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Lactobacillus johnsonii CRL1647, isolated from *Apis mellifera* L. bee-gut, exhibited a beneficial effect on honeybee colonies

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Abstract

Lactobacillus johnsonii CRL1647, isolated from the intestinal tract of a honeybee and selected due to its high lactic acid production, was assayed as a monoculture on bee colony performance. It was delivered to the bees on a one litre of 125 g/l sugar-cane syrup with a final concentration of 10^5 cfu/ml lactobacilli. The bees accepted the new nourishment, which was consumed within 24-48 h and was administered in two independent trials (every 14-15 days for 3 consecutive months in one case, and once a month for 13 consecutive months in the other). From late spring – early summer (2008) the photo-records and statistical analyses revealed significant differences in the open and the operculated brood areas in the treated group compared with the control. This stimulation was observed after the first administration of the lactobacilli and maintained throughout. Also, a higher number of bees were measured in the treated group (54%) and the control (18%) with respect to the initial bees' number. Furthermore, honey storage was higher, 40% and 19%, for the treated and control groups, respectively. From December 2008 to December 2009, a similar situation was observed even though, in this trial, the lactobacilli cells were administered once a month. The *in vivo* results of this study are promising and indicate that a *L. johnsonii* CRL1647 supplement to beehives favours mainly open and operculated brood areas, demonstrating a stronger stimulation of egg-laying and will become a natural product which will assist the beekeeper both in colony management and the creation of late nuclei and/or bee packages due to its beneficial effects in the beehive colony.

Keywords: probiotics, *Lactobacillus johnsonii*, beneficial microbes, honeybee

1. Introduction

Bee broods of *Apis mellifera* L. are infected by different pathogens than adults, and they are highly susceptible to bacterial diseases such as American and European foulbrood, caused by *Paenibacillus larvae* and *Melisococcus plutonious*, respectively (Ashiralieva and Genersch, 2006; Govan *et al.*, 1999; Williams, 2000). Furthermore, honeybees become ill not only due to contact with infectious agents (bacteria, fungal spores, viruses, parasites, etc.); environmental factors and beehive management can also act as predisposing causes. The intensive application of chemical substances in order to control their diseases has resulted in the development of resistance mainly in *P. larvae*, reduction in efficacy and their accumulation and subsequent detection in honey (Evans, 2003; Floris *et al.*, 2001; Miyagi *et al.*, 2000; Wallner, 1999). An excessive use of antibiotics, mainly oxytetracycline, to control and prevent bacterial diseases in beekeeping, has also been considered a possible predisposing condition for chalkbrood (Flores *et al.*, 2004; Heath, 1982).

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). They have been shown to balance the gut microbiota improving the resistance of indigenous microorganisms against colonisation of pathogens, properties not only observed in humans but

also in different animals (Fuller, 1989; Gatesoupe, 1999; Holzapfel and Schillinger, 2002; Salminen *et al*., 1998). Lactic acid bacteria take part in the majority of probiotic formulas and, in order to be considered probiotic, they must comply with one or more of the following criteria: to induce any desirable and measurable positive effect on host health, safe taxonomic identification, non-toxic, non-pathogenic, a producer of antimicrobial substances (short-chain fatty acids, lactic acid, bacteriocins or bacteriocin-like substances and biosurfactants). Also, they should not possess virulence genes or antibiotic resistance (Anadón *et al*., 2006; Gueimonde and Salminen, 2006; Klaenhammer and Kullen, 1999; Salminen *et al*., 1998).

For small beekeepers, like those in the north-west regions of the Argentinean Republic (for example, Jujuy), preparing late-bee nuclei is sometimes difficult as this activity coincides first with the summer rainy period and later with the absence of flowering periods between January and March. Thus, a good alternative would be a product that helps the beehive colonies during this special period and allows the beekeepers to obtain more honey and the bees to multiply their colonies.

Taking into account all these circumstances, this work studied the effect of the administration to the bees of *Lactobacillus johnsonii* CRL1647 in the bee colony's overall performance. This lactic acid bacterium was isolated by the group from the intestinal tract of a bee reared in Salta (a province in the northwest of Argentina) and selected due to its *in vitro* antimicrobial properties (Audisio, 2007; Audisio *et al*., 2011; Torres *et al*., 2009)

2. Materials and methods

Bees, hive location and environmental conditions

These experiments were carried out at a commercial apiary in San Antonio, Jujuy province (Argentinean Republic) at an approximate altitude of 1,345 m above sea level. The climate belongs to the Cwah type, which is characterised by a moderate climate, slightly rainy, with dry winters and hot summers (the hottest month >22 °C) and with an annual average temperature of >18 °C. The research was carried out with local *A. mellifera* L. bees kept in standard Langstroth hives.

Two independent assays were performed. A short-term study, from 12 November 2008 until 10 January 2009, and a long-term study from 21 December 2008 until 4 December 2009. The bee-colony used in these assays were nuclei prepared with an open brood frame, an operculated brood frame including the bees from this frame and finally, an open frame of honey. After 48 h, a royal chamber INTA-PROAPI with genetics was incorporated.

Bacterial strain and growth conditions

L. johnsonii CRL1647 was isolated in the province of Salta from the intestinal tract of an adult worker bee (Audisio, 2007; Torres *et al*., 2009). This bacterium was characterised by classical biochemical tests for the *Lactobacillus* genus and the typing of genus and species was corroborated by 16S ribosomal-DNA sequence analysis (GenBank accession number EU428007) (Audisio *et al*., 2011). It was cultured in *Lactobacillus*-selective deMan-Rogose-Sharpe (MRS) medium (Britania Laboratory, Buenos Aires, Argentina), at 37 °C for 12 h under microaerophilic conditions.

Lactobacillus johnsonii CRL1647 administration and its tolerance to osmolarity of syrup

L. johnsonii CRL1647 was delivered to the bees by a Doolittle-type feeder. The minimum sugar concentration, which did not alter bacterial viability and would be accepted by the bees, was previously determined in the laboratory as follows: the sugar-cane concentrations tested were 500 g, 250 g and 125 g, in a final volume of 1 l of tap water. The number of viable lactobacilli cells was determined by a plate count in MRS agar at different times (1, 3 and 24 h). The plates were incubated at 37 °C for 48-72 h under microaerophilic conditions (about 7% v/v O_2 and 14% v/v CO_2).

Dose

A dose of 1×10^5 cfu/ml of viable *L. johnsonii* CRL1647 cells was selected for administration in 1 l of syrup (125 g/l) and the number of cells was determined by a plate count on MRS agar. In the first assay *L. johnsonii* CRL1647 was administered 6 times every 14-15 days; in the second assay, the bacteria were administered every 27 or 30 days during 12 months. The lactobacilli were always administered at the same time of day (16:00).

Bee-colony parameters tested

The evolution of the treated colonies was monitored and any changes were always compared to control hives, which did not receive the lactic acid bacterium, but in all other conditions (weather, nourishment and supervision) were identical. The parameters considered to assess the general state of the colonies during the assays were as follows: number of bees in each frame, open and operculated brood areas and quantity of honey.

During each visit to the apiary, photographs were taken of each frame (control and treated hives) and were later analysed in the laboratory with software designed by the group. This image-analysis technique was developed taking into account the methods described by other authors but

with many modifications (Catalayud and Verdú, 1992; Fries *et al*., 1991). To determine the starting point (t0) of the hives, frames from the different hives were photographed and then the lactobacilli were administered.

The possible presence of lactobacilli cells in honey was also tested. At different times, equal amounts of honey and sterile distilled water were aseptically mixed and the microbiota were studied by plating 100 µl directly onto MRS agar (Britania Laboratory). The plates were incubated as explained before.

Statistics

Comparison of the average values of the following parameters: number of bees in each frame, open and operculated brood areas and quantity of honey, were carried out using the Student *t* distribution test for small samples. The paired average comparison test was used because the analysed samples are interdependent.

3. Results

Tolerance of *Lactobacillus johnsonii* to osmolarity of syrup

Different sugar-cane concentrations were assayed: 125 g/l, 250 g/l and 500 g/l, to determine which concentration would not affect *L. johnsonii* CRL1647 viability during the trial. As shown in Figure 1, lactobacilli cells were partially inhibited by 250 g/l syrup, while 500g/l sugar cane syrup completely inhibited the growth of this bacterium. However, 125 g/l of syrup kept the viability of *L. johnsonii* CRL1647 in cell numbers close to the register of the control and was selected for further analyses.

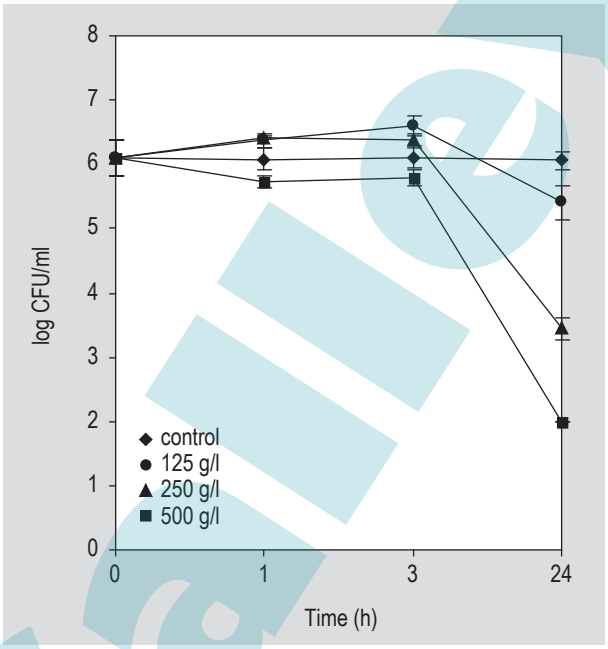

Figure 1. Survival growth curves of *Lactobacillus johnsonii* CRL1647 in the presence of different concentrations of sugarcane syrup at 37 °C under microaerophilic conditions.

Dose and administration

The bees' acceptance of the *L. johnsonii* CRL1647 cells administered in the sugar-cane syrup was key to being able to follow up with the different assays. The rate of consumption of 10^5 cfu/ml lactobacilli in 125 g/l sugar cane syrup was determined and estimated close to 24-48 h, as after this period the 1 l feeders were completely empty.

Effect of *Lactobacillus johnsonii* administration on bee-colony performance

The first trial was conducted from November 2008 to January 2009. Both the photo-register and statistic analyses revealed significant differences in the open and operculated brood areas in the treated group compared with the control (Figure 2). This stimulation was observed from the beginning of the trial, i.e. after the first administration of the lactobacilli (26 November 2008), was maintained throughout the trial and stimulation was notably greater at the end of the experiment (Figure 2). Also, a higher number of bees were measured in the treated group (54%) and the control (18%) with respect to the initial bees' number. Furthermore, honey storage was also higher, 40 and 19%, for the treated and control groups, respectively.

In the second trial, a similar situation to the first was observed even though in this case the lactobacilli cells were administered once a month and the assay was carried out for a whole year. After the first administration (15 December 2008) a significant difference in the open and operculated brood areas in the treated group compared with the control were measured (Figure 3 and Figure 4A and B). On the third month delivering the lactobacilli, the bee numbers were 20% higher in the treated group compared to the control. This situation was maintained at the end of the experiment (Figure 4C). The region where the *in vivo* trials were carried out has two flowering peaks, one in May-June and the other in December-January; in both situations a 16% increase in honey storage was observed in the treated group compared to the control (Figure 4D).

The statistical analyses of the data of the two trials revealed that with 1×10^5 viable cells of *L. johnsonii* CRL1647 the treated hives were different from the controls with a $P<<0.001$.

Also, it is important to note that in this work no lactobacilli cells were detected in the honey from the treated colonies.

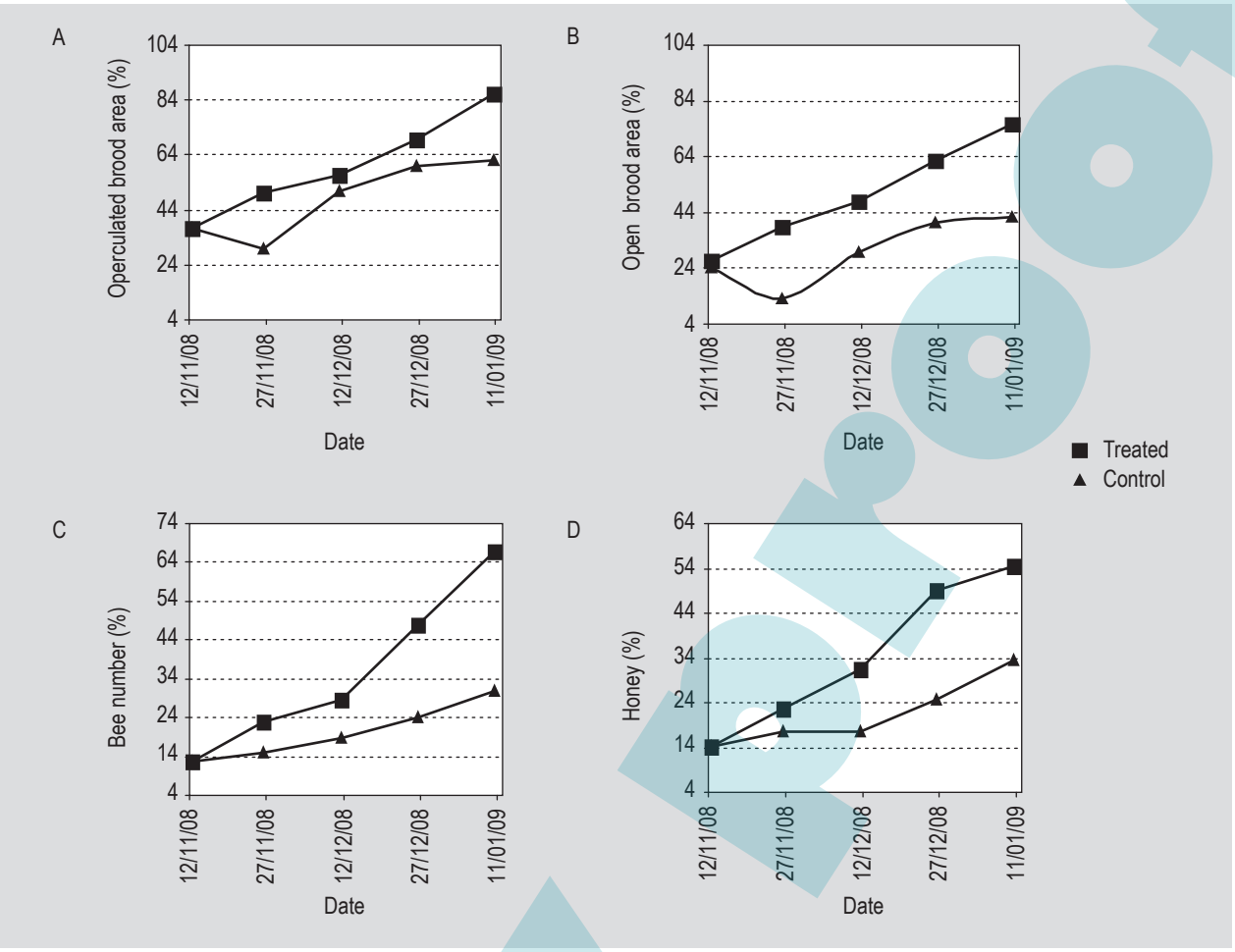

Figure 2. Evolution of different beehive parameters during the first assay applying *Lactobacillus johnsonii* CRL1647 (from November 2008 to January 2009): Operculated and open brood areas (A and B, respectively), bee number (C) and honey production (D).

Figure 3. Bee-colonies status after 4 weeks of administration of *Lactobacillus johnsonii* CRL1647. (A) control frame; (B) treated frame. Photographs were taken in the second trial.

4. Discussion

The use of different, old or new, commercial antibiotics can generate microorganisms that are unaffected by a suite of compounds (Kochansky *et al.*, 2001), which, in turn can affect the beekeeping industry, since many antibiotics leave residues in hive products (Feldlaufer *et al.*, 2004). The Argentinean Republic is an important international honey producer and exporter (Vazquez *et al.*, 2009). However, a few years ago, the presence of antibiotic residues in honey had a negative impact on its commercialisation. Therefore, natural ways to improve beehive health and strength would

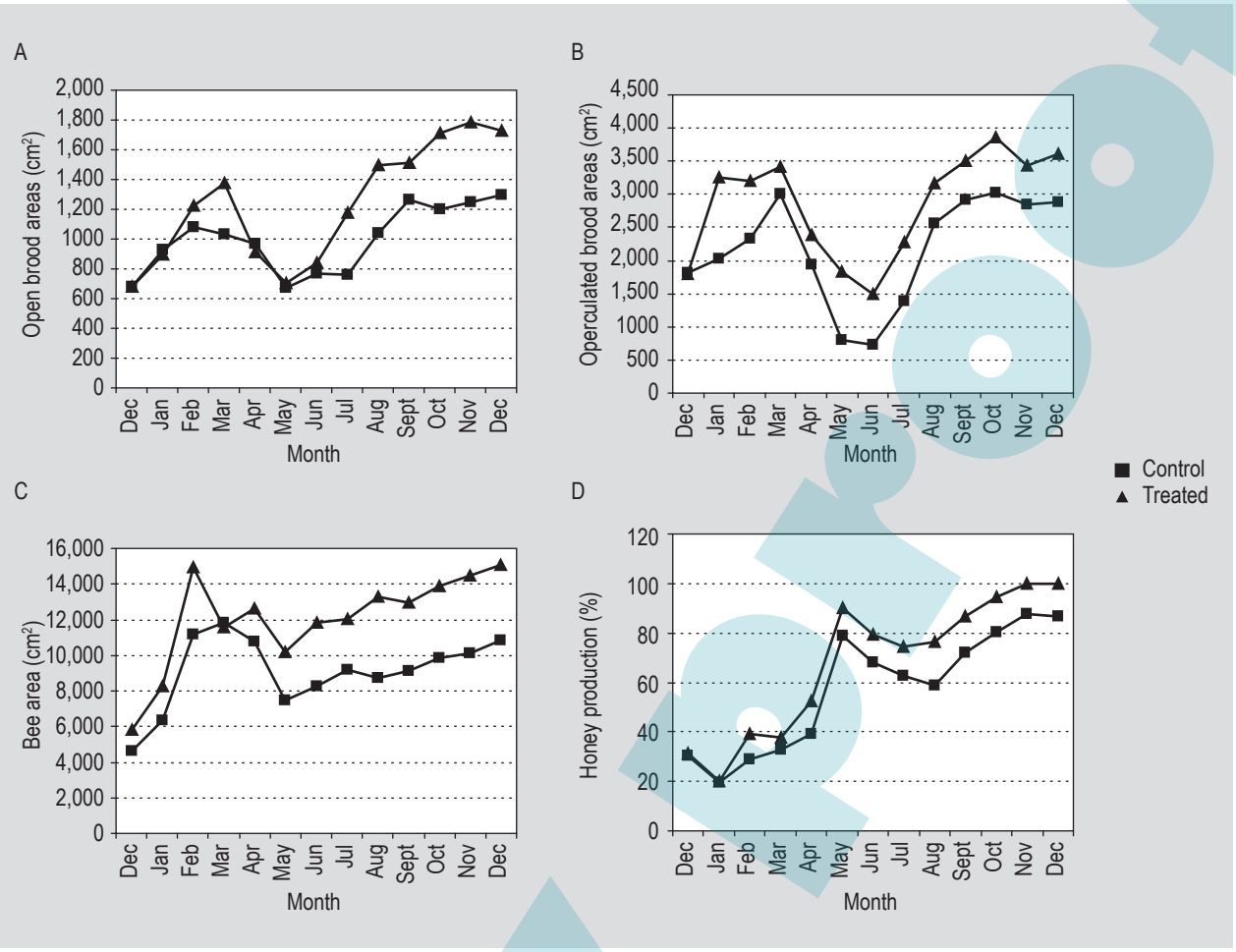

Figure 4. Evolution of different beehive parameters during the second trial using *Lactobacillus johnsonii* CRL1647 (from December 2008 to December 2009): (A) Open and (B) operculated brood areas, (C) bee populate areas and (D) honey production.

be a safe alternative. The ideal 'solution' must be harmless to brood and adult bees, should not leave any waste products in apiary produce, mainly honey, and should be easy to use.

One answer may be the application of probiotic supplements or competitive exclusion cultures, successfully used in humans and other animals such as birds, pigs, fish, etc. (Anadón *et al.*, 2006; Salminen *et al.*, 1998). With this in mind, *L. johnsonii* CRL1647, isolated from the gut of an adult worker bee was administered to common colonies. The aim of this study was to investigate whether the lactic acid bacterium *per se* had an impact on the normal development of a healthy bee colony and acted as a stimulant.

The bees' acceptance of the microorganism administered in the syrup was the first positive result and the sugar-cane concentration of this food was determined according to *L. johnsonii* CRL1647 cell viability. Knowledge of the following parameters, i.e. the time in which bees finished the feed and the sugar concentration, is an important point of consideration because if the bacterium is not viable, it cannot act properly as a potential probiotic supplement. Máchová *et al.* (1997) reported that different lactic acid bacteria, of different origins, lost viability when they were administered to colonies in a 50% sugar syrup. However, they did not determine which sugar concentration had no impact on cell viability and likewise were accepted by the bees. It is interesting to note that probiotic effects are always better or of a higher magnitude with viable cells, but also that dead cells or even components of bacteria can have positive health effects (immune stimulation) (Ouwehand *et al.*, 1999).

To analyse the evolution of colonies' parameters an adaptation was used of the method developed by Calatayud and Verdú (1992), who designed it to follow the evolution of the population of the ectoparasitic mite *Varroa jacobsoni* Oud in honeybee colonies. In this work, a photo-register was an important tool. The statistical analyses of the results confirmed the previous observations, i.e. stimulation of both the open and operculated brood. These changes generated a higher number of bees in the treated colonies and, consequently, more honey storage.

Thus, the *in vivo* results of this study are promising and indicate that a *L. johnsonii* CRL1647 supplement to hives favours mainly open and operculated brood areas, demonstrating greater stimulation of egg-laying. Therefore, this *Lactobacillus* administration to colonies as a natural product will assist the beekeeper in colony management and will result in pure uncontaminated honey, plus the creation of late nuclei and bee packages.

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