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Lactobacillus kunkeei strains decreased the infection by honey bee pathogens *Paenibacillus larvae* and *Nosema ceranae*

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RESEARCH ARTICLE

Abstract

Due to their social behaviour, honey bees can be infected by a wide range of pathogens including the microsporidia *Nosema ceranae* and the bacteria *Paenibacillus larvae*. The use of probiotics as food additives for the control or prevention of infectious diseases is a widely used approach to improve human and animal health. In this work, we generated a mixture of four *Lactobacillus kunkeei* strains isolated from the gut microbial community of bees, and evaluated its potential beneficial effect on larvae and adult bees. Its administration in controlled laboratory models was safe for larvae and bees; it did not affect the expression of immune-related genes and it was able to decrease the mortality associated to *P. larvae* infection in larvae and the counts of *N. ceranae* spores from adult honey bees. These promising results suggest that this beneficial microorganism's mixture may be an attractive strategy to improve bee health. Field studies are being carried out to evaluate its effect in naturally infected colonies.

Keywords: beneficial microorganisms, probiotics, honey bee health, American Foulbrood, nosemosis

1. Introduction

Honey bees (*Apis mellifera*) are among the main pollinators of commercial crops and wild flowers around the world (Klein *et al.*, 2007; Morse and Calderone, 2000). It has been estimated that 70 out of the 107 most important crops for the human diet are moderate to strictly dependent on animal pollination, being bees the most economically valuable species (Klein *et al.*, 2007).

Due to their social behaviour, honey bees are infected by different parasites and pathogens, including the mite *Varroa destructor*, the bacterium *Paenibacillus larvae*, the microsporidia *Nosema ceranae* and different RNA viruses (Genersch, 2010). *Paenibacillus larvae* is a gram-positive and spore-forming bacterium that causes American Foulbrood (AFB), the most severe bacterial disease that affects honey bee larvae (Genersch *et al.*, 2006; Hansen and Brodsgaard, 1999). *Nosema ceranae* is an obligate intracellular spore-forming parasite that belongs to the order *Microsporidia* (Fries *et al.*, 1996). It is the causative agent of Nosemosis type C, an important threat to the honey bee health (Higes *et al.*, 2013).

Honey bees have developed different individual strategies to fight against pathogen infections like mechanical, physiological and immunological defences (Evans, 2006; Evans and Spivak, 2010; Wilson-Rich *et al.*, 2008). Mechanical barriers include the insect cuticle and epithelial layers, which prevent microbes from adhering to or entering the body, whereas the physiological inhibitors include pH changes and other chemical conditions of the insect gut. The immunological defences consist of four interconnected pathways (Toll, Imd, Jak/STAT, and Jnk) which include proteins that recognise parasites, proteins that amplify the signal and effectors proteins (Evans and Spivak, 2010). Recently, Doublet *et al.* (2017) have identified a common set of genes that respond to the infection by different pathogens (*V. destructor, N. apis, N. ceranae* and RNA viruses), suggesting a common response to different infections. This common set of genes includes hymenoptaecin, defensin, abaecin and lysozyme, among

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others. Besides that, honey bees have evolved collective immune defences ('social immunity') which result from the behavioural cooperation among individuals (Cremer *et al.*, 2007; Evans and Spivak, 2010). However, these mechanisms are often not enough to overcome different disorders, so beekeepers frequently apply different products (antibiotics or acaricides) to prevent or control diseases.

The use of antibiotics and synthetic acaricides has several disadvantages since they can cause an imbalance on the enteric homeostasis, affecting as well the bee metabolism and the immune response. It is known as well that this practice can stimulate the generation of resistant organisms (Boncristiani *et al.*, 2012; Evans, 2003; Garrido *et al.*, 2013; Rosenkranz *et al.*, 2010). Besides that, those products can remain in honey and other bee products (Harriet *et al.*, 2017; Martel *et al.*, 2006). For these reasons, a healthy and integrated management strategy to improve honey bee health is needed.

The use of probiotics as food additives is a widely used approach to improve human and animal health (Chaucheyras-Durand and Durand, 2010; Reid, 1999). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). To be considered as probiotics, microorganisms must fulfil a series of biological requirements and safety criteria (to be non-toxic and non-pathogenic, to be normal inhabitants of the targeted host-species, to adhere to the gut epithelium, between others).

In the case of honey bees, there have been multiple attempts to identify and characterise bacteria belonging to the gut microbiota to be used as probiotics, which have been recently reviewed by Alberoni *et al.* (2016). These authors proposed to name these bacteria as 'beneficial microorganisms' instead of 'probiotics' since the requirements needed for a microorganism to be classified as a probiotic are not usually evaluated.

Multiple *in vitro* assays have shown that bacteria isolated from the hive and the gut microbiota can inhibit the growth of pathogens, such as *P. larvae, Melissococcus plutonius* or *Ascosphaera apis.* These bacteria are generally included in the genera *Lactobacillus, Bifidobacterium, Bacillus, Brevibacillus* and *Enterococcus*, among others (Alippi and Reynaldi, 2006; Audisio *et al.* 2011; Evans and Armstrong, 2006; Forsgren *et al.*, 2010; Killer *et al.*, 2014; Lee *et al.*, 2009; Sabaté *et al.*, 2009; Vásquez *et al.*, 2012; Wu *et al.*, 2013; Yoshiyama and Kimura, 2009). Regarding *in vivo* studies, the administration of different *Lactobacillus* and *Bifidobacterium,* strains to *P. larvae* infected larvae, significantly reduced their mortality (Forsgren *et al.*, 2010). Similar results were observed in the case of infection with the bacterial pathogen *M. plutonius* (Vásquez *et al.*, 2012; Wu *et al.*, 2014).

Other studies have focused on the ability of different bacterial strains to inhibit the growth of *N. ceranae.* Corby-Harris *et al.* (2016) evaluated the effect of the administration of *Parasaccharibacter apium* in healthy colonies. When honey bees were infected with *N. ceranae* spores under laboratory conditions, they observed fewer spores in treated bees compared to those that had not been treated (Corby-Harris *et al.*, 2016). Baffoni *et al.* (2016) also showed that the oral administration of *Bifidobacterium* and *Lactobacillus* strains to infected adult honey bees generated a decrease in the number of *N. ceranae* spores. Sabaté *et al.* (2012) and Audisio *et al.* (2015) also observed a decrease in the number of *N. ceranae* spores in honey bees orally fed with *Bacillus subtilis* or *Lactobacillus johnsonii* for several months under field conditions. Audisio (2017) reported that these strains also have beneficial effects at a colony level, increasing egg-laying by the queen and reducing the incidence of nosemosis and varroosis.

These results encourage the study of beneficial microorganisms as a strategy to decrease the infection by different pathogens and improve honey bee health. However, the selection of microorganisms should be carefully performed, since the use of inadequate products can lead to unwanted effects. As an example, the use of a commercial product based on *Lactobacillus rhamnosus* increased the susceptibility to nosemosis, de-regulated the insect immune systems and increased bee mortality (Ptaszyńska *et al.*, 2016).

The aim of this study was to isolate and characterise bacterial isolates from the honey bee gut microbial community, to evaluate their effect on the bee immune system and their effect against the bee pathogens *P. larvae* and *N. ceranae*.

2. Materials and methods

Honey bee samples and culture conditions

Nurse honey bees were collected from colonies located in the provinces of Colonia, Treinta y Tres, and Montevideo, Uruguay. Guts were aseptically extracted, homogenised in 1 ml of phosphate buffered saline (PBS) and cultured in De Man, Rogosa and Sharpe (MRS) and Rogosa agar (Merck, Darmstadt, Germany) at 37 °C for 48 h under microaerophilic conditions. Three to five bacterial colonies per plate were randomly selected and pure cultures were obtained. Isolates were maintained in MRS broth supplemented with 15% glycerol as stock cultures at -80 °C or as work cultures at -20 °C. Identification of these isolates were initially performed based on colony morphology, microscopic examination, Gram staining, catalase and oxidase reactions (Gerhardt *et al.*, 1994).

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Characterisation of bacterial isolates

In vitro assays to assess Paenibacillus larvae inhibition

Inhibitory activity of the bacterial isolates against *P. larvae* was investigated using an *in vitro* assay (Del Rosario Pascual Anderson and Calderón y Pascual, 1999). *P. larvae* strain 44 was used in this assay (Antúnez *et al.*, 2007). A fresh *P. larvae* culture (less than 48 h) in J agar (Hornitzky and Nicholls, 1993) was used to prepare a cell suspension in PBS adjusted according to the 0.5 McFarland standard which was spread by swabbing on J agar. Then, the selected bacterial isolates were inoculated by puncturing in the same plate. After incubation at 37 °C for 48 h under microaerophilic conditions, plates were examined for inhibition zones and the diameters were measured. Assays were carried out in triplicates.

Bacterial growth

A 96-flat-bottom microwell plate containing 180 µl of MRS broth per well was inoculated with 20 µl of a bacterial suspension in PBS with a turbidity equivalent to the 0.5 McFarland scale per well. Plates were incubated at 37 °C and absorbance (OD_{600}) was measured every 2 h. The doubling time and the growth rate were estimated (Gerhardt *et al.*, 1994). Three independent assays were performed.

Osmotic tolerance to sugar syrup

Viability under osmotic conditions was determined at two temperatures (4 and 28 °C) and two sugar syrup concentrations (1:1 or 2:1, 1000 or 2,000 g of sugar in 1 l of water, respectively). A bacterial suspension in PBS equivalent to the 4 McFarland scale was diluted in sugar syrup at a final concentration of 1×10^7 cfu/ml and was incubated at 4 or 28 °C for 72 h. The number of viable bacterial cells per treatment was determined by plate count on MRS agar at 0 and 72 h. Plates were incubated at 37 °C for 24 h under microaerophilic conditions.

Tolerance to different pH

To estimate bacterial survival at different acidity conditions, a modification of the method described by Jacobsen *et al.* (1999) was performed. The test was carried out in 96-flat-bottom microwell plates containing 180 µl of MRS broth at pH 3, 5 or 7, and inoculated with 20 µl of a bacterial suspension in PBS at 0.5 McFarland scale. Optical density at 600 nm (OD600) was measured at 0, 1, 2, 3, 4 and 24 h of incubation at 37 °C. The number of viable bacterial cells per treatment was determined by plate counting on MRS agar at 0 and 4 h. Plates were incubated at 37 °C for 48 h under microaerophilic conditions.

Preselection of isolates

Ten bacterial isolates showing the best biological and biotechnological characteristics (inhibition of *P. larvae*, osmotic tolerance to sugar syrup and tolerance to acid conditions) were selected and studied in detail.

Inhibition assays between isolates

A bacterial suspension in PBS at 0.5 McFarland scale of the ten preselected isolates was inoculated by swabbing in MRS agar and the rest of the bacterial isolates (9) were inoculated in the same plate by puncture, to rule out inhibition effects between the selected bacterial strains. Plates were incubated at 37 °C for 48 h under microaerophilic conditions. Three independent assays were performed.

DNA extraction and PCR reaction

Genomic DNA was extracted from overnight bacterial cultures of the ten preselected isolates using a commercial kit (Gen Elute Bacterial Genomic; Sigma, St. Louis, MO, USA). DNA was subjected to amplification of the 16SrRNA gene, using universal bacterial primers 27F and 1492R (Lane, 1991). PCR was carried out in a final volume of 25 µl containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP Mix, 0.5 µM of each primer, 1 µl of DNA and 1 U Taq DNA polymerase (Invitrogen, Life Technologies, São Paulo, Brazil). Amplification consisted of an initial denaturation step at 95 °C for 5 min, 29 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The PCR reactions were performed in a Multi Gene Opti Max Thermal Cycler (Labnet International, Edison, NJ, USA). PCR products were examined using agarose (1%) gel electrophoresis and visualized using GelRed (Biotium, Fremont, CA, USA) in a UV light transilluminator (Macro VueUvis-20, Hoefer Inc., Holliston, MA, USA). Amplicons were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced at Macrogen Inc. (Seoul, Korea). Obtained sequences were analysed using the Classifier tool of the RDP (Wang *et al.*, 2007) and BLASTn to compare with data in the GenBank database (NCBI; Altschul *et al.*, 1997).

Effect of bacterial isolates on larvae survival and *Paenibacillus larvae* infection

Toxicity of the bacterial isolates on *A. mellifera* larvae and their antibacterial effect against *P. larvae* were investigated using an *in vivo* larval model. Worker bee larvae from the progeny of a single wild-mated honey bee queen (a hybrid of *Apis mellifera mellifera* with *Apis mellifera scutellata*) maintained in a disease-free apiary at the IIBCE were used. One-day-old larvae were collected and maintained as described by Evans (2004). 22 groups of 12 larvae each were used.

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The ten preselected isolates were cultured on MRS agar and individual cell suspensions were prepared on MRS broth and incubated at 37 ° for 24 h under microaerophilic conditions. Then, an aliquot was taken and its absorbance at 600 nm was measured. The same amount of bacterial cells from each isolate was centrifuged, the supernatant was discarded and pellet was suspended in artificial diet, to a final concentration of 1×10^4 cell/µl.

Ten larval groups were fed *ad libitum* with this artificial diet supplemented with one of the ten isolates (groups 1, 22, 35, 37, 51, 67, 78, 110, 117, 122); other ten groups were fed with the above-mentioned diet supplemented with one preselected isolate and contaminated with *P. larvae* (1000 spores/µl, groups 1P, 22P, 35P, 37P, 51P, 67P, 78P, 110P, 117P, 122P); one control group of larvae was fed only with the artificial diet (L) and another control group was fed with the artificial diet contaminated with *P. larvae* (1000 spores/µl) (LP).

Larvae were fed with artificial diet (alone or supplemented with *P. larvae* spores or/and bacterial cells) for the first 48 h after grafting and then normal larval diet was used in all cases. Plates were incubated at 34.5 °C with high humidity for 6 days. Each day, larvae were taken out from the incubator and examined. Larvae were classified as dead when they lost their body elasticity or displayed a colour change to brownish. The number of dead larvae was recorded and surviving larvae were transferred to new wells filled with fresh food.

Effect of bacterial isolates on bee survival

An *in vivo* model was used to rule out possible deleterious effects of the bacterial isolates on adult bees. Frames of sealed brood from an *A. mellifera* healthy colony from the experimental apiary J.J. Nagera (Social Bees Research Centre, Mar del Plata, Argentina) were maintained at 35 °C in an incubator. New emerging worker bees were removed, confined into special cages, kept in the incubator and fed *ad libitum* with a sugar syrup solution (50% w/v sucrose in tap water). After two days of emergence, bees were divided into eleven groups of 15 bees each, starved for 3 h and fed as described by Rinderer (1976) and modified by Porrini *et al.* (2011).

Sugar syrup supplemented with bacteria was prepared as described for larval assays, but pellet was suspended in sugar syrup instead of artificial larval diet (final concentration of 1×10^4 cells/µl).

Bees from each group were individually fed with 20 µl of sugar syrup supplemented with one of the bacterial isolates (groups 1, 22, 35, 37, 51, 67, 78, 110, 117, 122), or sugar syrup without bacteria (control group, C). After ingesting all the inoculum, the bees were returned to their cages and fed with syrup *ad libitum*. The assay was carried out in triplicate. Cages were examined daily for 7 days, to record the mortality.

Preparation of the bacterial mixture

As different isolates may show different characteristics with additives or complementary effects on the host (Forsgren *et al.*, 2010), we decided to evaluate a mixture of bacterial isolates. Isolates that did not show growth inhibition between each other, were non-toxic to larvae and adult bees and that decreased larval mortality by *P. larvae* were finally selected to generate the bacterial mixture (BM). Isolates were cultured individually on MRS agar and individual cell suspensions were prepared on MRS broth and incubated at 37 ° for 24 h under microaerophilic conditions. Then, an aliquot was taken from each culture and its absorbance at 600 nm was measured. The same amount of bacterial cells from each isolate were mixed and centrifuged, the supernatant was discarded and suspended in artificial diet, PBS 1× or sugar syrup to a final concentration of about 1×10^4 cells/µl.

Effect of the bacterial mixture on larvae survival and *Paenibacillus larvae* infection

Toxicity of the bacterial mixture to *A. mellifera* larvae and antibacterial effect against *P. larvae* was investigated using the *in vivo* larval model described above. In this case, one larval group was fed *ad libitum* with artificial diet supplemented the bacterial mixture (1×10^4 cells/µl, LBM); another group was fed with the above-mentioned diet supplemented with the bacterial mixture and contaminated with *P. larvae* (1000 spores/µl, LBMP); one control group of larvae was fed only with the artificial diet (L) and another control group was fed with the artificial diet contaminated with *P. larvae* (1000 spores/µl, LP). Groups of 12 larvae were used in each case and three independents assays were carried out.

Effect of the bacterial mixture on bee survival

The effect of the bacterial mixture on bee survival was carried out using the *in vivo* bee model described above. Two groups of 25 bees each were starved for 3 h as described above. Then, bees from one group were individually fed with 20 µl sugar syrup supplemented with the bacterial mixture (2×10^4 cfu/µl, BM) and the bees from the second group were individually fed with 20 µl of sugar syrup (C). The assay was carried out in triplicate. Cages were examined daily for 10 days, to record the mortality.

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Effect of the bacterial mixture on *Nosema ceranae* infection

The antiparasitic activity of the bacterial mixture on *N. ceranae* was evaluated using the *in vivo* bee model described above. In this case, three groups of 50 bees each were prepared and starved for 3 h. Then, bees from one group were individually fed with 20 µl sugar syrup supplemented with the bacterial mixture (2×10^4 cfu/µl, NBM) and bees from the other two groups were individually fed with 20 µl of sugar syrup (N and C). Then the bees were placed into their cages and fed with syrup *ad libitum*. One day later (3 days after emerging) bees from the groups NBM and N were fed *ad libitum* with 4 ml of syrup 1:1 supplemented with 5×10^5 spores of *N. ceranae* (extracted from naturally infected bees; Fries *et al.*, 2013) during 24 h. After that period, the infected syrup was removed. The assay was carried out in triplicate. Bees were taken out of the incubator and examined daily, recording the mortality.

To evaluate the multiplication of *N. ceranae*, bees were sacrificed 7 days post-infection (10 days post-emergence), midguts of 30 bees per group were removed and homogenised individually in 1 ml sterile distilled water. Then quantification of spore suspensions was performed in Neubauer chamber and with an optical microscope at 400× (Cantwell, 1970).

Effect of the bacterial mixture on immune-related genes and gut microbiota

The effect of the bacterial mixture on immunity and gut microbiota was investigated using the *in vivo* bee model described above, but using bees from a healthy colony located at INIA La Estanzuela (Colonia, Uruguay). Two groups of 15 adult bees each were starved for 3 h. Then, one group (BM) was fed with 20 µl sugar syrup supplemented with the bacterial mixture (2×10^4 cfu/µl) and the control group (C) was fed only with 20 µl sugar syrup. The assay was carried out in triplicate. Cages were examined daily, to record the mortality.

Two days after application of treatments 20 bees of each treatment were randomly selected, removed from the incubator, and maintained at -80 °C for gene expression analysis. At the same time, 20 bees of each treatment were collected and stored at -20 °C for gut community analysis.

Gene expression analysis

Total RNA was isolated from 10 individual bees belonging to groups BM and C using the RNeasyPlus Mini Kit (Qiagen). RNA was immediately used to generate cDNA using Quantitec Reverse Transcription Kit (Qiagen). Both kits include steps to eliminate the contaminating genomic DNA.

To compare the immune response of bees subjected to different treatments, transcript levels for genes encoding antimicrobial proteins abaecin, hymenoptaecin, defensin, and the immunity-related enzymes glucose dehydrogenase, lysozyme and vitellogenin were assessed using previously described primers (Corona *et al.*, 2007; Evans, 2006; Johnson *et al.*, 2009; Yang and Cox-Foster, 2005). Transcript levels for ribosomal proteins RPS5 and β-actin were used as reference genes to normalise variations in cDNA levels (Evans, 2006; Yang and Cox-Foster, 2005).

Real-time PCR reactions were carried out in 96-well plates using the QuantiTec SYBR PCR Kit (Qiagen) using a BIO-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR was carried out in a final volume of 20 µl, comprising 1× SYBR (Power SYBR ® Green PCR Master Mix), 0.3 µM of each primer, 40 ng of DNA and RNAse free water. Two negative controls (without DNA) were included.

Cycling program consisted of an initial activation step at 50 °C for 2 min and 95 °C for 15 min, and 39 cycles of 94 °C for 15 sec, 52 °C for 30 sec and 72 °C for 30 sec. Melting curves were performed for the confirmation of gene-specific amplification.

The amplification results were expressed as the threshold cycle number (Ct), which represents the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. To accurately measure the levels of expression, normalisation to the mean of multiple reference genes is recommended rather than to a single gene (Vandesompele *et al.*, 2002). In this case, the reference genes RPS5 and b-actin were used. The geometric mean Ct of the reference genes was calculated and used for normalisation. The expression ratio was analysed as described by Pfaffl (2001).

16S rRNA amplicon sequencing

Ten bees belonging to groups BM and C were surface-sterilised by chlorine 1% solution and rinsed three times in distilled water (Engel *et al.*, 2013). Guts from bees from the same group were extracted and pooled together. Total DNA was extracted using the SDS-CTAB method. Samples were suspended in extraction buffer (100 mM Tris-HCl pH 8.0; 100 mM sodium EDTA pH 8.0; 100 mM sodium phosphate pH 8.0; 1.5 M NaCl; 1% CTAB (hexadecyltrimethylammonium bromide) and 100 ml of proteinase K 1 mg/ml) and incubated by shaking for 30 min at 37 °C. SDS 20% was added and samples were incubated at 65 °C for 2 h. Samples were centrifuged at 6,000×*g* for 10 min at room temperature and supernatants were transferred to sterile tubes. One volume of chloroform:isoamyl alcohol (24:1, v/v) was added and carefully mixed. The aqueous phase was recovered by centrifugation at 10,000×*g* for 10 min and precipitated with 0.6 volume of isopropanol at

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room temperature for 1 h. The samples were centrifuged at 50,000×*g* for 30 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionised water, to give a final volume of 200 ml. DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and concentrations were normalised to 20 ng/µl.

DNA was analysed by sequencing of V4 region of 16S rRNA gene using an Illumina MiSeq platform and 250 paired-end (PE) cycles (University of Texas, Austin, USA).

Quantitative PCR to estimate the total number of bacteria

DNA obtained from groups BM and C were subjected to qPCR to estimate the total number of bacteria. A fragment of the gene that encodes for the 16S rRNA was amplified using primers 1114F and 1275R (Denman and McSweeney, 2006). Reaction conditions and cycling were the same described for gene expression analysis. Reactions were performed in triplicate, in a BIO-RAD CFX96TM Real-Time system. Two negative controls (without DNA) were included.

Absolute quantification was based on a standard curve from DNA obtained from an *Escherichia coli* XL1 Blue culture. 10-fold serial dilutions of the DNA were performed, to obtain DNA corresponding to 10^3-10^7 cells per reaction. Cycle threshold (Ct) values of each sample were then compared to the standard curve to approximate the number of bacteria per sample. This protocol was based on the publication by Ott *et al.* (2004). Those authors quantified total bacteria in the human gut by using standard curves for 10 different bacterial strains with a broad range of 16S rDNA copy numbers, and found little variance when using different strains, suggesting the use of *Escherichia coli*.

Data analysis

The data from different experiments (osmotic tolerance to sugar syrup, viability under different pHs, gene expression, *N. ceranae* spores count) were analysed to determine if they fitted a normal distribution (Kolmogorov-Smirnov test) and whether their variance was homogeneous (Levene tests). The variation between groups was evaluated by ANOVA/ t test and where the data did not fit the parametric assumptions, the non-parametric Kruskal-Wallis and Mann Whitney test were applied. *P*-values below 0.05 were considered significant. Statistical analyses were performed using Past 3x version 2.17c (Hammer *et al.*, 2001).

Larval and bee survival

The effect of bacteria on larvae and bee survival was analysed using the Kaplan-Meier method and statistical differences were compared using the Gehan-Breslow test.

16S rRNA amplicon sequencing

Paired-end reads were joined using fastq-join method and demultiplexed with QIIME software package (Qiime.org) (Caporaso *et al.*, 2010).

Sequences were analysed using the QIIME software tool with default parameters for each step. Reads were screened for chimeras using the software program USEARCH 6.1. De novo operational taxonomic unit (OTU) picking was performed with the uclust option in QIIME (Edgar, 2010). Assignment of taxonomy to representative OTUs was carried out with the Greengenes database classifier (McDonald *et al.*, 2012; Werner *et al.*, 2012) at the default 97% sequence identity. Sequences matching plant chloroplast or mitochondrial 16 S rRNA were filtered from the dataset. Rarefaction curves of observed OTUs plotted against sampling depth were built. Alpha diversity index (Shannon) was computed with QIIME software.

3. Results

Honey bee samples and primary isolates identification

150 isolates were obtained from the gut of 36 honey bees. 65 isolates were randomly selected and after an initial screening, 52 were Gram-positive oxidase- and catalase-negative bacilli (potential *Lactobacillus* spp.).

Initial screening of bacterial isolates

Antimicrobial activity against *P. larvae* of the 65 randomly selected isolates was investigated using an *in vitro* inhibition assay. 57 isolates were able to inhibit the growth of *P. larvae*, generating inhibition zones from 60±0.4 mm to 260±0.6 mm (diameter). The generation time was similar in most cases. The average generation time was 116±48 min, ranging from 59 min to 289 min. When the osmotic tolerance to sugar syrup was tested, isolates showed a higher resistance at 4 °C in both sugar concentrations (1:1 and 2:1) than at 28 °C (Mann-Whitney test $P<0.01$ in both cases). No significant differences in bacterial viability were observed at different sugar concentrations, either at 4 or 28 °C (Mann-Whitney test $P>0.05$ in both cases). After 4 h incubation, all the isolates showed the ability to resist at pH 5 and 7, but only 27 were able to resist at pH 3.

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The 10 isolates that exhibited the most promising features associated with its use as beneficial microorganisms (*in vitro* inhibition of *P. larvae*, short generation time and tolerance to high sugar concentrations or acidity) were identified by sequencing the 16S rRNA gene, and most of them were identified as *L. kunkeei* (Supplementary Table S1).

To evaluate the toxicity and antimicrobial effect against *P. larvae in vivo*, these bacterial isolates and *P. larvae* spores were administered to *A. mellifera* larvae within the food (Supplementary Figures S1 and S2). No toxic effects were observed for most of the isolates. Larvae that received the bacterial isolates showed a similar mortality curve than the control larvae (L) (Supplementary Figure S1, Gehan-Breslow test, $P>0.05$), except for isolate 78 (Gehan-Breslow test, $P=0.03$).

On the other side, larvae that received isolates 35, 37, 51, 67, 78, 110, 117, 122 and were infected with *P. larvae* spores showed a lower mortality than infected larvae that did not receive bacteria (LP) (Supplementary Figure S2, Gehan-Breslow test, $P<0.05$ in all cases).

Toxicity of bacterial isolates on adult bees was also investigated by using an *in vivo* model. No deleterious effect was detected by any isolate (Supplementary Figure S3, Gehan-Breslow test, $P>0.05$ in all cases).

Inhibitory activity among the preselected isolates was assessed and 6 of the 10 isolates inhibited the growth of others (1, 22, 51, 78, 117 and 122) while 4 of them did not show any inhibitory influence on the other isolates (35, 37, 67 and 110). To obtain a bacterial mixture that could present different beneficial properties and coexist, these last 4 isolates were chosen (Supplementary Table S1). Although all of them were identified as *L. kunkeei*, they showed phenotypic and genotypic variations between them (data not shown), suggesting that they are different strains.

Effect of the bacterial mixture on larvae and bee survival

No toxic effects of the bacterial mixture (isolates 35, 37, 67 and 110) on larvae or adult bees were observed, since survival curves were similar in treated and control groups (Gehan-Breslow test, $P>0.05$ in all cases, Figure 1 and 2A). Even more, the administration of the bacteria increased the viability of larvae (LBMP vs L, Gehan-Breslow test, $P=0.03$). The behavior of the treated larvae and bees was normal, being active during inspections throughout the experiments.

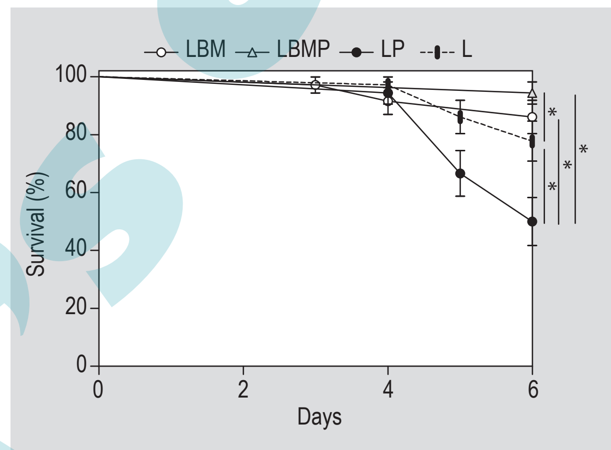

Figure 1. Survival curves of larvae fed *ad libitum* with an artificial diet supplemented with the bacterial mixture (LBM); fed with the above-mentioned diet contaminated with *Paenibacillus larvae* spores (LBMP); fed with the artificial diet only (L) and fed with the artificial diet contaminated with *P. larvae* (LP). Asterisks indicates significant differences ($P<0.05$).

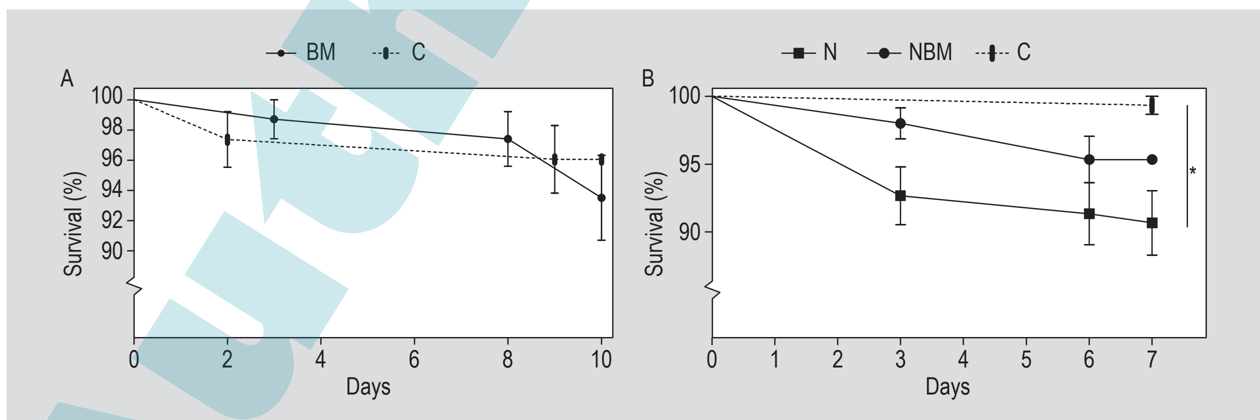

Figure 2. Survival curves of adult bees fed with the bacterial mixture. (A) Bees feed with the bacterial mixture (BM) and control (C). (B) Bees fed with the bacterial mixture and infected with *Nosema ceranae* spores (NBM), bees fed only with *N. ceranae* (N) and control bees (C). Asterisks indicates significant differences ($P<0.05$).

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Effect of bacterial mixture on *Paenibacillus larvae* or *Nosema ceranae* infection

Bacterial mixture reduced mortality of larvae infected by *P. larvae in vivo.* In particular, larvae that received the bacterial mixture and were infected with *P. larvae* spores had lower mortality than infected larvae that did not receive bacteria (LBMP vs LP, Gehan-Breslow test, P<0.01, Figure 1). Regarding the antiparasitic effect on *N. ceranae*, infected bees fed with the bacterial mixture had significantly fewer spores than the bees infected with *N. ceranae* that did not receive bacteria (NBM vs N, Mann-Whitney test, P<0.01, Figure 3). None of the bees belonging to control group (C) showed *Nosema* spp. spores.

Bee survival along seven days post infection was similar between the control- and *N. ceranae*-infected groups treated with the bacterial mixture (C vs NBM, Gehan-Breslow test, P>0.05, Figure 2B). The survival of bees infected with *N. ceranae* was significantly lower than survival of the control group bees (C vs N, Gehan-Breslow test, P<0.01).

Effects of the bacterial mixture on expression of immune-related genes

Feeding of honey bees with syrup supplemented with the bacterial mixture did not produce significant changes on the expression of antibacterial peptides, immunity-related enzymes or vitellogenin compared to the control bees (BM vs C, Mann-Whitney test, P>0.05 in all cases, Figure 4).

Effects of the bacterial mixture on bacterial gut community

A preliminary study of the composition and diversity of the bacterial gut community of bees fed with sugar syrup supplemented with bacterial mixtures or sugar syrup alone was performed through 16S rRNA amplicon sequencing. A single MiSeq PE run of 250 cycles resulted in 112,268 reads, of which 112,030 (99%) passed stringent quality thresholds. The data set was rarefied (i.e. subsampled to the size of the smallest library) to 45,953 sequences, and after alignment and clustering we identified a total of 114 OTUs (at 97% identity) across the entire data set.

Bees fed on bacterial mixture showed higher OTUs diversity than control bees according to rarefaction curves (Supplementary Figure S4) and Shannon diversity index (3.32 and 3.16, respectively). The dominant gut microbial community was composed by *Bifidobacteriales* (*Bifidobacterium* spp.), *Lactobacillales* (*Lactobacillus* spp.), *Rhohospirillales* (*Bartonella apis* and *Acetobacteria* spp.), *Neisseriales* (*Snodgrassella alvi*.) and *Pasteurellales*/*Orbales* (*Gilliamella apicola, Frischella perrara*), but their relative abundance varied among treatments (Supplementary Figure S5). In the bees that received the bacterial mixture the abundance of *Lactobacillales* and *Rhodospirillales* was higher compared to bees that received only sugar syrup. On the other side, the abundance of *Enterobacteriales* and *Neisseriales* was lower. The total number of bacteria was similar in the guts from both bee groups ($1.42\times10^7\pm8.44\times10^6$ and $2.47\times10^7\pm1.15\times10^7$ bacteria per bee, from bees of BM and C groups, respectively).

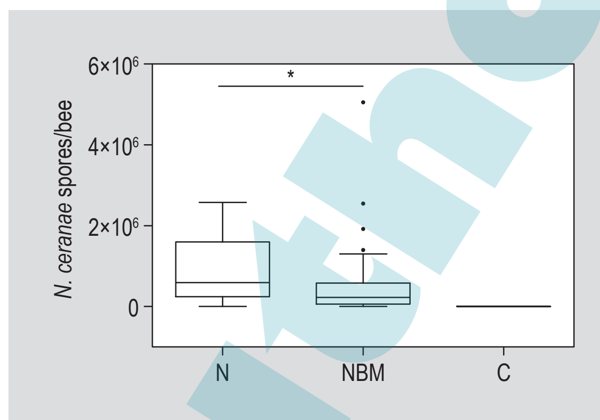

Figure 3. Infection level of *Nosema ceranae* spores in artificially infected bees (N), bees fed with the bacterial mixture and infected with *N. ceranae* (NBM), and control bees (C). Results are shown as box plots, where the horizontal line indicates the median, the box the first quartile of the data above and below the median (• outliers). Asterisk indicates significant differences (*P*<0.05).

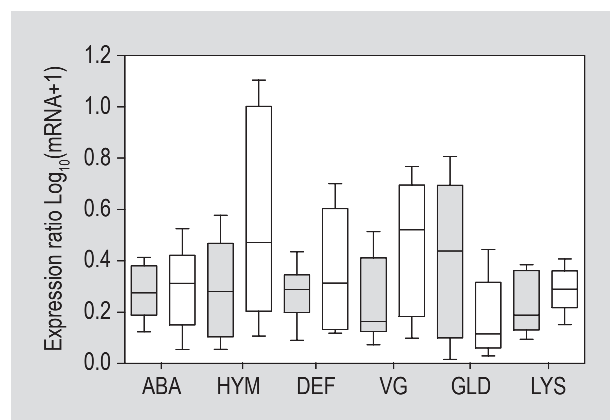

Figure 4. Effect of feeding adult bees with the bacterial mixture on the relative expression of mRNA coding for genes related to immunity (ABA = abaecin; HYM = hymenoptaecin; DEF = defensin; VIT = vitellogenin; GLD = glucose dehydrogenase and LYS = lysozyme). Results are shown as box plots, where the horizontal line indicates the median, the box the first quartile of the data above and below. Control group are shown in white squares and group fed with the bacterial mixture in grey squares. Significant differences between groups were not observed.

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4. Discussion

In the present study, we isolated and characterised bacterial strains obtained from the native gut microbiota of honey bees and evaluated their ability to inhibit *in vitro* and *in vivo* growth of *P. larvae* and *N. ceranae.*

Bacterial isolates that were able to survive at high sugar concentrations and acidic conditions were selected since it was expected that the microorganisms would be administered in sugar syrup (1000 or 2,000 g/l). Besides that, they were expected to survive the passage through the larval gut (pH close to 7.5) and resist high sugars concentrations (e.g. sucrose, glucose, fructose, levulose, dextrose) and low pH of honey and nectar (pH 3.4-6.1) (Bignell and Heath, 1985; White *et al.*, 1962). Based on these characteristics, their safety for larvae and bees and their ability to coexist, four isolates were selected. Although all of them belonged to the same species, *L. kunkeei,* probiotic characteristics vary among strains exerting additive or complementary effects (Forsgren *et al.*, 2010; Jacobsen *et al.,* 1999). For that reason we decided to work with a mixture of bacterial isolates.

L. kunkeei has frequently been isolated from the stomach of *A. mellifera* and *A. dorsata* (Olofsson and Vásquez, 2008; Tajabadi *et al.,* 2011). It has also been isolated as a spoilage organism associated with grape juice fermentations (Edwards *et al.,* 1998). The presence of *L. kunkeei* inside the colony could prevent the growth of fungi that can spoil the nectar (Vásquez and Olofsson, 2009) preserving the organoleptic and nutritional characteristics of the honey.

The mixture of selected *L. kunkeei* isolates did not cause deleterious effects in larvae or adult bees. Even more, they induced an increased larvae viability. Similar results were reported by Yoshiyama *et al.* (2013) and Corby-Harris *et al.* (2016) when they analysed the effect of the administration of other lactic acid bacteria and *Parasaccharibacter apium,* respectively.

The administration of the bacterial mixture did not alter the expression of antimicrobial proteins or immune-related genes in adult honey bees, probably because those isolates belong to the native microbiota. This is an important point to consider since the down-regulation of these genes caused by a particular *L. rhamnosus* strain (at 3.7×10^3 cfu/ml) favoured the development of *N. ceranae* (Ptaszyńska *et al.,* 2016). On the other side, an immune response up-regulation (mainly antimicrobial proteins) could lead to changes in the microbial composition, exerting the growth of a pathogenic commensal, as it was described in *Drosophila melanogaster* (Ryu *et al.,* 2008).

In this case, the bacterial mixture did not affect the number of bacteria present in the gut bacterial community although it seemed to affect the relative abundance of different groups. Dominant community was composed by *Bifidobacteriales* (*Bifidobacterium* spp.), *Lactobacillales* (*Lactobacillus* spp.), *Rhohospirillales* (*Bartonella apis* and *Acetobacteria* spp.), *Neisseriales* (*Snodgrassella alvi*) and *Pasteurellales*/*Orbales* (*Gilliamella apicola, Frischella perrara*), coinciding with previous studies (Kwong and Moran, 2016). The bacterial mixture tended to increase the abundance of *Lactobacillales*, suggesting that it can potentially stimulate beneficial microorganisms. Nevertheless, it is important to consider that the obtained results are preliminary and evaluating the gut bacterial community after two days of administration. A detailed analysis should be performed monitoring the dynamics during a longer period.

An important finding of this study was that the bacterial mixture decreased the mortality of larvae infected with *P. larvae* from 50 to 6%. In previous studies Forsgren *et al.* (2010) and Hamdi and Daffonchio (2011) also reported that the administration of beneficial bacteria in the larval food significantly reduced the mortality by *P. larvae* from 70 to 55% or from 70 to 22%, respectively. Future studies will be performed on naturally infected apiaries to assess the biological relevance of this bacterial mixture.

Regarding *N. ceranae* infection, the bacterial mixture reduced the spore number in infected honey bees and tended to reduce bee mortality. The reduction in bee mortality was not statistically significant possibly due the short time of the duration of the experiment (only seven days).

It would be interesting to elucidate the mechanisms involved in this antibacterial and antiparasitic activity. Possibly, the administration of beneficial microorganisms has direct effects on pathogens through the production of antimicrobial compounds, such as formic, lactic and acetic acid, hydrogen peroxide, diacetyl, benzoate, bacteriocins (Olofsson and Vásquez, 2008; Porrini *et al.,* 2010) or to the stimulation of the host immune response (Evans and Lopez, 2004). This point would be especially useful in the case of infection by *N. ceranae* since it has been reported that this pathogen can depress the honey bee immune system (Antúnez *et al.,* 2009).

The administration of the beneficial microorganisms can also facilitate the generation of a protective barrier that physically separates the intestinal lumen from the infection target cell. It was demonstrated by Vásquez *et al.* (2012) that the honey bee microbiota (including *L. kunkeei* strains) form biofilms and networks by which they attach to the wall of the gut and thus decreases the infection caused by pathogens such as *P. larvae* or *Melissococcus plutonius* (Vásquez *et al.,* 2012).

In conclusion, the mixture of *L. kunkeei* isolates obtained from the bee midgut proved to be a beneficial microorganism's mixture that exerts positive effects on

larvae and adult bees under laboratory conditions. Field studies are being carried out to evaluate its effect in naturally infected colonies. Further studies should be performed to elucidate its mechanisms of action and their effect in the promotion of honey bee colony health.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2017.0075.

Table S1. *In vitro* characterisation of probiotic properties of ten preselected bacterial isolates obtained from the honeybee microbial gut community.

Figure S1. Survival curves of larvae fed *ad libitum* with an artificial diet supplemented with bacterial isolates (1, 22, 35, 37, 51, 67, 78, 110, 117, 122) and fed with the artificial diet only.

Figure S2. Survival curves of larvae fed *ad libitum* with an artificial diet supplemented with bacterial isolates and contaminated with *Paenibacillus larvae* spores (1P, 22P, 35P, 37P, 51P, 67P, 78P, 110P, 117P, 122P); fed with the artificial diet only and fed with the artificial diet contaminated with *P. larvae*.

Figure S3. Survival curves of adult bees fed with diet supplemented with bacterial isolates (1, 22, 35, 37, 51, 67, 78, 110, 117, 122) or without bacteria.

Figure S4. Observed operational taxonomic units rarefaction plots of gut bacterial community of honey bees feed with bacterial mixture or control bees.

Figure S5. Composition of gut bacterial community of honey bees feed with bacterial mixture or control bees.

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