host. In contrast, they can irreversibly perturb community assembly, hindering the establishment of a robust and microbiota in young adult bees'. Finally, Schwarz *et al.* (2016) determined that if a strain of *Snodgrassella alvi* was administered to honeybee colonies, they had greater susceptibility to parasitic infections. All three aforementioned studies failed to mention a key point, which is the careful selection of the bacterium strain in order to yield positive results. Bee probiotics should not be dismissed. The challenge is to select the correct strain. Our results are proof of such selection.

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