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RESEARCH ARTICLE

Supplementation of honey bee production colonies with a native beneficial microbe mixture

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Abstract

Honey bee colonies form a complex superorganism, with individual and social immune defences that control overall colony health. Sometimes these defences are not enough to overcome infections by parasites and pathogens. For that reason, several studies have been conducted to evaluate different strategies to improve honey bee health. A novel alternative that is being studied is the use of beneficial microbes. In a previous study, we isolated and characterised bacterial strains from the native gut microbiota of honey bees. Four *Apilactobacillus kunkeei* strains were mixed and administered in laboratory models to evaluate their potential beneficial effect on larvae and adult bees. This beneficial microbe mixture was safe; it did not affect the expression of immune-related genes, and it was able to decrease the mortality caused by *Paenibacillus larvae* infection in larvae and reduced the *Nosema ceranae* spore number in infected adult honey bees. In the present study, we aimed to delve into the impact of the administration of this beneficial microbe mixture on honey bee colonies, under field conditions. The mixture was administered in sugar syrup using lyophilised bacterial cells or fresh cultures, by aspersion or sprayed and feeder, once a week for three consecutive weeks, in autumn or spring 2015, 2017 and 2019. Colony strength parameters were estimated before the administration, and one and three months later. Simultaneously different samples were collected to evaluate the infection levels of parasites and pathogens. The results showed that administering the beneficial microbe mixture decreased or stabilised the infection by *N. ceranae* or *Varroa destructor* in some trials but not in others. However, it failed to improve the colony's strength parameters or honey production. Therefore, field studies can be a game-changer when beneficial microbes for honey bees are tested, and meticulous studies should be performed to test their effectiveness.

Keywords

probiotics – honey bee health – beneficial microorganisms – *Nosema* spp. – *Varroa destructor*

1 Introduction

Western honey bees (*Apis mellifera*) are important pollinators of commercial crops and wildflowers, having a crucial role in food production (reviewed by Potts *et al.*, 2016). However, honey bees are threatened since large-scale colony loss episodes have been reported worldwide (Gray *et al.*, 2021; Requier *et al.*, 2018, Tang

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et al., 2020). The infection by pests and pathogens, including the microsporidia *Nosema ceranae*, the mite *Varroa destructor*, trypanosomatids and different RNA viruses, is one of the most critical drivers of those losses (reviewed by Goulson *et al.*, 2015 and Steinhauer *et al.*, 2018).

Nosema ceranae and *Nosema apis*, causative agents of nosemosis, are obligate intracellular spore-forming microsporidia that develop exclusively in the ventricle epithelial cells of adult bees (Fries *et al.*, 1996; Higes *et al.*, 2013). This parasite alters the gut physiology, immune response and other vital functions. In the field, Nosemosis caused by *N. ceranae* has been associated with reduced honey production, weakness and increased colony mortality (reviewed by Martin-Hernández *et al.*, 2018). *L. passim* is the most common trypanosomatid in honey bees, living in their intestinal lumen (Buendía-Abad *et al.*, 2022; Schwarz *et al.*, 2015). It may cause harm to honey bees when combined with *N. ceranae* (Arismendi *et al.*, 2020). *Varroa destructor* is another threat to honey bees. This obligatory parasite spends its life inside the colony and reproduces within the capped cells. The mite feeds on the fat body tissue, reducing the lifespan of the bees (reviewed by Nazzi and Le Conte, 2016; Ramsey *et al.*, 2019). Besides the direct damage of varroa to honey bees, the mites can transmit different RNA viruses, which can contribute significantly to honey bee colony losses (reviewed by Beaurepaire *et al.*, 2020; Traynor *et al.*, 2020). Another important threat for honey bees is *Paenibacillus larvae*, a Gram-positive and spore-forming bacterium that affects honey bee larvae, and causes the disease American Foulbrood (Genersch *et al.*, 2010). The use of antibiotics and synthetic acaricides to control those pests and pathogens is not recommended since it can stimulate the generation of resistant organisms (Evans, 2003; Huang *et al.*, 2013; Rosenkranz *et al.*, 2010) or remain in honey and other bee products affecting their quality (Harriet *et al.*, 2017; Martel *et al.*, 2006, 2007). Therefore, effective alternative strategies are needed to improve the health of honey bees.

Probiotics are widely used to boost human and animal health (Chaucheyras-Durand and Durand, 2010; Reid, 1999). However, before their approval, those microorganisms must fulfil a series of safety and biological requirements, including being non-toxic and non-pathogenic, belonging to the microbiota of the targeted host species, adhering to the gut epithelium, and inhibiting the growth of microbial pathogens, among others (Reid, 1999).

Many researchers have been developing probiotics based on native bacteria to control pests and pathogens that affect honey bees (reviewed by Alberoni *et al.*, 2016). First attempts included the isolation of bacteria from the colony and the gut microbiota, which were able to inhibit *in vitro* the growth of pathogens such as *P. larvae* or *Ascosphaera apis*, among others (Alippi and Reynaldi, 2006; Audisio and Benitez-Ahrendts, 2011; Audisio *et al.*, 2015; Evans and Armstrong, 2006; Forsgren *et al.*, 2010; Sabaté *et al.*, 2009; Vásquez *et al.*, 2012; Wu *et al.*, 2014; Yoshiyama and Kimura, 2009). However, just a few studies advanced in the impact of beneficial bacteria on bee health using *in vivo* assays. For example, administering different *Lactobacillus* and *Bifidobacterium* strains to *P. larvae*-infected larvae significantly reduced larval mortality (Forsgren *et al.*, 2010). In addition, the administration of *Parasaccharibacter apium*, *Bifidobacterium* spp. or *Lactobacillus* spp. strains decreased the infection by *N. ceranae* in adult bees (Baffoni *et al.*, 2016; Corby-Harris *et al.*, 2016).

Despite the promising results of probiotics in vitro or under laboratory conditions using larvae and adult bees, scarce studies support their use at the field level on naturally infected colonies. As an example, Sabaté *et al.* (2012), Audisio and Benítez-Ahrendts (2011), and Audisio *et al.* (2015) studied the administration of *Bacillus subtilis* or *Lactobacillus johnsonii* CRL1647. The administration of these strains for several months under field conditions decreased the number of *N. ceranae* spores and increased egg-laying (Audisio and Benítez-Ahrendts, 2011; Audisio *et al.*, 2015). In addition, Sabate *et al.* (2012) verified that the administration of *B. subtilis* reduced *V. destructor* infestation levels in the field. On the other hand, the use of commercial probiotics (not specifically designed for honey bees) may increase the susceptibility to pathogens (Ptaszyńska *et al.*, 2016) or fail to establish in the worker bees' guts (Motta *et al.*, 2022).

In a previous study, we isolated and characterised bacterial strains obtained from the native gut microbiota of honey bees. Selected bacterial isolates survived at high sugar concentrations and acidic conditions, which was a requisite since they are expected to be administered in sugar syrup and must survive through the passage along the larval/adult gut. Furthermore, they inhibited the growth of *P. larvae in vitro* and did not alter the expression of different genes associated with immunity in adult bees. Four *Apilactobacillus kunkeei* strains were selected for further studies, and a mixture was generated (Arredondo *et al.*, 2018). Since probiotic characteristics vary among strains, the mixture

of strains may exert additive or complementary effects (Forsgren *et al.*, 2010; Jacobsen *et al.*, 1999). The administration of the beneficial microbe mixture was safe for larvae and adult bees and decreased the mortality of larvae infected with *P. larvae* from 50 to 6%. Finally, it reduced the *N. ceranae* spore number in infected adult honey bees and tended to reduce the mortality associated with Nosemosis (Arredondo *et al.*, 2018). In the present study, we evaluated the impact of the administration of the beneficial microbe mixture previously developed, on honey bee colonies health and strength. In particular, we carried out four field studies and evaluated bacterial administration in different conditions (lyophilised or fresh cultures suspended in sugar syrup) administered by spraying or spraying and in a feeder, in autumn or spring.

2 Materials and methods

Bacterial isolates and culture conditions

The beneficial microbe mixture was prepared as described by Arredondo *et al.* (2018) with modifications. Briefly, four *A. kunkeei* isolates (35UY, 37UY, 67UY and 110UY, Arredondo *et al.*, 2018) were cultured in semi-selective De Man Rogosa Sharpe (MRS) agar or broth and incubated at 37 °C for 24 h under microaerophilic conditions. From those pure cultures, individual cell suspensions at 1 Mc Farland were prepared, inoculated into 25 ml of MRS broth and incubated for 24 h under agitation and microaerophilic conditions. Then, 4 ml of the overnight cultures were inoculated into 300 ml of MRS broth at 37 °C with agitation for 24 h (four flasks per strain cultured). Next, the entire volume from the MRS broth cultures of each *A. kunkeei* isolate was centrifuged at 10,000 × g for 10 min. Then, two alternative procedures were followed for the preparation of the product:

- *Lyophilisation*. Pellets were suspended in 10% skim milk and mixed in equal proportions. Subsequently, they were incubated at –80 °C for 2 h and placed in a Vertical Freezing dryer BW-18 (Bluewave Industry Co. Ltd., Shanghai, China) for 24 h. The tubes containing the lyophilised beneficial microbe mixture were stored in the dark at –4 °C until use. Then, bacteria were suspended in 1:1 sucrose syrup (50% sugar in 50% water) and immediately used.

- *Fresh pellets* were re-suspended in 1:1 sucrose syrup and immediately used.

Viability of the beneficial microbe mixture

The number of viable *A. kunkeei* cells in the beneficial microbe mixture used in different field assays was determined by plate count on MRS agar before and after administration in the field. For this, 100 µl of the syrups containing the beneficial microbe mixture were taken, diluted on 1× sterile phosphate-buffered saline (PBS) and swabbed in MRS agar in triplicate. All the plates were incubated at 37 °C for 24 h under microaerophilic conditions.

Field experiments set-up

Four field assays were carried out between 2015 and 2019 to evaluate the effect of the beneficial microbe mixture on bee health and colony strength (Supplementary Figure S1). In all cases, acaricide treatments (organic or synthetic) were used to eliminate *V. destructor* as a confounding factor and applied in the presence of brood.

Assay 1: effect of lyophilised beneficial microbe mixture combined with liquid oxalic acid in autumn

The experiment was performed on three nearby apiaries with production colonies under standard commercial management in the 2015 autumn (March to July). The apiaries were located in Paso Severino (34°15′47.05′S, 56°15′25.69′W) in Florida Department (Uruguay). In March, colonies were distributed into 4 groups and subjected to different treatments: (1) oxalic acid 6% (OC, n = 15); (2) beneficial microbe mixture combined with oxalic acid 6% (OBM, n = 15); (3) Control group without treatment (C, n = 15) and (4) amitraz (AC, n = 45).

The oxalic acid, an organic acaricide, was used at 6% (60 g of oxalic acid in a litre of 1:1 sugar syrup) by dripping in the frames head once a week for three consecutive weeks. The beneficial microbe mixture was prepared using the lyophilised cells and applied by spraying on the brood chamber (50 ml per colony, approximate dose of 1×10^7 cfu/ml) once a week for three consecutive weeks, coinciding with oxalic acid applications. Finally, amitraz, a commercially available synthetic acaricide, was used as a control. It was applied in strips and left for forty days in the colonies, according to the manufacturer's suggestions.

Assay 2: effect of lyophilised beneficial microbe mixture combined with liquid oxalic acid in spring

The second assay was carried out in spring 2015 (September to December) using 39 colonies that received amitraz in autumn. Colonies were distributed into two groups, and the treatments were applied: (1)

oxalic acid 6% (OC, n = 19); (2) beneficial microbe mixture combined with oxalic acid 6% (OBM, n = 20).

The oxalic acid and the beneficial microbe mixture were prepared and used as described in the first assay. Amitraz was not used as it is not recommended to use synthetic acaricides in spring.

Assay 3: effect of fresh beneficial microbe mixture combined with amitraz strips in autumn

The experiment was performed in autumn of 2017 (March to July) on production colonies located near Marindia (34°45′37.04′S, 55°49′21.01′W) in Canelones Department (Uruguay). As oxalic acid was not effective to control varroa in autumn (result of assay 1), all colonies were treated with amitraz. First, a preliminary sampling of nurse bees from each colony was conducted to determine the infection levels of *N. ceranae* and *V. destructor*. Then, the colonies were divided into three standardised groups (n = 15) based on the number of *N. ceranae* spores in the preliminary sampling.

In March, colonies were distributed into 3 groups and subjected to different treatments: (1) beneficial microbe mixture combined with amitraz (ABM, n = 15); (2) sugar syrup used as a vehicle for BM combined with amitraz (AV, n = 15) and (3) amitraz (AC, n = 15).

In this case the beneficial microbe mixture was prepared from fresh cultures and applied at an approximate concentration of 1×10^7 cfu/ml by spraying in each brood chamber (50 ml) and in a feeder (200 ml). The vehicle was applied by spraying in each frame (50 ml) and in a feeder (200 ml). Both treatments were applied once a week for three consecutive weeks.

Assay 4: effect of fresh beneficial microbe mixture combined with oxalic acid strips in autumn

The experiment was performed on two nearby apiaries located in a field near Villa Vieja (34°4′43.41′S, 56°25′18.93′W) in San José Department (Uruguay) in late autumn 2019 (May to August).

First, a preliminary sampling of nurse bees from each production colony was conducted to determine the infection level with *N. ceranae* and *V. destructor*. Then, the apiary was divided into three standardised groups (n = 11) based on the number of *N. ceranae* spores in the preliminary sampling.

In May, colonies were distributed into three groups and subjected to different treatments: (1) beneficial microbe mixture combined with oxalic acid (OBM, n = 11); (2) sugar syrup used as a vehicle for BM combined with oxalic acid (OV, n = 11) and (3) oxalic acid (OC, n = 11).

In this case, the beneficial microbe mixture was prepared from fresh cultures and applied at an approximate concentration of 1×10^7 cfu/ml by spraying in each brood chamber (50 ml) and a feeder (500 ml). The vehicle was applied by spraying in each frame (50 ml) and in a feeder (500 ml). Both treatments were applied once a week for three consecutive weeks. Colonies also received oxalic acid for varroa control, but strips were used instead of liquid oxalic acid in this assay. Strips are safe for colony development, and effective against varroa, even in the presence of brood (Maggi *et al.*, 2016).

Sampling

Samplings were carried out in the four assays, on the day of the first administration of the treatments, one and three months later. All the colonies were individually sampled and honey bees were placed in independent plastic containers with alcohol 95% until analysis. From each colony, one-hundred nurse bees were collected from at least three unsealed brood combs to detect and quantify the infestation rate by *V. destructor*, and at least 70 honey bees from peripheral combs (named foragers) were collected to detect *N. ceranae* and *L. passim*. Besides that, about 60 nurse bees were collected alive. Those samples were sent immediately to the laboratory, where they were stored at –80 °C to prevent RNA degradation. Samples from the first, second and third assays were used for the quantification of RNA viruses, while samples from the fourth assay were used for microbiota analysis.

Colony strength estimation

The colony's strength was estimated by visual inspection of honey bee population, brood area and honey reserves by two specialised technicians according to Delaplane *et al.* (2013). Adult honey bee population and honey reserves were estimated as the number of covered frames and expressed in cm^2 considering that each side of the frame measures 880 cm^2, Brood area was estimated as quarters of frame side and expressed in cm^2. Clinical signs were also recorded. In addition, honey bee colony survival was registered during the assays.

Sanitary status of the colonies

The impact of different treatments on the infestation level of *V. destructor*, *Nosema* spp., *L. passim*, and the viral load of *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Deformed wing virus* (DWV) and *Sacbrood virus* (SBV) was evaluated one and three months after the first administration. All samples were individually analysed.

Detection and quantification of *Nosema* spp.

The infection level of *Nosema* spp. in forager bees was determined as the number of spores in a pool of 60 abdomens per sample, as described in Fries *et al.* (2013).

Detection and quantification of *Varroa destructor*

The detection and quantification of mites in nurse bees were performed as described in Dietemann *et al.* (2013) from 100 bees per colony.

Virus detection and quantification

Twenty bees per colony were homogenised, and RNA extraction was carried out using 200 µl of the supernatant with the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. After elution, 1 µl was digested with the DNaseI Amp grade kit (Invitrogen), and reverse transcription was performed with the Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The viral loads were quantified using specific primers for each virus (Supplementary Table S1): ABPV (Johnson *et al.*, 2009), BQCV (Kukielka *et al.*, 2008), DWV (Kukielka *et al.*, 2008) and SBV (Johnson *et al.*, 2009). In addition, RPS5 (Evans, 2006) and β-actin (Yang and Cox-Foster, 2005) were used as reference genes. Reactions were carried out in a CFX96 Real-Time System thermal cycler (Bio-Rad, Hercules, CA, USA). 5 µl of tenfold diluted cDNA were mixed with 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems) containing 0.6 µM of each primer and 4.76 µl of RNase-free water. Amplification was performed with the following program: 95 °C for 10 min, and 39 cycles of 95 °C for 15 s, 50 °C for 30 s and 60 °C for 30 s. The specificity of the reaction was verified including a denaturation curve of the amplified products from 65 to 95 °C. The Pfaffl (2001) method was used to normalise and estimate the expression of the viruses. Virus detection and quantification were performed in the first three assays.

Detection of *Lotmaria passim*

DNA extraction was carried out using the pellets obtained above. First, the pellets were resuspended in 400 µl of sterile distilled water. Subsequently, 200 µl of the suspension were taken, and DNA purification was carried out using the commercial PureLink™ Viral RNA/DNA Mini Kit (Invitrogen), following the manufacturer's recommendations. The PCR was performed as described by Arismendi *et al.* (2016), using Lp2 forward and reverse primers (Supplementary Table S1). Amplification was carried out in a MultiGene Optimax Thermal cycler (Labnet International, Edison, NJ, USA). The visualisation of the amplicons was carried out on a 1% agarose gel, stained with GelRed (Biotium™, Fremont, CA, USA). This analysis was only carried out in samples from the first assay (March and July 2015).

Honey bee gut microbiota

DNA extraction

Twenty nurse bees per colony, from eight colonies per treatment from the fourth assay (May and August, 2019), were externally sterilised using a 1% chlorine solution (Engel *et al.*, 2013). Honey bee guts were dissected and homogenised in 500 µl of PBS using ceramic beads and a FastPrep-24™ at 6.0 m/s for 40 s. Samples were centrifuged at 5,000 ×*g* for 1 min, and the supernatants were collected and used for DNA extraction by the SDS-CTAB method as described in Arredondo *et al.* (2018). DNA was quantified using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and concentrations were normalised to 10 ng/µl.

16S rRNA amplicon sequencing

The V3-V4 region of the 16S rRNA gene was amplified and sequenced from gut DNA pools using the primers V3F_Nextera (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG-3) and Meta_V4_806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAT-3′). Genomic DNA was sent to the University of Minnesota Genomics Center, and sequencing was performed using Illumina MiSeq 2 × 300 bp paired-end reads using standard workflows.

Sequence processing

Bioinformatic analysis of honey bee gut microbiota was carried out in QIIME2 version 2020.2 (Bolyen *et al.*, 2019). From 46 experimental samples, 3,847,639 reads were obtained with a mean ± standard error of 83,644.33 ± 16,906.49 reads per sample. Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin. Then, reads were filtered and trimmed based on length, forward reads between 40 and 300 nucleotides and reverse reads between 20 and 278 nucleotides. Representative sequences were denoised, and chimeric reads were removed using DADA2 (Callahan *et al.* 2016). Afterwards, paired reads were merged, and the resulting amplicon sequence variants (ASVs) were classified by 'classify-sklearn' (Bokulich *et al.*, 2018) using the BEExact database (Daisley and Reid, 2021). Finally, the feature table was converted to

a '.biom' file and extracted to a '.csv' file for statistical analysis.

Statistical analysis

The data resulting from the colony strength estimation or the sanitary status were analysed to determine if they fitted a normal distribution (Kolmogorov-Smirnov test) and whether their variance was homogeneous (Levene tests). The adult population data fulfilled these assumptions and it was analysed by ANOVA and Sidak's multiple comparisons tests. The other parameters studied did not fulfil the parametric assumptions. Therefore, Kruskal-Wallis and Dunn's multiple comparisons or Mann-Whitney test were used. In the case of *L. passim*, when only the presence/absence of the pathogen was recorded, a comparison between infected or not infected colonies was performed using Fisher's exact test. In all cases, *P*-values ≤0.05 were considered significant and $P \leq 0.1$ indicated a trend. Normality analyses and box-plot plots were performed using GraphPad Prism 8.0 for iOs (La Jolla, CA, USA).

Statistical analysis of the honey bee gut microbiota was performed with R Studio v1.4.1717 (RStudio Team 2021) using the phyloseq package v1.36.0 (McMurdie and Holmes, 2013). 'Subset_taxa' function was used to exclude reads belonging to mitochondria, chloroplast and eukaryotes. ASVs with at least 1% relative abundance in a minimum of 1 sample were retained with the 'filterfun_sample' function on the Genefilter package v1.74.0 (Gentleman *et al.*, 2021). Next, alpha and beta diversity were calculated using the Vegan package v2.5-7 (Oksanen *et al.*, 2020). The alpha diversity was calculated with the number of observed ASVs and the Shannon index using the 'estimate_richness' function (Oksanen *et al.*, 2020). Then, beta diversity was evaluated by Bray-Curtis, Jaccard, UniFrac weighted (by the relative abundance of ASVs), and UniFrac unweighted indexes (Oksanen *et al.*, 2020). Permutational multivariate analysis of variance with the 'adonis' function was used to test the effect of treatments on community structure on beta diversity data. We then used the function 'betadisper' to test for homogeneity of multivariate dispersions (Anderson, 2006; Anderson *et al.*, 2006) and compared the distances of individual samples to group centroids in multidimensional space using 'permutest'. The 'metaMDS' function was used for plot ordinations. Then, differences between the relative abundance of different ASVs among treatments were examined using the DESeq2 package v1.36.0 (Love *et al.*, 2014).

3 Results

Beneficial microbe mixture viability

Bacterial viability was evaluated before and after the fieldwork in the four assays. In all cases, the initial dose of the beneficial microbe mixture was about 10^7 cfu/ml, and after 8 h of fieldwork, it dropped one order of magnitude to 10^6 cfu/ml (Supplementary Table S2).

Colony strength

At the beginning of each assay, brood, adult honey bee population, and honey reserves were similar between groups ($P > 0.05$ by Sidak's multiple comparisons test or Dunn's multiple comparisons test, in all cases, Supplementary Figures S2-S4).

In autumn assays (first, third and fourth), the brood and adult population decreased along the year, as expected, as the colonies prepared for wintering and the queens slowed egg-laying (Supplementary Tables S3-S4). In the first assay, the administration of the beneficial microbe mixture with oxalic acid (group OBM) delayed this decrease in the adult honey bee population compared to group AC (amitraz control, $P < 0.001$ by Sidak's multiple comparisons test, Supplementary Figure S2). However, no difference was observed compared to group OC (oxalic acid control), $P > 0.05$ by Sidak's multiple comparisons test, Supplementary Figure S2), suggesting that the effect is generated by the oxalic treatment and not by the beneficial microbe mixture. Honey reserves decrease from autumn to winter in the first and fourth assay, meanwhile, it increases during spring ($P < 0.05$ by Sidak's multiple comparisons test, Supplementary Figure S4).

The administration of the beneficial microbe mixture by itself did not affect the adult bee population, brood or honey reserves compared to the other groups in any of the assays ($P > 0.05$ by Dunn's multiple comparisons test, in all cases, Supplementary Figures S2-S4). Similar results were obtained in autumn and spring, in different environments, using the beneficial microbe mixture lyophilised or fresh cells.

Sanitary status of the colonies

At the beginning of the autumn assays (first, third and fourth), *Nosema* spp. spore loads were low and similar among the different groups (less than 1×10^5 spores/bee or close to zero, Figure 1).

In the first assay, the administration of BM did not affect the number of *N. ceranae* spores ($P > 0.05$ by

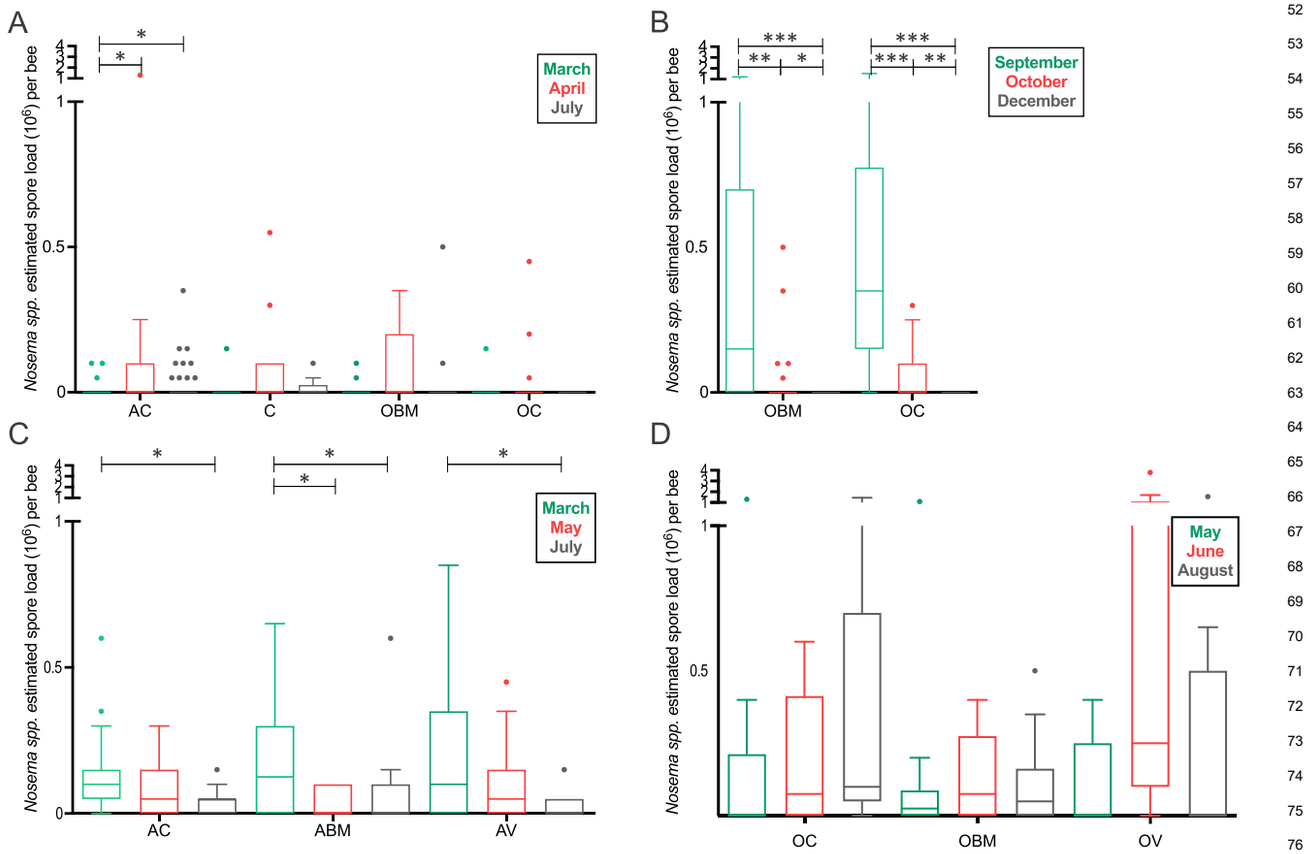

FIGURE 1 Number of *Nosema* spp. spores per bee: (A) first assay, (B) second assay, (C) third assay, (D) fourth assay. The results are shown as box-plots. Significant differences within treatments in the Mann-Whitney test are indicated with asterisks ($P < 0.05$ =*, $P < 0.01$ =**, $P < 0.001$***). The months are represented in different colours: time 0 (green), one month (red), and three months after (grey).The different groups are: amitraz control (AC), oxalic acid control (OC), oxalic acid combined with beneficial microbes mixture (OBM), Control without any treatment (C, there is only one), amitraz combined with beneficial microbes mixture (ABM), amitraz combined with the vehicle of the beneficial microbes mixture (syrup 1:1, AV). Oxalic acid combined with the vehicle of the beneficial microbes mixture (syrup 1:1, OV).

Dunn's multiple comparisons test, in all cases, Figure 1A, Supplementary Table S6). In spring (second assay), at the beginning of the assay, the *N. ceranae* spore load was similar between the groups OC and OBM ($P > 0.05$ by Dunn's multiple comparisons test). The spore number decreased from September to October in both groups ($P \leq 0.05$ by Dunn's multiple comparisons test). Regardless, the administration of the BM did not influence the *N. ceranae* spore load since there were no differences between group OC and OBM in October or December ($P > 0.05$ by Dunn's multiple comparisons test, Figure 1B).

On the other hand, in the third assay, this administration quickly reduced the number of spores between March and May (Mann-Whitney test: $P = 0.02$, Figure 1C) in the ABM group, while in the control groups (AV and AC) this reduction was observed two months later (July, Mann-Whitney test: $P = 0.02$). However, no significant differences were observed using Dunn's multiple comparisons test ($P > 0.05$).

Finally, in the fourth assay, the number of spores in the OBM group remained low while it increased in the other groups, although the differences were not significant ($P > 0.05$ by Dunn's multiple comparisons test, in all cases, Figure 1D).

Regarding *Varroa destructor*, the percentage of infestation varied depending on the acaricide used and the month they were applied (Supplementary Figure S5). In the first assay (autumn of 2015), the three groups showed similar infestation percentages at the beginning of the study (Supplementary Figure S5A). One and three months later, the infestation percentage was significantly lower in group AC, treated with amitraz, compared to the rest of the groups ($P \leq 0.05$ by Dunn's multiple comparisons test in all cases, Supplementary Figure S5A). No effect of the administration of BM was observed on this parameter in this assay.

In the second assay (spring 2015), the *V. destructor* infestation percentage was similar between the groups subjected to different treatments at specific time points (Supplementary Figure S5B). However, the administration of BM maintained a stable percentage of infestation with *V. destructor* in group OBM from September to December, while in group OC, a significant increase in the mite infestation percentage was observed from October to December ($P \leq 0.05$ by Dunn's multiple comparisons test, Supplementary Figure S5B, Supplementary Table S7).

The same effect occurred in the third assay. The percentage of infestation with *V. destructor* remained stable through time in the group ABM, which received the beneficial microbe mixture with amitraz. However, in the groups that received amitraz alone or amitraz with sugar syrup without bacteria (AC and AV) the percentage of infestation significantly increases from March to July ($P < 0.01$ by Dunn's multiple comparisons test, Supplementary Figure S5C, Supplementary Table S7).

The infestation percentage with *V. destructor* was low across the fourth assay, and no significant differences were found among the treatments ($P > 0.05$ by Dunn's multiple comparisons test, Supplementary Figure S5D).

The BM administration did not boost the survival of the colonies in any of the assays ($P > 0.05$ by Log-Rank, Supplementary Table S8). Besides, higher mortality was observed at the end of the first assay in the group C (not treated with acaricides) compared to AC or OC ($P < 0.05$ by Log-Rank in both cases). For that reason, in the subsequent assays, all colonies were treated with acaricides, and no differences in mortality were observed between treatments.

In 2015, all the studied RNA viruses were detected in the colonies. Nevertheless, in 2017 only ABPV and DWV were detected. The administration of the BM mixture did not produce significant changes in the infection levels of any studied virus ($P > 0.05$ by Dunn's multiple comparisons test, Supplementary Figures S6-S9).

The presence of *L. passim* was studied in the first assay of the 2015 autumn. Although this parasite was not evenly distributed among the treatments in March or July, no significant differences were observed (χ^2: $P > 0.05$). However, all groups showed a significant increase between those months (χ^2: $P < 0.01$ in all cases, Supplementary Figure S10).

Impact of the beneficial microbe mixture administration on the honey bee gut microbiota

In order to evaluate the impact of the beneficial microbe mixture administration on the gut microbiota of honey bees, we used deep amplicon sequencing of the V3-V4 region of the bacterial 16S rRNA gene. The three groups of colonies of the fourth assay were studied (OBM, OV and OC) before BM administration in May and in August, three months after the first administration. We obtained 3,847,639 raw reads from 46 samples, averaging 83,644 reads per sample. Two control samples at time 0 were discarded due to a low coverage. Rarefaction curves based on the alpha diversity metrics demonstrated that the sequencing depth was enough to capture the bacterial diversity in the honey bee guts. In addition, 1,979,897 reads passed the quality control and the Chimera check, obtaining an average of 43.041 joint reads per sample. The taxonomic assignment of the 46 samples produced 584 ASVs based on the BEExact database. In all samples, the gut microbiota included the core members *Lactobacillus* spp., *Gilliamella* spp. *Snodgrassella* sp., *Bartonella* spp. and *Bifidobacterium* spp., among other species (Supplementary Figure S12).

The administration of the BM did not affect the gut microbiota. At the beginning of the assay, the alpha and beta diversities were similar between treatments (Observed ASV's index: ANOVA $P > 0.05$; Bray-Curtis dissimilarities, weighted and unweighted UniFrac, and Jaccard index: $P > 0.05$ in all cases) except for the Shannon diversity index which was significantly lower in the OBM group compared with the OC group (Shannon index: ANOVA $P = 0.03$). After three months (August) alpha and beta diversity remained similar between treatments (Observed ASV's and Shannon indexes: ANOVA $P > 0.05$; Bray-Curtis, Jaccard, weighted and unweighted UniFrac indexes: PERMANOVA $P > 0.05$ in all cases) (Figure 2A,B).

On the other hand, time was a major factor affecting gut microbiota. The comparison within each treatment showed that diversity decreased from May (autumn) to August (winter) for alpha and beta diversity indexes (Observed ASV's and Shannon indexes: ANOVA $P < 0.05$; Bray-Curtis, Jaccard, weighted and unweighted UniFrac indexes: PERMANOVA $P < 0.05$ in all cases, Supplementary Table S14, Figure 2C). Regarding the gut microbiota core members, the administration of the BM nor the time affected the relative abundance of different ASVs, while changes were observed in the groups OV and OC according to the DESeq2. Particularly, the abundance of the *Lactobacillus* species increased in August in both groups (OV and OC), moreover, in the group OC the abundance of species from *Bifidobacterium*, *Snodgrassella* and *Giliamella* also increased in August (Supplementary Figure S11).

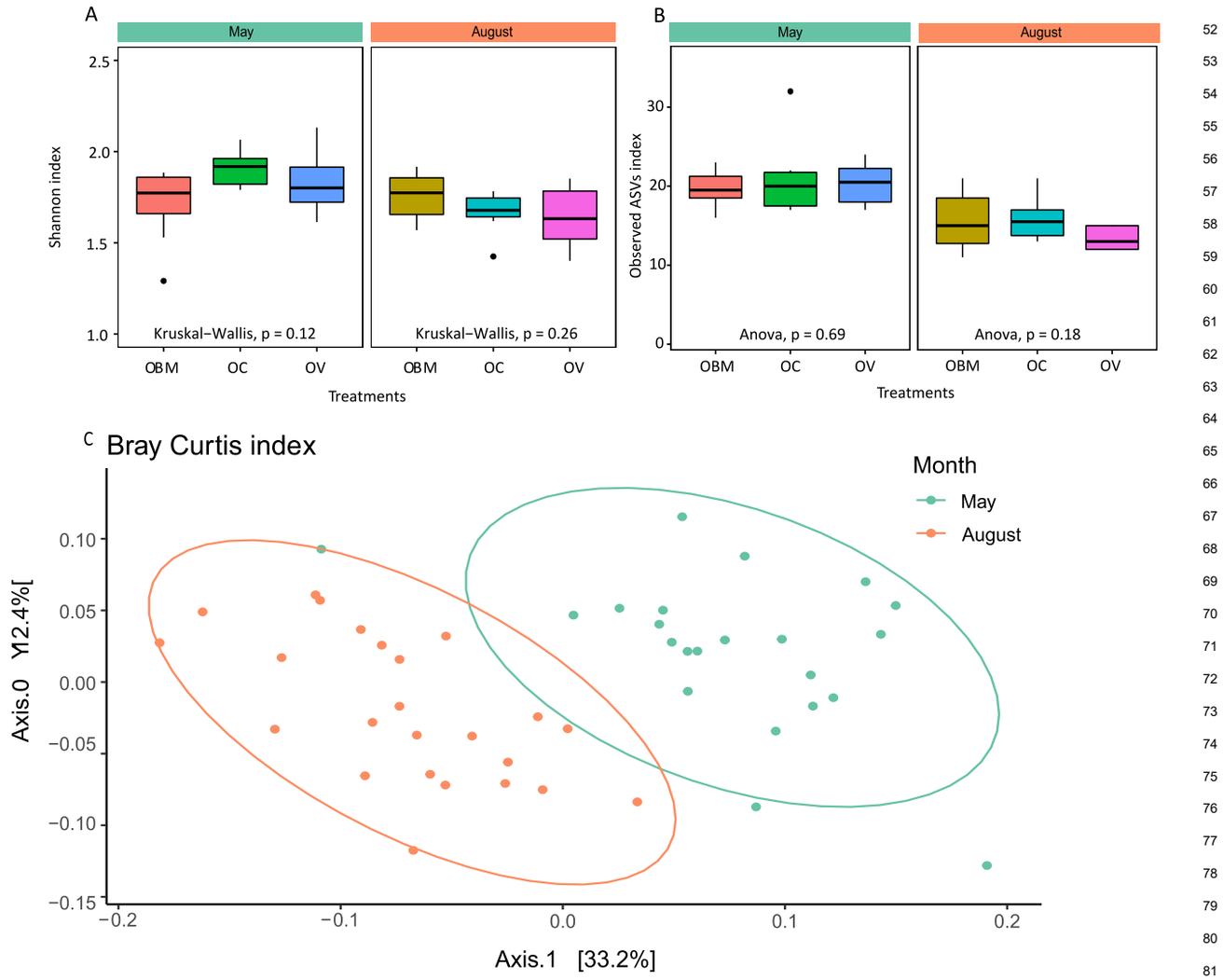

FIGURE 2 Honey bee gut microbiota under different treatments at field level (fourth assay, autumn 2019). Eight colonies per treatment were sampled two times, before treatments (May) and three months after (August). (A) Shannon index, (B) observed ASVs, (C) principal coordinates analysis (PCoA) plot of Bray-Curtis dissimilarity among samples shows a significant effect of time (determined by PERMANOVA tests). Oxalic acid was applied to the three groups: control (OC), beneficial microbe mixture (OBM) and the vehicle of the beneficial microbes mixture (OV).

4 Discussion

Supplementation of honey bee colonies with selected native bacteria could promote an increase in the brood area, the adult population and the amount of honey and pollen in the colony (Alberoni *et al.*, 2018; Audisio and Benitez-Ahrendts, 2011; Audisio *et al.*, 2015; Corby-Harris *et al.*, 2016; Sabate *et al.*, 2012; Tejerina *et al.*, 2020). However, poorly selected bacteria can deregulate their defences, potentially increasing mortality (Ptaszyńska *et al.*, 2016). Therefore, a beneficial microbe mixture must meet specific criteria when looking for tools to improve honey bee health. The microorganism or microorganisms must be native to the bee, the colony, or the flowers, be safe for all stages of development, be easy to administer and beneficial in some aspects for the colonies (Corby-Harris *et al.*, 2016).

Previous works had confirmed that the beneficial microbe mixture of *A. kunkeei* strains developed in our laboratory from honey bee native strains was safe for larvae and bees (Arredondo *et al.*, 2018). Furthermore, its administration in honey bee colonies under field conditions also resulted safe, as no deleterious effects were observed.

However, no positive effects on colony strength parameters were detected, even when different administration strategies were evaluated. The beneficial microbe mixture was used lyophilised, recovered from a fresh culture, administered by spraying or sprayed and in a feeder in autumn and spring. Different results were reported by Alberoni *et al.* (2018), who observed that

the administration of a bacterial mixture composed of three *Bifidobacteria* spp. and three *Lactobacillus* spp. increased pollen and honey area in summer. Meanwhile, other authors observed good outcomes by administering only one bacterial strain, e.g. higher honey storage and the honey bee population (Audisio and Benitez-Ahrendts, 2011; Audisio *et al.*, 2015; Sabate *et al.*, 2012) or lower parasite and pathogens loads (Audisio *et al.*, 2015; Corby-Harris *et al.*, 2016; Sabate *et al.*, 2012; Tejerina *et al.*, 2020).

In the first two assays, the freeze-dried beneficial microbe mixture in skim milk was used because this preservation technique confers an advantage for the production and long-term storage of the product. However, skim milk could be risky for honey bees. It has been reported that some pollen supplements containing galactose or lactose at a concentration as low as 2% in syrup or nectar can decrease the lifespan of bees (Barker, 1977). Although we did not observe a negative impact on colony strength in those assays, we decided to modify the strategy since we neither observed a positive result. Therefore, the beneficial microbe mixture was used as a fresh culture in the third and fourth assays as in all the laboratory tests carried out before with larvae and bees (Arredondo *et al.*, 2018). Anyway, positive effects on colony strength were neither detected.

Regarding the effect of the beneficial microbe mixture on the colony sanitary status, administration by spraying induced a fast decrease in the number of *Nosema* spp. spores in the colonies. This result agrees with a previous work where the administration of this beneficial microbe mixture reduced the number of *N. ceranae* spores in adult honey bees under laboratory conditions (Arredondo *et al.*, 2018). All together, result suggests that there could be a direct interaction between *N. ceranae* and the beneficial microbe mixture within the guts of adult bees. If the *A. kunkeei* strains colonise the guts before the pathogen internalisation in the gut epithelial cells, those microorganisms could prevent the infection of *N. ceranae*, or at least the subsequent multiplication within the intestinal epithelium. The decrease in the number of *N. ceranae* spores in the colony coincides with that reported by other authors, who have observed that the administration of metabolites, microorganisms or mixtures of native microorganisms can decrease the level of infection with *N. ceranae* in the field (Audisio *et al.*, 2015; Baffoni *et al.*, 2016; Corby-Harris *et al.*, 2016; Maggi *et al.*, 2013; Sabate *et al.*, 2012; Tejerina *et al.*, 2020). Although these results are promising, this decrease was significant only in the third assay, again indicating that particular environmental conditions could influence the impact of the treatment.

Surprisingly, our work showed that the administration of the beneficial microbe mixture delayed the multiplication and decreased the level of *V. destructor* infestation in the second and third assays. In a previous study, Saccà and Lodesani (2020), found that a strain of *A. kunkeei* was able to eliminate varroas by contact in laboratory experiments; they suggest that this may be due to the ability of this bacterium to acidify the environment in which they live, or by the production of bioactive metabolites. A similar hypothesis emerges from our field results; perhaps spraying the beneficial microbe mixture in the colonies eliminates the phoretic varroas by contact, reducing the number of mites that reach the next generation. Another possible mechanism of action could be that after being ingested, the beneficial microbes from the mixture secrete metabolites that reach the bee's surface by the haemolymph. These substances repel or eliminate the varroas, reducing their reproduction chances and thus reducing the population. Finally, although perhaps less likely, it could be that the administration of the beneficial microbe mixture promotes the hygienic behaviour of the bees, damaging varroas and, therefore, reducing the number of mites circulating in the colony. Several studies have shown the beneficial effect of native strains that reduced *V. destructor* infestation percentages in field trials. These strains were *L. johnsonii* CRL 1647, *B. subtilis* subsp. *subtilis* Mori2, *Ligilactobacillus salivarius* A3iob (Audisio and Benitez-Ahrendts, 2011; Audisio *et al.*, 2015; Sabate *et al.*, 2012; Tejerina *et al.*, 2020) or metabolites of *L. johnsonii* CRL 1647 and AJ5, *Enterococcus faecium* SM21 (De Piano *et al.*, 2020; Maggi *et al.*, 2013).

The field assays allowed, in parallel, to generate knowledge about the use of different acaricides for the control of *V. destructor*. Three acaricidal treatments were evaluated, and it was confirmed that colonies died if they did not receive any treatment, if the treatment was inadequate, or if it was applied at the wrong time (Rosenkranz *et al.*, 2010; Steinhauer *et al.*, 2018). Furthermore, it was observed that commercial oxalic acid strips were more effective than the homemade oxalic acid applied by dripping into the frame heads. It should be noticed that the time of year and the amount of brood may have influenced the result obtained since the efficacy of organic acaricides decreases in the presence of large quantities of brood (Marcangeli and García, 2004; Rosenkranz *et al.*, 2010). Therefore, none of the acaricidal treatments applied in this work could eliminate the mites. This finding coincides with other

authors' previous reports (Beaurepaire *et al.*, 2017; Maggi *et al.*, 2011; Rosenkranz *et al.*, 2010).

The beneficial microbe mixture administration did not affect the viral loads of the studied viruses. A high variability of viral loads per colony was observed regardless of the group to which they belonged. All these viruses can be transmitted horizontally within the same species (reviewed by Beaurepaire *et al.*, 2020), or between species, including native bees, such as bumble bees (Alger *et al.*, 2019; Bravi *et al.*, 2019), stingless bees and solitary bees (Alvarez *et al.*, 2018; Lucia *et al.*, 2014; Ueira-Vieira *et al.*, 2015). Thus, trophallaxis and visits to the flowers can facilitate horizontal virus transfer and continuous reinfection of colonies, explaining the high variability observed in the viral loads.

L. passim presence in the colonies was not affected by the administration of the beneficial microbe mixture. This trypanosomatid showed a low prevalence in late summer and increased towards winter, with a seasonality similar to that found in Germany (D'Alvise *et al.*, 2019). On the other hand, although the BM was effective under laboratory conditions in reducing the mortality caused by *P. larvae* in larvae (Arredondo *et al.*, 2018), we could not test its effectiveness in the field since nowadays, this pathogen has a low prevalence in our country (Palacios, unpublished data).

Finally, the administration of the beneficial microbe mixture did not affect the honey bee gut microbiota. Core honey bee gut microbiota is composed of *Lactobacillu*s Firm 4 and 5, *Gilliamella apicola*, *Snodgrassella alvi* and *Bifidobacterium* spp. (reviewed by Kwong and Morán, 2016). Besides them, other bacteria may vary in abundance with particular hosts or ecological conditions, e.g. *Bartonella* and *Frischella* (reviewed by Kwong and Morán, 2016; Raymann and Morán, 2018; Subotic *et al.*, 2019). All these bacteria were present in all samples and did not change between treatments. In a previous work under laboratory condition, the administration of this BM mixture increased the abundance of Lactobacillales, suggesting that it can potentially stimulate beneficial microorganisms (Arredondo *et al.*, 2018), nevertheless that was not observed in this study. On the other hand, gut microbiota changed in all groups from autumn to winter, nonetheless no differences in the alpha diversity indexes or ASV's were observed. Seasonal variations on gut microbiota have already been described by several authors (Castelli *et al.*, 2022; Kešnerová, *et al.*, 2020; Li *et al.*, 2022; Subotic *et al.*, 2019).

Probiotics are widely used to boost animal health under gastrointestinal dysbiosis or disease (Chaucheyras-Durand and Durand, 2010; Reid, 1999). As it was reviewed by Motta *et al.* (2022), no beneficial microbe formulations have been demonstrated to be reliably effective in honey bee colonies. It should be considered a minimum colony number in future studies to confirm the success of the beneficial microbe administration since sometimes in the literature, the number of colonies per group is low ($n \leq 5$). Although our results were inconclusive, perhaps we would have a more reliable effect in colonies with high levels of parasitation with *Nosema* spp. or *V. destructor* or under other stressors exposure as toxins or poor nutrition.

5 Conclusions

The administration of the beneficial microbe mixture induced the decrease of *V. destructor* and *Nosema* spp., although the results were variable in different trials. Besides, bacteria failed to improve the colony's strength parameters or honey production. Although the administration of beneficial microbes can represent a promising strategy to improve honey bee health, meticulous studies must confirm that they work under field conditions. Those studies should be long-term assays, including high numbers of colonies, different doses, years and regions.

Supplementary Material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.23507757

Figure S1. Schematic representation of the different field assays.

Figure S2. Adult population: (A) first assay, (B) second assay, (C) third assay, D (fourth assay).

Figure S3. Brood population: (A) first assay, (B) second assay, (C) third assay, (D) fourth assay.

Figure S4. Honey reserves: (A) first assay, (B) second assay, (C) third assay, (D) fourth assay.

Figure S5. Infestation level with *V. destructor*: (A) first assay, (B) second assay, (C) third assay, (D) fourth assay.

Figure S6. ABPV infection level: (A) first assay, (B) second assay, (C) third assay.

Figure S7. BQCV infection level: (A) first assay, (B) second assay.

Figure S8. DWV infection level: (A) first assay, (B) second assay, (C) third assay.

Figure S9. SBV infection level: (A) first assay, (B) second assay. The

Figure S10. Cumulative bar graph where the number of colonies infected and non-infected with *L. passim* is observed.

Figure S11. Differential abundance of ASVs of honey bee gut bacterial communities.

Figure S12. Relative abundance of the different bacterial genera in each colony.

Table S1. Primers used for PCR and qPCR-based quantification of viruses in nurse honey bee samples.

Table S2. Beneficial microbe mixture dose administered in each assay.

Table S3. Statistics of adult population comparisions within treatments in each assay.

Table S4. Statistics of brood population comparisions within treatments in each assay.

Table S5. Statistics of honey reserve comparisions within treatments in each assay.

Table S6. Statistics of *Nosema* spp. spores load comparisions within monts in each assay.

Table S7. Statistics of *Varroa destructor* infestation percentaje comparisions within treatments in each assay.

Table S8. Statistics of colony mortality in each assay.

Table S9. Statistics of *Acute bee paralysis virus* (ABPV) infection comparisions within treatments in each assay.

Table S10. Statistics of *Black queen cell virus* (BQCV) infection comparisions within treatments in each assay.

Table S11. Statistics of *Deformed wing virus* (DWV) infection comparisions within treatments in each assay.

Table S12. Statistics of *Sacbrood virus* (SBV) infection comparisions within treatments in each assay.

Table S13. Statistics of *Lotmaria passim* within treatments in the first assay.

Table S14. Impact of the time in the microbial gut microbiota on field assays.

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Conflict of interest

The authors declare no conflict of interest.

Data availability

The sequence datasets generated in the current study are available in the NCBI under BioProject ID PRJNA967750).

References

Alberoni, D., Baffoni, L., Gaggìa, F., Ryan, P.M., Murphy, K., Ross, P.R., Stanton, C. and Di Gioia, D., 2018. Impact of beneficial bacteria supplementation on the gut microbiota, colony development and productivity of *Apis mellifera* L. Beneficial Microbes 9: 269-278.

Alberoni, D., Gaggìa, F., Baffoni, L. and Di Gioia, D., 2016. Beneficial microorganisms for honey bees: problems and progresses. Applied Microbiology and Biotechnology 100: 9469-9482.

Alger, S.A., Burnham, P.A., Boncristiani, H.F. and Brody, A.K., 2019. RNA virus spillover from managed honeybees (*Apis mellifera*) to wild bumblebees (*Bombus* spp.). PLoS One 14: e0217822.

Alippi, A.M. and 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. Journal of Invertebrate Pathology 91: 141-146.

Alvarez, L.J., Reynaldi, F.J., Ramello, P.J., Garcia, M.L.G., Sguazza, G.H., Abrahamovich, A.H. and Lucia, M., 2018. Detection of honey bee viruses in Argentinian stingless bees (*Hymenoptera*: *Apidae*). Insectes Sociaux 65: 191-197.

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersions. Biometrics 62: 245-253.

Anderson, M.J., Ellingsen, K.E. and McArdle, B.H., 2006. Multivariate dispersion as a measure of beta diversity. Ecology Letters 9: 683-693.

Arismendi, N., Bruna, A., Zapata, N. and Vargas, M., 2016. PCR-specific detection of recently described *Lotmaria passim* (*Trypanosomatidae*) in Chilean apiaries. Journal of Invertebrate Pathology 134: 1-5.

Arismendi, N., Caro, S., Castro, M.P., Vargas, M., Riveros, G. and Venegas, T., 2020. Impact of mixed infections of gut parasites *Lotmaria passim* and *Nosema ceranae* on the lifespan and immune-related biomarkers in *Apis mellifera*. Insects 11: 420.

Arredondo, D., Castelli, L., Porrini, M.P., Garrido, P.M., Eguaras, M.J., Zunino, P. and Antúnez, K., 2018. *Lactobacillus kunkeei* strains decreased the infection by honey bee pathogens *Paenibacillus larvae* and *Nosema ceranae*. Beneficial Microbes 9: 279-290.

Audisio, M.C. and Benitez-Ahrendts, M.R., 2011. *Lactobacillus johnsonii* CRL1647, isolated from *Apis mellifera* L. bee-gut, exhibited a beneficial effect on honeybee colonies. Beneficial Microbes 2: 29-34.

Audisio, M.C., Sabate, D.C. and Benítez-Ahrendts, M.R., 2015. Effect of *Lactobacillus johnsonii* CRL1647 on different parameters of honeybee colonies and bacterial populations of the bee gut. Beneficial Microbes 6: 687-695.

Baffoni, L., Gaggìa, F., Alberoni, D., Cabbri, R., Nanetti, A., Biavati, B. and Di Gioia, D., 2016. Effect of dietary supplementation of *Bifidobacterium* and *Lactobacillus* strains in *Apis mellifera* L. against *Nosema ceranae*. Beneficial Microbes 7: 45-51.

Barker, R.J., 1977. Some carbohydrates found in pollen and pollen substitutes are toxic to honey bees. Journal of Nutrition 107: 1859-1862.

Beaurepaire, A., Krieger, K.J. and Moritz, R.F., 2017. Seasonal cycle of inbreeding and recombination of the parasitic mite *Varroa destructor* in honeybee colonies and its implications for the selection of acaricide resistance. Infection, Genetics and Evolution 50: 49-54.

Beaurepaire, A., Piot, N., Doublet, V., Antunez, K., Campbell, E., Chantawannakul, P., Chejanovsky, N., Gajda, A., Heerman, M., Panziera, D., Smagghe, G., Yañez, O., de Miranda, J.R. and Dalmon, A., 2020. Diversity and global distribution of viruses of the western honey bee, *Apis mellifera*. Insects 11: 239.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A. and Caporaso, J.G., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6: 1-17.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R. and Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37: 852-857.

Bravi, M.E., Alvarez, L.J., Lucia, M., Pecoraro, M.R., García, M.L.G. and Reynaldi, F.J., 2019. Wild bumble bees (*Hymenoptera*: *Apidae*: *Bombini*) as a potential reservoir for bee pathogens in northeastern Argentina. Journal of Apicultural Research 58: 710-713.

Buendía-Abad, M., García-Palencia, P., de Pablos, L.M., Alunda, J.M., Osuna, A., Martín-Hernández, R. and Higes, M., 2022. First description of *Lotmaria passim* and *Crithidia mellificae* haptomonad stages in the honeybee hindgut. International Journal for Parasitology 52: 65-75.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. and Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13: 581-583.

Castelli, L., Branchiccela, B., Romero, H., Zunino, P. and Antúnez, K., 2022. Seasonal dynamics of the honey bee gut microbiota in colonies under subtropical climate: seasonal dynamics of honey bee gut microbiota. Microbial Ecology 83: 492-500.

Chaucheyras-Durand, F. and Durand, H., 2010. Probiotics in animal nutrition and health. Beneficial Microbes 1: 3-9.

Corby-Harris, V., Snyder, L., Meador, C., Naldo, R., Mott, B. and Anderson, K., 2016. *Parasaccharibacter apium*, gen. nov., sp. nov., improves honey bee (*Hymenoptera*: *Apidae*) resistance to *Nosema*. Journal of Economic Entomology 109: 537-543.

Daisley, B.A. and Reid, G., 2020. BEExact: a metataxonomic database tool for high-resolution inference of bee-associated microbial communities. mSystems 6: e00082-21.

D'Alvise, P., Seeburger, V., Gihring, K., Kieboom, M. and Hasselmann, M., 2019. Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecology and Evolution 9: 10241-10252.

De Piano, F.G., Maggi, M.D., Meroi Arceitto, F.R., Audisio, M.C., Eguaras, M. and Ruffinengo, S.R., 2020. Effects of bacterial cell-free supernatant on nutritional parameters of *Apis*

mellifera and their toxicity against *Varroa destructor*. Journal of Apicultural Science 64: 55-66.

Delaplane, K.S., Van Der Steen, J. and Guzman-Novoa, E., 2013. Standard methods for estimating strength parameters of *Apis mellifera* colonies. Journal of Apicultural Research 52: 1-12. https://doi.org/10.3896/IBRA.1.52.1.03.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P. and Ellis, J.D., 2013. Standard methods for varroa research. Journal of Apicultural Research 52: 1-54.

Engel, P., James, R.R., Koga, R., Kwong, W.K., McFrederick, Q.S. and Moran, N.A., 2013. Standard methods for research on *Apis mellifera* gut symbionts. Journal of Apicultural Research 52: 1-24. https://doi.org/10.3896/IBRA.1.52.4.07.

Evans, J.D., 2003. Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*. Journal of Invertebrate Pathology 83: 46-50.

Evans, J.D., 2006. Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. Journal of Invertebrate Pathology 93: 135-139.

Evans, J.D. and Armstrong, T.N., 2006. Antagonistic interactions between honey bee bacterial symbionts and implications for disease. BMC Ecology 6: 4.

Forsgren, E., Olofsson, T.C., Váasquez, A. and Fries, I., 2010. Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie 41: 99-108.

Fries, I., Chauzat, M.P., Chen, Y.P., Doublet, V., Genersch, E., Gisder, S., Higes, M., McMahon, D.P., Martín-Hernández, R., Natsopoulou, M., Paxton, R.J., Tanner, G., Webster, T.C. and Williams, G.R., 2013. Standard methods for *Nosema* research. Journal of Apicultural Research 52: 1-28. https://doi.org/10.3896/IBRA.1.52.1.14.

Fries, I., Feng, F., daSilva, A., Slemenda, S.B. and Pieniazek, N.J., 1996. *Nosema ceranae* n sp (*Microspora*, *Nosematidae*), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (*Hymenoptera*, *Apidae*). European Journal of Protistology 32: 356-365.

Genersch, E., 2010. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. Journal of Invertebrate Pathology 103: S10-S19.

Gentleman, R., Carey, V.J., Huber, W. and Hahne, F., 2021. genefilter: genefilter: methods for filtering genes from high-throughput experiments. R package version 1.74.0.

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347: 1255957.

Gray, A., Adjlane, N., Arab, A., Ballis, A., Brusbardis, V., Charrière, J.-D., Chlebo, R., Coffey, M.F., Cornelissen, B., Amaro da Costa, C., Dahle, B., Danihlík, J., Dražić, M.M., Evans, G., Fedoriak, M., Forsythe, I., Gajda, A., de Graaf, D.C., Gregorc, A., Ilieva, I., Johannesen, J., Kauko, L., Kristiansen, P., Martikkala, M., Martín-Hernández, R., Medina-Flores, C.A., Mutinelli, F., Patalano, S., Raudmets, A., Martin, G.S., Soroker, V., Stevanovic, J., Uzunov, A., Vejsnaes, F., Williams, A., Zammit-Mangion, M. and Brodschneider, R., 2020. Honey bee colony winter loss rates for 35 countries participating in the COLOSS survey for winter 2018-2019, and the effects of a new queen on the risk of colony winter loss. Journal of Apicultural Research 59: 744-751.

Harriet, J., Campá, J.P., Grajales, M., Lhéritier, C., Pajuelo, A.G., Mendoza-Spina, Y. and Carrasco-Letelier, L., 2017. Agricultural pesticides and veterinary substances in Uruguayan beeswax. Chemosphere 177: 77-83.

Higes, M., Meana, A., Bartolomé, C., Botías, C. and Martín-Hernández, R., 2013. *Nosema ceranae* (*Microsporidia*), a controversial 21st century honey bee pathogen. Environmental Microbiology Reports 5: 17-29.

Huang, W.F., Solter, L.F., Yau, P.M. and Imai, B.S., 2013. *Nosema ceranae* escapes fumagillin control in honey bees. PLoS Pathogens 9: e1003185.

Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Møller, P.L., Michaelsen, K.F., Pærregaard, A., Sandström, B., Tvede, M. and Jakobsen, M., 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Applied and Environmental Microbiology 65: 4949-4956.

Johnson, R.M., Evans, J.D., Robinson, G.E. and Berenbaum, M.R., 2009. Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). Proceedings of the National Academy of Sciences of the USA 106: 14790-14795.

Kešnerová, L., Emery, O., Troilo, M., Liberti, J., Erkosar, B. and Engel, P., 2020. Gut microbiota structure differs between honeybees in winter and summer. ISME Journal 14: 801-814.

Kukielka, D., Esperón, F., Higes, M. and Sánchez-Vizcaíno, J.M., 2008. A sensitive one-step real-time RT-PCR method for detection of deformed wing virus and black queen cell virus in honeybee *Apis mellifera*. Journal of Virological Methods 147: 275-281.

Kwong, W.K. and Moran, N.A., 2016. Gut microbial communities of social bees. Nature Reviews Microbiology 14: 374-384. https://doi.org/10.1038/nrmicro.2016.43

Li, C., Tang, M., Li, X. and Zhou, X., 2022. Community dynamics in structure and function of honey bee gut bacteria in response to winter dietary shift. Host Microbial Interactions 13: e01131-22.

Love, M.I., Huber, W. and Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 131.

Lucia, M., Reynaldi, F.J., Sguazza, G.H. and Abrahamovich, A.H., 2014. First detection of deformed wing virus in *Xylocopa augusti* larvae (*Hymenoptera*: *Apidae*) in Argentina. Journal of Apicultural Research 53: 466-468.

Maggi, M., Negri, P., Plischuk, S., Szawarski, N., De Piano, F., De Feudis, L., Eguaras, M. and Audisio, C., 2013. Effects of the organic acids produced by a lactic acid bacterium in *Apis mellifera* colony development, *Nosema ceranae* control and fumagillin efficiency. Veterinary Microbiology 167: 474-483.

Maggi, M., Tourn, E., Negri, P., Szawarski, N., Marconi, A., Gallez, L., Medici, S., Ruffinengo, S., Brasesco, C., De Feudis, L., Quintana, S., Sammataro, D. and Eguaras, M., 2016. A new formulation of oxalic acid for *Varroa destructor* control applied in *Apis mellifera* colonies in the presence of brood. Apidologie 47: 596-605.

Maggi, M.D., Ruffinengo, S.R., Mendoza, Y., Ojeda, P., Ramallo, G., Floris, I. and Eguaras, M.J., 2011. Susceptibility of *Varroa destructor* (*Acari*: *Varroidae*) to synthetic acaricides in Uruguay: varroa mites' potential to develop acaricide resistance. Parasitology Research 108: 815-821.

Marcangeli, J. and García, M.C., 2004. Efecto de la cantidad de cría de abeja *Apis mellifera* (*Apidae*) sobre la eficacia del Oxavar® para el control del ácaro *Varroa destructor* (*Varroidae*). Revista de la Sociedad Entomológica Argentina 63: 35-38.

Martel, A.C., Zeggane, S., Aurières, C., Drajnudel, P., Faucon, J.-P. and Aubert, M., 2007. Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar or Asuntol. Apidologie 38: 534-544.

Martel, A.-C., Zeggane, S., Drajnudel, P., Faucon, J.-P. and Aubert, M., 2006. Tetracycline residues in honey after hive treatment. Food Additives and Contaminants 23: 265-273.

Martín-Hernández, R., Bartolomé, C., Chejanovsky, N., Le Conte, Y., Dalmon, A., Dussaubat, C., García-Palencia, P., Meana, A., Pinto, M.A., Soroker, V. and Higes, M., 2018. *Nosema ceranae* in *Apis mellifera*: a 12 years postdetection perspective. Environmental Microbiology 20: 1302-1329.

McMurdie, P.J. and Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8: e61217.

Motta, E.V., Powell, J.E., Leonard, S.P. and Moran, N.A., 2022. Prospects for probiotics in social bees. Philosophical Transactions of the Royal Society B 377: 20210156.

Nazzi, F. and Le Conte, Y., 2016. Ecology of *Varroa destructor*, the major ectoparasite of the western honey bee, *Apis mellifera*. Annual Review of Entomology 61: 417-432.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecsand, E. and Wagner, H., 2020. vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29: e45.

Potts, S.G., Imperatriz-Fonseca, V., Ngo, H.T., Aizen, M.A., Biesmeijer, J.C., Breeze, T.D., Dicks, L.V., Garibaldi, L.A., Hill, R., Settele, J. and Vanbergen, A.J., 2016. Safeguarding pollinators and their values to human well-being. Nature 540: 220-229.

Ptaszyńska, A.A., Borsuk, G., Zdybicka-Barabas, A., Cytryńska, M. and Małek, W., 2016. Are commercial probiotics and prebiotics effective in the treatment and prevention of honeybee nosemosis C? Parasitology Research 115: 397-406.

Ramsey, S.D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J.D., Cohen, A., Lim, D., Joklik, J., Cicero, J.M., Ellis, J.D., Hawthorne, D. and Van Engelsdorp, D., 2019. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. Proceedings of the National Academy of Sciences of the USA 116: 1792-1801.

Raymann, K. and Morán, N.A., 2018. The role of the gut microbiome in health and disease of adult honey bee workers. Current Opinion in Insect Science 26: 97-104. https://doi.org/10.1016/j.cois.2018.02.012

Reid, G., 1999. The scientific basis for probiotic strains of *Lactobacillus*. Applied and Environmental Microbiology 65: 3763-3766.

Requier, F., Antúnez, K., Morales, C.L., Aldea Sánchez, P., Castilhos, D., Garrido, P.M., Giacobino, A., Reynaldi, F.J., Rosso Londoño, J.M., Santos, E. and Garibaldi, L.A., 2018. Trends in beekeeping and honey bee colony losses in Latin America. Journal of Apicultural Research 57: 657-662.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B., 2010. Biology and control of *Varroa destructor*. Journal of Invertebrate Pathology 103: S96-119.

RStudio Team, 2021. RStudio: integrated development environment for R. RStudio, PBC, Boston, MA, USA. Available at:http://www.rstudio.com/

Sabaté, D.C., Carrillo, L. and Audisio, M.C., 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Research in Microbiology 160: 193-199.

Sabaté, D.C., Cruz, M.S., Benítez-Ahrendts, M.R. and Audisio, M.C., 2012. Beneficial effects of *Bacillus subtilis* subsp. *subtilis* Mori2, a honey-associated strain, on honeybee colony performance. Probiotics Antimicrobial Proteins 4: 39-46.

Saccà, M.L. and Lodesani, M., 2020. Isolation of bacterial microbiota associated to honey bees and evaluation of potential biocontrol agents of *Varroa destructor*. Beneficial Microbes 11: 641-654.

Schwarz, R.S., Bauchan, G.R., Murphy, C.A., Ravoet, J., de Graaf, D.C. and Evans, J.D., 2015. Characterization of two species of *Trypanosomatidae* from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. Journal of Eukaryotic Microbiology 62: 567-583.

Steinhauer, N., Kulhanek, K., Antúnez, K., Human, H., Chantawannakul, P. and Chauzat, M.P., 2018. Drivers of colony losses. Current Opinion in Insect Science 26: 142-148.

Subotic, S., Boddicker, A.M., Nguyen, V.M., Rivers, J., Briles, C.E. and Mosier, A.C., 2019. Honey bee microbiome associated with different hive and sample types over a honey production season. PLoS ONE 14: e0223834.

Tang, J., Ma, C., Shi, W., Chen, X., Liu, Z., Wang, H. and Chen, C., 2020. A national survey of managed honey bee colony winter losses (*Apis mellifera*) in China (2013-2017). Diversity 12: 318.

Tejerina, M.R., Benítez-Ahrendts, M.R. and Audisio, M.C., 2020. *Lactobacillus salivarius* A3iob reduces the incidence of *Varroa destructor* and *Nosema* spp. in commercial apiaries located in the northwest of Argentina. Probiotics and Antimicrobial Proteins 12: 1360-1369.

Traynor, K.S., Mondet, F., De Miranda, J.R., Techer, M., Kowallik, V., Oddie, M.A., Chantawannakul, P. and McAfee, A., 2020. *Varroa destructor*: a complex parasite, crippling honey bees worldwide. Trends in Parasitology 36: 592-606.

Ueira-Vieira, C., Almeida, L.O., de Almeida, F.C., Amaral, I.M.R., Brandeburgo, M.A.M. and Bonetti, A.M., 2015. Scientific note on the first molecular detection of the acute bee paralysis virus in Brazilian stingless bees. Apidologie 46: 628-630.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L. and Olofsson, T.C., 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS ONE 7: e33188.

Wu, M., Sugimura, Y., Iwata, K., Takaya, N., Takamatsu, D., Kobayashi, M., Taylor, D., Kimura, K. and Yoshiyama, M., 2014. Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. Journal of Insect Sciences 14: 129.

Yang, X. and Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences of the USA 102: 7470-7747.

Yoshiyama, M. and Kimura, K., 2009. Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus larvae*, the causal agent of American foulbrood. Journal of Invertebrate Pathology 102: 91-96.