Original Article

EFFECTS OF *LACTOBACILLUS JOHNSONII* AJ5 METABOLITES ON NUTRITION, *NOSEMA CERANAE* DEVELOPMENT AND PERFORMANCE OF *APIS MELLIFERA* L.

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Received: 10 May 2016; accepted: 13 February 2017

A b s t r a c t

The European honey bee (*Apis mellifera* L.) is known to be affected by such stress factors as pathogen load, poor nutrition and depressed immunity. *Nosema ceranae* is one of the main parasites that affect colony populations. The relationship between the stress factors and honey bee-bacteria symbiosis appears as an alternative to enhance bee health. The aim of this study was to evaluate the effect of the oral administration of bacterial metabolites produced by *Lactobacillus johnsonii* AJ5 on nutritional parameters, the *N. ceranae* development and the performance of *A. mellifera* colonies. Laboratory assays were performed and demonstrated that the bacterial metabolites did not have a toxic effect on bees. Field trial showed an increase of colonies population over time. Also, a decreasing trend of fat bodies per bee was detected in all colonies but there were no evident changes on abdomen protein content at the end of the assay. Lastly, *N. ceranae* prevalence showed a tendency to reduce with the organic acids. Future studies should be performed to increase our knowledge of the physiological effects of bacterial metabolites on the health of bee colonies.

Keywords: *Apis mellifera*, bacterial metabolites, *Lactobacillus johnsonii* AJ5, *Nosema ceranae*, nutrition

INTRODUCTION

The beekeeping world is facing a complex scenario. A variety of health and nutritional aspects has generated adverse conditions that negatively affect the evolution and normal development of bee colonies. A recent trouble associated with this situation is colony loss which is closely related to chronic stressors, including poor nutrition, increased pathogen loads and genetic diversity (Naug, 2009; vanEngelsdorp et al., 2009; Neumann & Carreck, 2010). The composition and function of honey bee microbiota is a critical factor to reduce nutritional stress and both affect the host's immune system even though have not yet been fully characterized (Dillon & Dillon, 2004; Mattila et al., 2012; Vásquez et al., 2012).

The European honey bee is affected by several pathologies. One of them is Nosemosis which has a great impact on the development of not only the insect (Fries, 1988; Higes et al., 2007) but also the colony (Higes et al., 2008; Paxton, 2010). This disease is caused by two microsporidia species, *Nosema apis* and *Nosema ceranae* (Fries et al., 1996; Higes, Martín, & Meana, 2006; Forsgren & Fries, 2010), which are both obligate intracellular parasites of the ventricular epithelial cells of adult bees (Fries, 1988; Higes et al., 2007). *N. ceranae* is a microsporidia more virulent than *N. apis* (Forsgren & Fries, 2010). Apart from causing ventricular epithelium lesions, it suppresses humoral and cellular defenses (Alaux et al., 2010a) and produces a decrease in vitellogenin expression (Antúnez et al., 2009). These nutritional and immunological depressions result in decreased worker longevity, a precocious onset of foraging and a loss of foragers in the field. Thus, *N. ceranae* is considered one of the factors that contribute to colony depopulation also associated with the recent colony collapse disorder (CCD) (Oldroyd, 2007; Higes et al., 2008; Paxton, 2010).

In order to overcome the pathologies, continuous chemical applications have been employed incorrectly producing undesirable results such as resistant strains (Maggi et al., 2009, 2010, 2011) and residues on beehive products (Bogdanov,

2006; Medici, 2010; Simion et al., 2011). Consequently, antibiotics have been banned in most EU member states. In this context, natural and non-contaminant alternatives have been incorporated to strengthen nutrition and immune response in colonies and indirectly parasite tolerance. Adequate nutrition of *A. mellifera* is known to contribute to colony development (Brodschneider & Crailsheim, 2010) and enhance their individual and social immunocompetence (Alaux et al., 2010b). There has been a special emphasis on the study of mutualistic relationships between bees and their coexisting microorganisms (Mattila et al., 2012; Vásquez et al., 2012). These are involved in many aspects of host life, physiology and evolution, including nutrition, reproduction, immune homeostasis, defence and speciation (Crotti et al., 2012).

Eight bacterial species clusters dominate *A. mellifera* workers' gut, comprising over 95% of the whole community (Moran, 2015). Even if the species-level diversity is low, the strain diversity depicts more metabolic functions that benefit hosts (Alberoni et al., 2016). Microbial gut symbionts of social insects have the enzymatic capability (i.e., cellulases, hemicellulases and lignase) to uptake the energy from a plant-based diet (Engel et al., 2012; Newton et al., 2013). Besides, these microorganisms produce fatty acids, amino acids, metabolites (Gündüz & Douglas, 2009) and vitamins required for honey bees (Brodschneider & Crailsheim, 2010). Whereas the functions above are related to nutrition, comparative analysis of gene contents suggests that microbiota contributes to host interaction, biofilm formation (Engel et al., 2012; Vásquez et al., 2012) and antimicrobial action (Alberoni et al., 2016). There is strong evidence of an increase of antimicrobial peptide (AMPs) production induced by honey bee gut bacteria (Evans & Lopez, 2004; Jefferson et al., 2013; Yoshiyama et al. 2013; Janashia & Alaux, 2016). Several in vitro trials confirmed the hability of lactobacilli and bifidobacteria to inhibit honey bee pathogens, in particular *Paenibacillus larvae*, *Melissococcus plutonius* and *Ascosphaera apis* (Forsgren et al., 2009; Sabaté et al., 2009; Yoshiyama & Kimura, 2009; Audisio

et al., 2011; Vásquez et al., 2012). Similarly, a reduction in *N. ceranae* intensity was reported when a *Bacillus* spp. strain culture (Sabaté et al., 2012) or metabolic products of specific *Bacillus* spp. (Porrini et al., 2010) and Lactobacillus spp. (Maggi et al., 2013) were administered to bees. Over the last years, honey bee researchers have been working hard to analyze the composition and function of honey bee microbiota (Engel et al., 2012; Mattila et al., 2012; Vásquez et al., 2012; Alberoni et al., 2016) to use it as an alternative to deal with nutritional and immunological problems (Crotti et al., 2012; Moran, 2015). Furthermore, much effort has been focused on developing natural formulations to improve colony performance and consequently mitigate the negative effects of major diseases. In this sense a specific lactic acid bacteria (LAB) isolated from bee gut was selected on the base of the potential probiotic properties on animals of this bacterial group (Audisio, 2016). The aim of this study was to determine the effect of the oral administration of bacterial metabolites produced by *Lactobacillus johnsonii* AJ5 on the performance of *A. mellifera* colonies, the *N. ceranae* development and nutritional parameters.

MATERIAL AND METHODS

1. Biological material: bees, hive location and experimental conditions

The field trial was carried out in an experimental apiary located 10 km away south of Mar del Plata, Argentina (37°55'48"S 57°40'59"O). Experiments were conducted in spring, between October and December 2012. The experiments were carried out on colonies of *A. mellifera* L. kept in standard Langstroth hives. A total of ten hives, previously standardized, was used for the assays. Each one consisted of five combs covered with adult bees, one open brood comb, two sealed brood combs and one open frame full of honey and pollen. The queen was one-year old. Colonies inspections were inspected at midday.

2. Bacterial metabolites synthesis

The metabolites synthesized by *Lactobacillus johnsonii* AJ5 (Genbank access code EU428008) were recovered in the cell-free supernatant (CFS) according to Audisio et al. (2011). This lactic acid bacterium was grown in MRS broth (MRS, Britania, Argentina) for 24 h at 37°C under microaerophilic conditions (7% v/v O_2 and 14% v/v CO_2). After that, the CFS was retrieved by centrifuging (10,000 g, 10°C, 15 min), filter-sterilized (0.22 μm) and kept at 4°C until its use.

3. Organic acids characterization and quantification

The characterization and the concentration of the organic acids produced by *L. johnsonii* AJ5 was performed by HPLC (Audisio et al., 2011). The CFS from an MRS culture was deproteinized and filter-sterilized (0.45 μm) before the HPLC analyses. The sample amount injected was 20 μL. The column temperature was 55°C and the flow rate of the H_2SO_4 10 mM mobile phase was 0.6mL/ min. Detection was carried out by determining the refraction index using a 2142LKB Differential Refractometer. The chromatography column (Rezec Organic Acid, Phenomenex) had a diameter of 7.8 mm and a length of 300 mm. Peak profiles, integration, and quantification were obtained with a CR601 Shimadzu chromatopac integrator (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan). Samples were duplicated.

4. Toxicity of bacterial metabolite on honey bees

Honey bees were confined individually inside plastic containers (length: 3 cm; diameter: 3 cm) with an internal feeder, a sponge soaked in water (65 μl/day) and adequate ventilation. They were daily supplied with 80 μl of each following dose of CFS on syrup 2:1 (water:sugar): 1, 6, 20 and 40% v/v. Two controls were performed: i) sugar syrup 2:1 and ii) a solution of MRS broth and syrup (6.25% v/v). Each treatment was replicated 30 times. Individual bee mortality per treatment was recorded at 24, 48 and 72 h. Incubation conditions were 33°C ± 1.5 and 70% ± 3 relative humidity (RH). All tests were conducted with nurse bees between 24 and 48 h which were obtained from sealed brood combs

Table 1

Summary of the cell-free supernatant (CFS) and MRS broth application (1, 2, 3 and 4) and time of sampling for the variables analyzed (X). T_0: initial time, T_1: one week after the start of trial, T_2: two weeks after the start of trial, T_3: three weeks after the start of trial, T_4: five weeks after the start of trial

	T_0	T_1	T_2	T_3	T_4
CFS and MRS application	1	2	3	4	
Sampling of colony development	X		X		X
Sampling for fat bodies	X		X		X
Sampling for soluble protein	X		X		X
N. ceranae sampling	X		X		X

in the incubator. The emerged bees endured 3-4 h of starvation.

5. CFS administration and time of sampling on bee colonies

Treatments were administrated in syrup 2:1 by a Doolittle feeder. Five colonies (treated group) received the CFS and another five colonies (control group) sterile MRS broth. Colonies received a first dose (attack dose, T0: initial time) of 50ml followed by three weekly applications of 10 ml (T1: second application, T2: third application, T3: fourth application), all of them in 500 ml of syrup 2:1 (Tab. 1). To evaluate the effects of CFS administration on colony, bee-colony parameters, fat bodies, soluble protein and *N. ceranae* load, samples were taken before each treatment application (T_0 and T_2) and one week after the last application (T_4, Tab. 1).

6. Colony bee: parameters tested

The parameters considered to qualify the general state of the colonies during the CFS in field application (Tab.1) were: i) number of combs fully covered with bees (estimated as number of combs covered with adult bees), ii) open and sealed brood areas (according to Branco, Kid & Pickard, 1999) and iii) quantity of honey and pollen (estimated as number of combs covered with honey or pollen, respectively).

7. Determination of fat bodies and soluble protein

Samples of 25 nurse bees per colony, under each treatment and at each sample time (n=25), were collected from the brood nest and frozen until fat bodies and soluble protein were determined

(Tab. 1). Fat bodies were measured according to Wilson-Rich et al. (2008). Abdomens were severed from thoraces and dried for three days at room temperature and then were weighed and washed in ethyl ether for 24 h until the fat as dissolved. The abdomens were dried for three days and weighed again. The fat body was calculated as the percentage change in abdominal weight after the ethyl ether wash (Ellers, 1996).

Soluble protein content was measured according to protocol by Bowen-Walker & Gunn (2001). For this, the abdomens were individually subjected to ground-glass homogenization in 1 ml distilled water and then centrifuged at 1500 g for 5 min. Aliquots of supernatant were tested for soluble protein by the dye-binding method of Bradford (1976) using BioRad Dye Reagent (BioRad Labs. GmbH) and bovine serum albumin as standard (Sigma [fractionV, 98%]) (Anon, 1979).

8. *Nosema ceranae* development

Forager bees were sampled outside the entrance of the hives at midday (time of sampling in Tab. 1). The entrance of each hive was closed with a foam rubber so that foraging bees could be collected inside a flask with ethanol 70°. In the laboratory, the abdomens of 30 bees per combination of each colony, treatment and time were individually homogenized in 1ml of distilled water and checked for the presence of *Nosema* spores to obtain prevalence (percentage of infected bees *per* hive, colony level; adapted from Smart & Sheppard, 2012) and intensity (number of mature spores *per* bee, individual

level; Undeen & Vávra, 1997). The homogenates were observed in a Neubauer hemocytometer under a compound microscope (x400) to quantify the number of mature spores (Cantwell, 1970).

9. Statistical analysis

A Restricted Maximum Likelihood (REML) analysis and residual plots were used to select Linear Mixed Effects (LME) models with the best fitting to each variable. The hive was considered by random effects and treatment, time and their interaction by fixed effects. Bees were included as a random effect to the analysis of soluble proteins and fat body mass. Where necessary, a different variance function was used to model the variance structure of the errors within each group. The effects of treatments were analyzed by ANOVA using R software (version 3.1.1, 2014). Mean values of variables, standard deviation and least significant differences were calculated using the best-fit model. In the case in which treatment did not present significant differences, respect to the control the mean between both groups was considered. Differences among treatments were evaluated using the studentized range Tukey method (p < 0.05).

RESULTS

1. Bacterial metabolites characterization and quantification

The organic acids produced by *L. johnsonii* AJ5 present in the CFS were detected by HPLC. The compounds identified and their concentrations were lactic acid (275 ± 8 mM), phenyl-lactic acid (0.4 ± 0.1 mM) and acetic acid (40 ± 5 mM).

2. Toxicity of bacterial metabolite on honey bees

The administration of CFS under laboratory conditions did not produce considerable bee mortality so no statistical analyses were performed. After 72 h of exposure, none of the cases, had a mortality rate over 10%. Both controls showed a mortality rate of 3% at 72 h while the highest doses of CFS treatment reached only 6%.

3. Effects of CFS on colony development

Regarding the colony development parameters, it was not necessary to model the variance structure of the errors within group. Treatment and interaction treatment x time did not produce significant changes in each studied parameter (ANOVA, p>0.05). An increase in adult bee population from 5.0 ± 0.3 to 8.9 ± 0.3 was observed from T0 to T4. Similarly, sealed brood combs increased from 2.34 ± 0.19 to 3.95 ± 0.19 (from T0 to T4). Considering open brood, the mean varied throughout the field trial from 1.66 ± 0.25 to 2.85 ± 0.25. An increase over time could be observed in both groups for the variables except honey and pollen storage (ANOVA, p=0.74, p=0.09 respectively), with significant differences over time but not between treatments (Tab. 2).

3.4. Effects of CFS on fat bodies of worker bees

For fat bodies, the heterogeneity of the fitted values was modeled. Fat body development decreased with time (ANOVA, p=0.008) without any differences between CFS and MRS administrations at the end of the trial (T_4, ANOVA, p=0.13). Worker bees decreased their fat bodies

Table 2

Colony development parameters. Values are mean ± standard error. Different letters represent differences between times (p<0.05). T_0: initial time, T_2: two weeks after the start of trial, T_4: five weeks after the start of trial

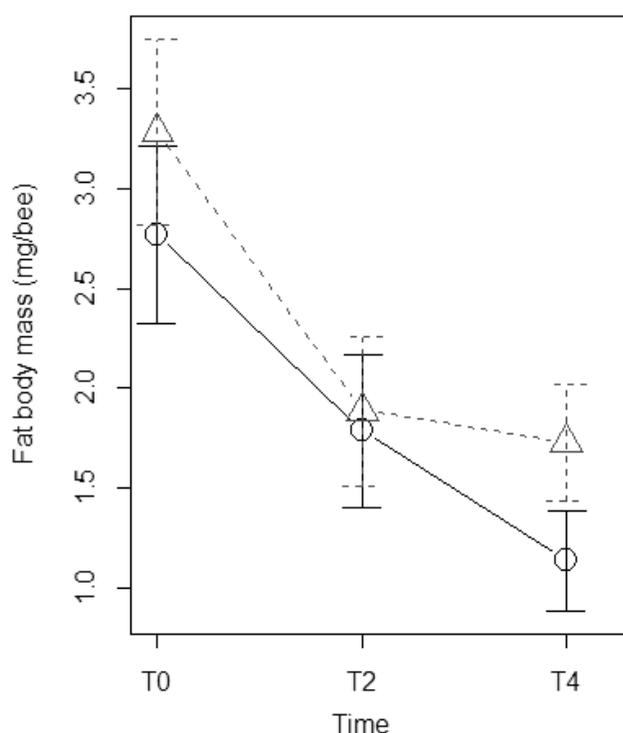|	T_0	T_2	T_4
Adult bees	5.00 ± 0.30 a	7.25 ± 0.30 b	8.95 ± 0.30 c
Sealed brood	2.34 ± 0.19 a	3.14 ± 0.19 b	3.95 ± 0.19 c
Open brood	1.66 ± 0.25 a	2.40 ± 0.25 ab	2.85 ± 0.25 b
Honey	0.55 ± 0.14 a	0.64 ± 0.14 a	0.70 ± 0.14 a
Pollen	0.35 ± 0.12 a	0.39 ± 0.12 a	0.52 ± 0.12 a

Fig. 1. Fat body per bee for different treatments and sampling time. Circles: means of bees which received MRS plus syrup 2.1; triangles: means of bees which received CFS plus syrup 2:1. T0: initial time, T2: two weeks after the start of trial, T4: five weeks after the start of trial. Bars indicate mean standard error.

reserves from 2.9 ± 0.4 (T_0) to 1.7 ± 0.3 mg/bee (T_2). Fat bodies reserves decreased towards the last sampling time (T_4, 1.3 ± 0.2 mg/bee, Fig. 1).

5. Effects of CFS on soluble protein content of worker bees

For the protein content, the heterogeneity of time factor covariance was modeled. CFS did not produce a significant change on soluble protein content compared with the control group (ANOVA, p=0.11). Soluble protein content had significant differences only among times (T_0, T_2 and T_4) (ANOVA, p<0.01). A decreasing trend was observed from 0.013 ± 0.0006 mg/mg (T_0) to 0.009 ± 0.0004 mg/mg (T_2). After the time of T_2 sampling, both groups presented an increased in soluble protein content up to 0.013 ± 0.0007 mg/mg (T_4).

6. Effects of CFS on *N. ceranae* development

The modelling of the structure of fitted values variance was necessary for *N. ceranae* intensity but not for *N. ceranae* prevalence. It was similar for treated and control groups of colonies. At the beginning of the trial, it was 74 % for both

groups and by the end of the experimental period (T_4) it had decreased on average to 42% (ANOVA, p<0.05). Significant differences were not observed between treatments (ANOVA, p=0.46), but a trend to a major reduction was observed for CFS administration (Fig. 2). *N. ceranae* intensity decreased over time in both groups of colonies. The average of number of *Nosema* spores at the initial level (T_0) was $1.7E+06 \pm 1.6E+05$ in the control group and $1.0E+06 \pm 1.1E+05$ in the treated hives (ANOVA, p<0.05). At the end of the experiment (T_4), colonies fed with the CFS decreased the number of spores to $1.4E+05 \pm 5.2E+04$ and colonies fed with the MRS culture to $1.5E+05 \pm 5.3E+04$ (ANOVA, p<0.05).
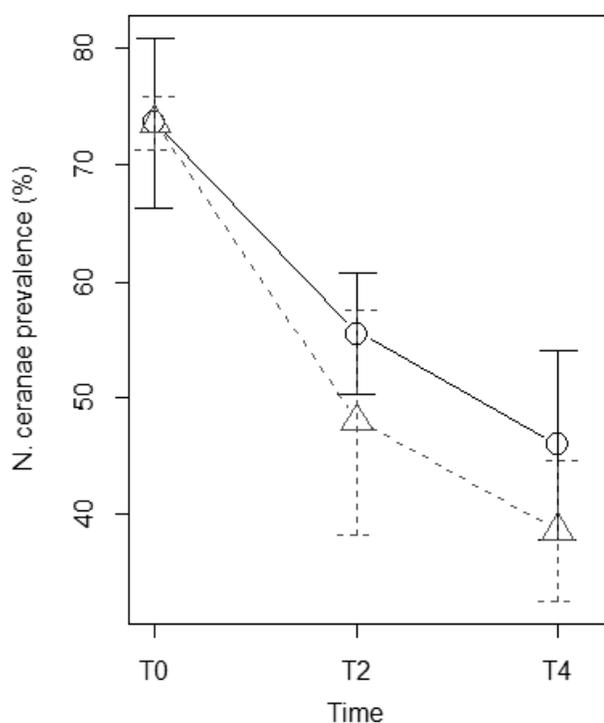

Fig. 2. *Nosema ceranae* prevalence (percentage of infected bees per hive) for different treatments and sampling time. Circles: means of percentage of infected bees per colony which received MRS plus syrup 2.1; triangles: means of percentage of infected bees per colony which received CFS plus syrup 2:1. T0: initial time, T2: two weeks after the start of trial, T4: five weeks after the start of trial. Bars indicate mean standard error.

DISCUSSION

Knowledge on the composition and functions of the bee gut microbiota and the link between a balanced gut microbiota and health status has been increasing over the last years together with the continuous decline of honey bees' colonies (Alberoni et al., 2016; Mattila et al., 2012). In this regard, some bacterial strains and products of their metabolism have begun to receive special scientific attention. Some studies have reported beneficial effects of microbiota on bees' health (Mattila et al., 2012; Janashia & Alaux, 2016) and undesirable effects particularly on microbial threats (Forsgren et al., 2009; Vásquez et al., 2012; Baffoni et al., 2015; Audisio, 2016).

Our study was focused on assessing the effect of bacterial metabolites produced by a LAB (*L. johnsonii* AJ5) on nutritional parameters, *N. ceranae* development and performance of *A. mellifera* colonies. LAB belonging to honey bee gut microbiota was selected on the basis of its production of organic acids (Audisio et al., 2011) and its beneficial effects on honey bees (Audisio & Benítez, 2011; Maggi et al., 2013; Audisio et al., 2015).

We observed that CFS in high doses is not lethal after 72 h of bee exposure. All populations' parameters increased at the end of the trial without treatments effects. This result differed from previous reports in which not only bee gut bacteria had been administered inside beehives but also their metabolites. Maggi et al. (2013) had found that colonies fed with a CFS produced by *Lactobacillus johnsonii* CRL1647 increased their beehive population compared with untreated colonies. One of the differences between these two studies was the use of distinct strains of the same bacterial species. Even though both are LAB and behave differently, they produce the same main organic acids but in different concentrations. Apart from that, the responses of colonies to the CFS could have been influenced by the application season. In the mentioned study, a higher organic acid dose than this trial was supplied at the beginning of winter. In summary, the main difference between the results of both studies could have been caused by the concentration of CFS administered and secondly the application season. Furthermore, beneficial effects were observed in colonies if the direct bacterium administration instead of its metabolites is considered. An increase in honey yield and a reduction in nosemosis and varroosis incidence were detected by the administration of a *Lactobacillus johnsonii* CRL1647 culture (Audisio et al., 2015). Also, Sabaté et al. (2012) reported an increase in the honey bee population and a decline in bee diseases worldwide when colonies were fed with a Gram-positive bacterium found in honey (*Bacillus subtilis* subsp. *subtilis* Mori2). These effects could have been influenced by the interaction between the microorganisms belonging to the bee microbiota (Mattila et al., 2012; Vásquez et al., 2012; Audisio, 2016).

The nutritional status of individual bees was also analyzed consisting of two parameters: fat bodies and soluble protein content on the abdomen. The former corresponded to an organ in bees that was related with the storage of lipids and proteins required for different metabolic activities (de Oliveira & da Cruz-Landim, 2003), synthesis of different proteins like vitellogenin (Amdam & Omholt, 2002; Corona et al., 2007) and humoral immune by the synthesis of antimicrobial peptides (Brown, Moret & Schmid-hempel, 2003; Wilson-Rich, Dres & Starks, 2008). The major component of fat body is lipid representing more than 50 % of dry weight and being a measure of bee health (Arrese & Soulages, 2010). We found no differences in the lipid mass on bees being fed SLC and MRS but a decreasing trend was observed over time. This result differs from the increase on fat body mass found by Maggi et al. (2013) in bees supplemented with bacterial metabolites. As mentioned before, these differences were probably based on the lower concentration of organics acids administered in this research. The content of soluble proteins in abdomen was the other parameter of nutritional status of bees and its content did not change between treatments or overtime. This suggests that bacterial metabolites utilized in this study maintained the nutrition status of bees at this specific CFS concentration in spring. Further assays including wide doses of bacterial metabolites would be necessary in order to

clarify this response.

Regarding to *N. ceranae* presence, colony and individual levels were studied, and results showed that the administration of CFS did not affect the individual level of the disease. There was a decrease in the number of *Nosema* spores *per* bee over time with no significant differences between CFS and MRS supplementation. However, a reduction trend of CFS was observed at colony level when compared to MRS treatment. Literature has shown enough evidence about the effect of bacteria or their metabolites on *Nosema* spp. viability. A combination of two non-pathogenic bacteria, bifidobacteria and lactobacilli, administered to *N. ceranae*-infected bees showed a reduction in parasitism level (Baffoni, et al., 2015). In the same way, Corby-Harris et al. (2016) discovered an increased resistance to *Nosema* infection by bees fed a hive bacterium. Concerning bacterial metabolites, Porrini et al. (2010) suggested that a surfactin could damage the spore external structure decreasing their viability. Maggi et al. (2013) as well proposed a hypothesis about the alteration of microsporidium envelope by an organic acid.

In summary, some studies indicate that a mutualistic relationship between bee and microbiota is essential in the development of colonies (Mattila et al., 2012; Vásquez et al., 2012). The current study shows a tendency of clarifying this knowledge since the concentration of CFS administered was not ideal. Taking it into account and considering the beneficial properties of nonpathogenic bacteria and their metabolites, more assays would be necessary to adjust the concentration of CFS supplied in order to detect significant effects on the parameters evaluated. In this sense, other physiological parameters should be assessed for the best understanding of the relationship between bacterial metabolites and the nutritional and immune status of the bees.

ACKNOWLEDGMENTS

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