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Effects of the organic acids produced by a lactic acid bacterium in *Apis mellifera* colony development, *Nosema ceranae* control and fumagillin efficiency

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ABSTRACT

The European honey bee *Apis mellifera* is known to be affected by many parasites and pathogens that have great impact over the insect development. Among parasites affecting bee health, *Nosema ceranae* is one of the main biotic factors affecting colony populations. As honey bee populations decline, interest in pathogenic and mutualistic relationships between bees and microorganisms has increased. The main goal of the current study was to assess the effect of the oral administration of the metabolites produced by Lactobacillus johnsonii CRL1647 (mainly organic acids) supplemented in syrup, on: (I) *N. ceranae* sporulation dynamics before and after fumagillin application, and (II) performance of *A. mellifera* colonies. Different experiments were conducted to evaluate the effects of these bacterial metabolites on bees: *in vitro* administration revealed no toxic effects against bees. Colonies fed with the lactic acids incremented their beehive population and also the amount of fat bodies per bee. Finally, the organic acids reduced the intensity of the pathogen after the second application of treatment as well as enhanced the fumagillin efficiency. This study provides important information for the development of new control substances against nosemosis.

1. Introduction

Apicultural economic development strongly relies on the health status of honey bee colonies. The European honey bee *Apis mellifera* is known to be affected by many parasites and pathogens that have great impact over insect development and represent a serious threat to the ecosystem, agriculture and apiculture. Among parasites affecting bee health, the parasitic mite *Varroa destructor* is one of the main pests affecting bee colonies, and was postulated to be partly responsible for worldwide colonies losses observed (Levy, 2011). Another serious threat to *A. mellifera* is the American foulbrood caused by *Paenibacillus larvae*, and also Nosemosis caused by the microsporidia *Nosema apis* and *N. ceranae*. Recently it has been proposed that the combined effects of all these parasites and/or pathogens, along with habitat loss and pesticide poisoning, could have drastic effects on honey bee colonies (Levy, 2011).

By far, chemical insecticides are used most commonly to control parasites and pathogens affecting honey bees.

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This application is usually performed when symptoms of the different diseases are observed in bee colonies, and sustaining these strategies over time could lead to pesticide residues on commercial bee products and resistance phenomena in bee pests (Maggi et al., 2011). In the last few years, there was increasing evidence of different ways to ensure bee health and indirectly, parasite tolerance. As honey bee populations decline, interest in pathogenic and mutualistic relationships between bees and their pathogens has increased. Microorganisms associated with *A. mellifera* have received special attention as a new option for integrated pest management (IPM). In this example, strains of bacteria and their products have become an interesting field for experimentation as they are commonly isolated from the hive environment and bees' digestive tracts (Audisio et al., 2011). Their potential effects on bees have been evaluated in three different ways. First, bacteria strains have been selected and studied as probiotic supplements for bee consumption, testing for effects on colony development. In these studies the administration of *Lactobacillus* and *Bacillus* strains helped the development of bee colonies by enhancing the brood and also honey yield (Sabaté et al., 2012). Second, bacteria (or their metabolites) were tested as alternative control methods for bee parasites. Sabaté et al. (2012) have documented a negative impact against *N. ceranae* and *V. destructor* when bacterial administration was applied on beehives in field conditions. Also, Porrini et al. (2010) have reported that particular surfactins can alter spore structure. Third, bacterial strains were studied as activators of immune-competence in bees. Evans and Lopez (2007) have demonstrated activation of antibacterial peptide expression when bees were fed bacteria. These authors also proposed that nonpathogenic bacteria could be used as a probiotic supply to enhance honey bee humoral immunity.

Nosema ceranae is an emergent parasite of *A. mellifera* apparently more virulent than *N. apis*. Currently, *N. ceranae* infections seem to be correlated with declining populations of honey bees in Spain (Higes et al., 2008). Taking into account that the major commercial medication available for *Nosema* control is based on the antibiotic fumagillin (Williams et al., 2008) and that this drug is not legal in most countries, alternative management strategies should be developed to confront this microparasite. Moreover, recent reports have provided controversial results about fumagillin efficacy (Williams et al., 2008) and also about its undesirable effects related to residues generated in honey (Lopez et al., 2008). In this way, much effort in the last few years has focused on alternatives to control this bee disease, most of them involving alternatives to chemical substances. Even though great progress has been achieved and extensive efforts are being made in the study of these compounds properties, more research is still needed.

The goal of the current study is to assess the effects of the oral administration of the metabolites produced by *Lactobacillus johnsonii* CRL1647, mainly identified as organic acids (Audisio et al., 2011), on: (I) *N. ceranae* sporulation dynamics before and after fumagillin application, and (II) performance of *A. mellifera* colonies.

2. Materials and methods

2.1. Biological material

In vitro experiments and laboratory measurements were conducted in the Arthropods Laboratory of the National University of Mar del Plata. Field trials were carried out in an experimental apiary located in Santa Paula farm (National Rout 226, 10 km south of Mar del Plata, Argentina). Experiments were performed between February and September 2012. Beehive inspections were performed at midday.

2.2. Bacterial metabolites

The antimicrobial compounds synthesized by *L. johnsonii* CRL1647 were recovered in the cell-free supernatants (CFS) as explained elsewhere (Audisio et al., 2011). Briefly, this lactic acid bacterium was grown during 24 h in MRS broth (MRS, Britania, Argentina) at 37 °C under microaerophilic conditions. Then, CFS was recovered by centrifuging (10,000 × *g*, 15 min, 10 °C), filtering (0.22 μm mesh), and kept at 4 °C until used.

2.3. Organic acids quantification by HPLC

Concentration of all organic acids produced by *L. johnsonii* CRL1647 was determined by HPLC according to Audisio et al. (2011). The CFS from a MRS culture was deproteinized and filter-sterilized (0.45 mm) 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 0.6 ml/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. Peakprofiles, integration, and quantification were obtained with a CR601 Shimadzu chromatopac integrator (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan). All tests were carried out in duplicate.

2.4. Assay I: toxicity effects of bacterial metabolite against honey bees

Before this experiment, bees remained without food for 4 h to produce starvation. Then, each bee was confined inside a plastic tube (length: 3 cm; diameter: 1 cm) and fed by mean of a micropipette tip (200 μl) with different doses of pure metabolite (20; 30; 40 and 60 μl *per* bee) (Fig. 1). Each treatment was replicated 20 times. Syrup 2:1 (sugar:water) and sterile MRS broth was used as control treatments. Once the metabolite dose was consumed, candy (mixture of powdered sugar and water) was placed inside each tube to provide food resource to the bees. Individual bee mortality *per* treatment was recorded at 24, 48 and 72 h. Bees were kept under incubator conditions during toxicity experiments (33 ± 0.79 °C; 60 ± 3.3% HR).

2.5. Assay II: effects of metabolite administration on colony development

Field research was carried out with a local *A. mellifera* ecotype (*A. mellifera mellifera* × *A. mellifera ligustica*)

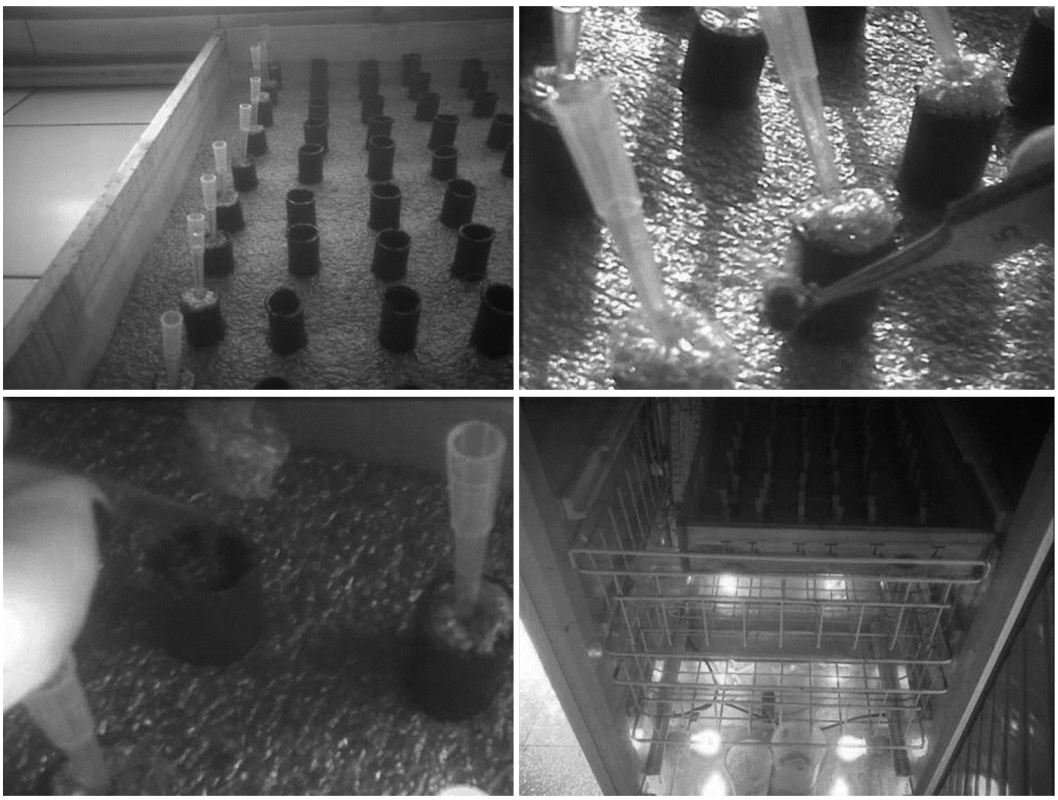

Fig. 1. Systemic administration of the bacterial metabolite *in vitro* conditions. Each tube was embedded into a styrofoam base. One single worker bee *per* tube was introduced and fed by mean of a micropipette tip (200 μl) with different doses of pure metabolite (20; 30; 40 and 60 μl *per* bee). Each treatment was replicated 20 times. Syrup 2:1 (sugar:water) and sterile MRS broth was used as control treatments. Once the metabolite dose was consumed, candy was placed inside each tube to provide food resource to the bees. Individual bee mortality per treatment was recorded at 24, 48 and 72 h. Bees were kept at 33 ± 0.79 °C; 60 ± 3.3% HR.

(Abrahamovich et al., 2007), kept in standard Langstroth hives. Prior to these studies, colonies had received parasitic control treatment against *V. destructor* (Flumevar®, two strips *per* hive with a total of 1 g of flumethrine). Hives were naturally infected with *N. ceranae*. Molecular diagnoses based on samples collected in Santa Paula apiary were performed and recently published (Genebank AN FJ425736; Medici et al., 2012). In addition, *N. ceranae* was the only microsporidium found in the southeast of Buenos Aires province (Medici et al., 2012). Colonies used in these assays were comprised of 15 nuclei prepared with sister queens (obtained from a commercial bee house), one non operculated brood comb, two operculated brood combs plus the bees present in these, and finally, one frame full of honey. Bacterial metabolite administration was carried out by a Doolittle feeder. Five colonies (Group A = LM) received 50 ml of pure metabolite + 200 ml of syrup 2:1 (sugar:water). Five colonies (Group B, control 1 = CM) received 50 ml of culture sterile media + 200 ml of syrup 2:1, and another five colonies (Group C, control 2 = syrup) received 250 ml of syrup 2:1. Treatments were provided respectively to each group (A, B and C), five times at intervals of five days (time-points T_0, T_1, T_2, T_3 and T_4) (Table 1).

The progress of the hives treated with the bacterial metabolite was monitored prior to each treatment application (time-points T_0, T_1, T_2, T_3 and T_4) and also one week after the fifth application (designated as time-point T_5) (Table 1). As an example: at T_0, the colonies were inspected and afterword the first treatment application was performed. Any change was compared with the untreated control hives. All other conditions (weather, nourishment and supervision) were identical. The parameters to quantify the general state of the colonies during the evaluation were as follows: number of combs fully covered with bees, open and sealed brood areas (following the methodology of Branco et al., 1999) and quantity of honey and pollen (estimated as box surface covered with honey or pollen respectively). A final quantification of colony development was performed on September 15th (end of winter). Here, we established the number of frames covered with adult bees as an estimator of the strength of each colony.

2.6. Assay III: effects of metabolite administration on fat bodies of workers bees

Just before the first treatment application (control, T_0) and the same day of the third and fifth ones (prior to its application: T_2 and T_4 respectively), a sample of 50 nurse bees *per* colony was collected from the brood nest (Table 1). Bees were frozen until laboratory assays.

To measure fat bodies the protocol of Wilson-Rich et al. (2008) was followed. Adult abdomens were severed from thoraces and dried for three days at room temperature. Abdomens were weighed and washed in ethyl ether for 24 h to dissolve fat. Larvae and pupae were not included in

Table 1
Variables analyzed for each sampling time-point.

	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Sampling of colony development	x	x	x	x	x	x	–	–
N. ceranae sampling	x	–	x	–	x	x	x	x
Fat bodies	x	–	x	–	x	–	–	–
Fumagillin application	–	–	–	–	x	x	x	–
Metabolite application	x	x	x	x	x	–	–	–

this assay because of their lack of hardened cuticle. Abdomens were then dried for three days and weighed again. The fat body was calculated as the percent change in abdominal weight after the ethyl ether wash.

2.7. Assay IV: effects of the bacterial metabolite on Nosema sporulation and fumagillin efficacy and efficiency

To assess the effect of the *L. johnsonii* CRL1647 metabolite administration on the development of *N. ceranae*, samples of bees from each colony were taken: (a) prior the first treatment application (control, time-point T_0); (b) the same day the third treatment application, prior to apply it (time-point T_2) and (c) the same day of the fifth treatment application, prior to apply it (time-point T_4) (Table 1).

To evaluate the effect of the metabolite on fumagillin efficacy and efficiency, the same day of the fifth treatment application (time-point T_4), colonies also were treated with 2% fumagillin (Nosemix-B® Solemar SA), according to label instructions. Each colony received 6 mg fumagillin *per* bee-covered comb, three times at intervals of seven days (time-points T_4, T_5 and T_6). Bee samples were taken from each colony after each fumagillin application, and also one week after the last drug application (time-points T_5, T_6 and T_7 respectively) (3 times) (Table 1).

Sampling was carried out always at midday, since Meana et al. (2010) reported that bees collected for measuring *Nosema* spore loads should be collected at the same time of day to be comparable. The entrance of every hive was closed with foam rubber so that foraging bees piled up and a representative sample group of more than 50 individuals could be collected and put into a flask with ethanol 70%. One sample group from each colony was taken at each time-point "*T*" as indicated above (T_0, T_2, T_4, T_5, T_6, and T_7) (see also Table 1, Figs. 5 and 6). In the laboratory, the abdomens of 50 bees from each sample group were individually homogenized in 2 ml of double distilled water and checked for the presence of *Nosema* spores under a compound microscope (400× and 1000×) to obtain infection levels (percentage of infected bees *per* hive, colony level, adapted from Smart and Sheppard, 2011). Later on, ten of these positive homogenates were randomly selected; spore amounts were quantified using an improved Neubauer haemocytometer, yielding the number of mature spores *per* bee (intensity, individual level). In sample groups without ten positive cases, all positives were quantified by haemocytometer. Table 1 summarizes the sampling time-points (from T_0 to T_7) and all the variables analyzed.

2.8. Statistics

Two-way ANOVA was performed to analyze the effects of treatments and time on colony development and fat bodies. Comparison of the average values was carried out using Tukey test ($p < 0.05$). A non-parametric test was performed to analyze *N. ceranae* prevalence and intensity among treatments and time of sampling (Kruskal–Wallis test). Comparison of the average values was carried out using Student–Nueman–Keuls test ($p < 0.05$).

3. Results

3.1. Bacterial metabolites quantification by HPLC analyses

The concentration in the CFS of the organic acids produced by *L. johnsonii* CRL1647 is reported in Table 2. Three acids were detected: lactic acid, phenyl-lactic acid and acetic acid.

3.2. Assay I: toxicity effects of bacterial metabolite against honey bees

The *in vitro* administration of the CFS, containing mainly organic acids, did not produce bee mortality during treatment, even in high doses and after 72 h of exposure (60 μl of bacterial metabolite produced 10% mortality). Table 3 depicts bee mortality (in percentage) for the doses tested. Both control treatments reached only 10% bee mortality after 72 h.

3.3. Assay II: effects of metabolite administration on colony development

Adult bee populations at the end of the test increased in the different treatments by (I) 39.5% when the bacterial *L.*

Table 2
Organic acid production by *Lactobacillus johnsonii* CRL1647.

	Lactic acid (mM)	Phenyl-lactic acid (mM)	Acetic acid (mM)
CRL1647	128.1	0.3	38.0

Table 3
Honey bee mortality (%) after 24, 48 and 72 h for different doses (μl) of organic acids isolated from *Lactobacillus johnsonii* CRL1647.

	0 μl	20 μl	30 μl	40 μl	60 μl
24 h	0	0	0	0	0
48 h	10	0	4	0	10
72 h	10	0	10	0	10

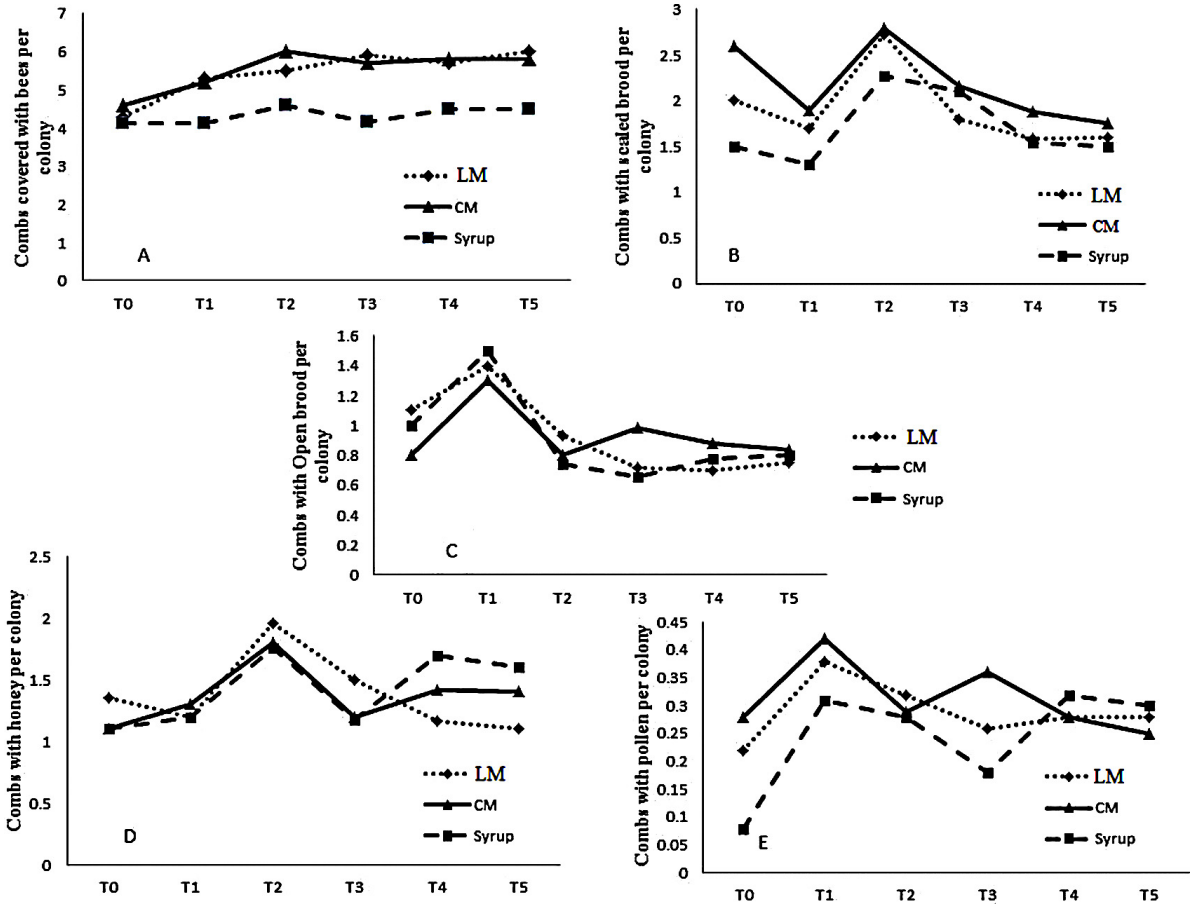

Fig. 2. Evolution of different beehive parameters during the field trial. (A) Depicts the progress of adult bee population. (B–E) Do the same for total combs with sealed brood *per* colony, total combs with open brood *per* colony, total combs with honey *per* colony and total combs with pollen *per* colony respectively. At time-point 0, all parameters were recorded from the hive. Then, first application of the three treatments was performed and repeated four more times every five days (time-points T_1, T_2, T_3 and T_4). LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar:water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

johnsonii CRL1647 metabolite (LM) was provided (from 4.3 ± 0.44 combs covered with bees at the beginning of the field trial to 6 ± 0.97 at the end); (II) 26.0% when culture media (CM) was provided (from 4.6 ± 0.65 combs covered with bees at the beginning of the field trial to 5.8 ± 0.67 at the end) and (III) 12.0% when colonies received syrup alone (syrup) (from 4.1 ± 0.54 combs covered with bees at the beginning of the field trial to 4.5 ± 0.7) (Fig. 2). Considering statistical analyses, significant differences were observed in the adult bee population *per* colony among treatments and time ($p < 0.05$, Table 4). Brood population also varied through the field trial. A continuous reduction of sealed brood for LM treatment could be observed after the third dose application (T_2) explaining the increase of adult bees on beehives (from 2 ± 0.5 combs with sealed brood at the beginning of the field trial to 1.6 ± 0.4 at the end) (Fig. 2, $p < 0.05$ and Table 4). Pollen stores were not affected by treatments ($p > 0.05$). However, statistical differences were detected in honey storage for the three groups over time ($p < 0.05$, Table 4) but no differences were detected among treatments at the end of the experiment ($p > 0.05$). After winter, clear differences were observed for colony strength among treatments. One of the five colonies from the control group died during the winter. These results are represented in Fig. 3.

3.4. Assay III: effects of metabolite administration on fat bodies of worker bees

Metabolite administration/delivery (LM) on beehives induced a significant increase in fat body production compared with culture media (CM) and syrup administration: after T_2, worker bees exposed to LM increased their fat bodies stores from 2.48 to 5.76 mg (Fig. 4 and Table 5). After four applications of LM, a pronounced decrease was observed: fat bodies decreased from 5.76 (T_2) to 1.06 mg *per* bee at the end of the field trial (T_4). In the control groups, the CM treatment did not produce significant

Table 4
Statistical output for the two way ANOVA conducted to test the differences among treatments on colony performance.

Colony variable	Treatment		Time	
	p value	*F* value	*p* value	*F* value
Adult bees	<0.001	16.6	<0.001	4.63
Sealed brood	0.04	3.15	<0.001	4.34
Open brood	0.97	0.02	0.01	3.29
Pollen	0.57	0.88	0.37	1.08
Honey	0.88	0.12	<0.001	4.24

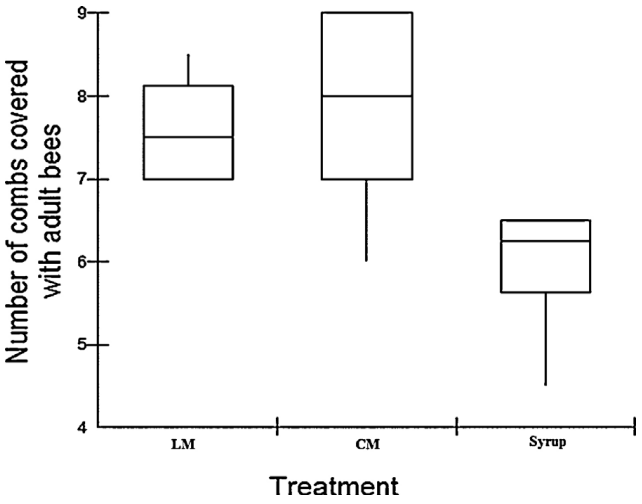

Fig. 3. Boxplot for the mean number of combs covered with adult bees after winter for each group of colonies involved on the field trial.

changes in fat bodies *per* bee over time ($p > 0.05$). However, an increase in fat body production was observed in workers sampled from colonies fed only with syrup: from 2.58 at T_0 to 5.74 mg *per* bee at T_4.

3.5. Assay IV: effects of the bacterial metabolite on Nosema sporulation

3.5.1. Nosemosis at colony level

Prevalence of *N. ceranae* over time was similar for the three groups of colonies involved in the field trial. Infection levels ranged from 16.4 to 25.6% at the beginning of the trial. By the end of the treatment applications, these values decreased to 0.4–1.2% (Fig. 5 and Table 6). Although the fumagillin application was successful in the control of the parasitosis, no improvement on its final efficacy due the administration of the bacterial metabolite was detected: all groups reached levels of *Nosema* prevalence close to 0% (Fig. 5 and Table 6).

Table 5
Mean values of fat bodies (mg) *per* honey bees and standard deviation (between brackets) measured over time for each treatment. Different letters indicate statistical differences (two way ANOVA, $p < 0.05$) inside time and treatment and also between them. T_0 represents the time before the first treatment application (control) and show the basal status of the mean fat body/bee for each group of colonies. T_2 represents the status of the mean fat body/bee after two treatment application. T_4 represents the status of the mean fat boy/bee after four treatment application. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar and water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

Treatment	T_0	T_2	T_4
LM	2.48 (±1.14) a	5.76 (±2.9) b	1.06 (±0.32) a
MC	2.06 (±0.6) a	3.17 (±2.45) a	2.4 (±1.73) a
Syrup	2.58 (±1.21) a	3.23 (±0.78) a	5.74 (±2.76) b

3.5.2. Nosemosis at individual level

Those colonies which were fed with bacterial metabolites showed a decrease in the average number of spores *per* bee: after the fourth application of the organic acids, *Nosema* intensity decreased from 5.52 to 2.61 $\times$ 10^6 spores *per* bee (Fig. 6 and Table 7). Moreover, both control group colonies showed an increase in the intensity of *N. ceranae* after T_0: from 1.74 to 4.28 $\times$ 10^6 spores *per* bee in the CM group, and from 3.14 to 4.65 spores *per* bee in the syrup group (Fig. 6 and Table 7). In this way, the administration of

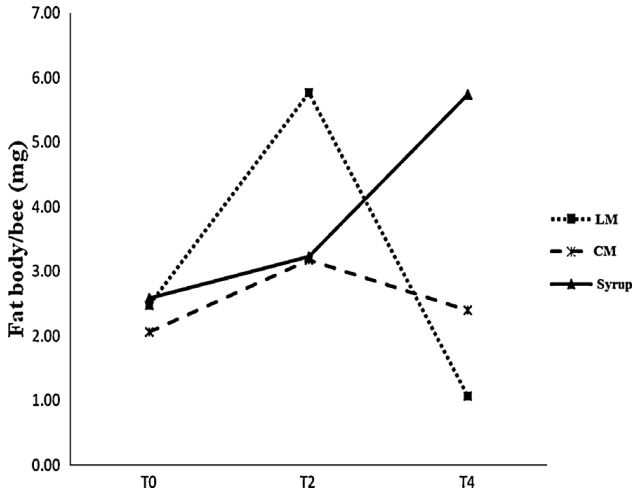

Fig. 4. Variation of fat body *per* bee among treatments and time. T_0: time before the first treatment application (control). Basal status of the mean fat body/bee for each group of colonies. T_2: status of the mean fat body/bee after two treatment applications. T_4: status of the mean fat body/bee after four treatment applications. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar:water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

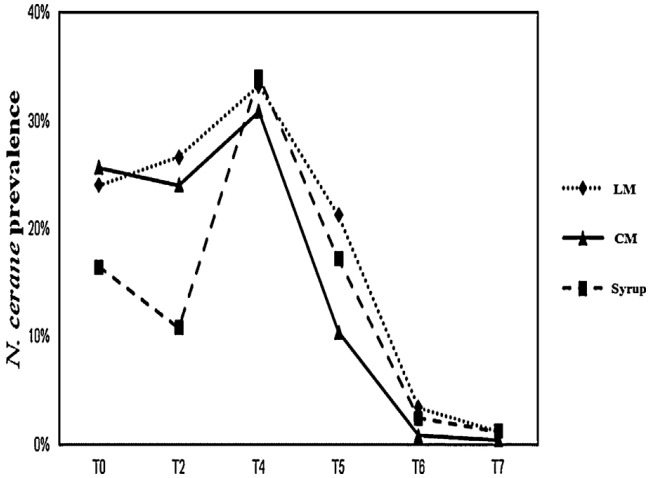

Fig. 5. Mean prevalence of *Nosema ceranae* (%) over time for the three groups of colonies involved on the field trial. T_0: time before the first treatment application (control). Basal status of the mean prevalence of *N. ceranae* for each group of colonies. T_2 and T_4: status of the mean prevalence of *N. ceranae* after two and four treatment applications respectively. T_5, T_6 and T_7: effects of the first, second and third fumagillin application against *N. ceranae* prevalence. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar:water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

Table 6
Mean prevalence of *Nosema ceranae* (%) and standard deviations (between brackets) over time for the three groups of colonies involved on the field trial. Different letters indicate statistical differences (Kruskal–Wallis test, $p < 0.05$) inside time and treatment and also among them. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar and water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

Treatment	T_0	T_2	T_4	T_5	T_6	T_7
LM	24 (±5.8) a	26.6 (±6.2) a	33.2 (±8.1) a	21.2 (±11.1) a	3.4 (±3.4) c	1.2 (±1.1) c
CM	25.6 (±7.5) a	24 (±11) a	30.8 (±14.6) a	10.4 (±5.5) b	0.8 (±1.1) c	0.4 (±0.9) c
Syrup	16.4 (±7.9) b	10.8 (±7.7) b	34 (±25.2) a	17.2 (±7.3) b	2.4 (±1.7) c	1.2 (±1.8) c

Table 7
Mean intensity of *Nosema ceranae* and standard deviations (between brackets) over time for the three groups of colonies involved on the field trial. Different letters indicate statistical differences (Kruskal–Wallis test, $p < 0.05$) inside time and treatment and also between them. Values were expressed in millions of spores *per* bee. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar and water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

Treatment	T_0	T_2	T_4	T_5	T_6	T_7
LM	5.52 (±5.3) a	2.8 (±2.1) b	2.61 (±1.7) b	2.51(±1.5) b	2.21(±1.1) b	2.77 (±3.7) b
CM	1.74 (±1.15) b	4.08 (±2.9) a	4.28 (±2.3) a	3.39 (±2.6) a	4.43 (±1.5) a	3.85(±3.1) a
Syrup	3.14 (±3.2) a	5.4 (±4.1) a	4.65 (±4.2) a	2.84 (±2.7) b	6.13 (±4.1) a	4.45 (±2.3) a

the bacterial metabolite improved the final efficiency of fumagillin when single infected bees were analyzed. Fig. 6 and Table 7 depicts these effects: those colonies which received the administrations of the bacterial metabolite maintained lower levels of *N. ceranae* intensity during fumagillin application and afterwards (from 2.51 at T_5 to 2.77×10^6 spores *per* bee at T_7). However, colonies belonging to the control groups experienced a reinfestation (from 2.84 at T_5 to 4.45×10^6 spores *per* bee at T_7).

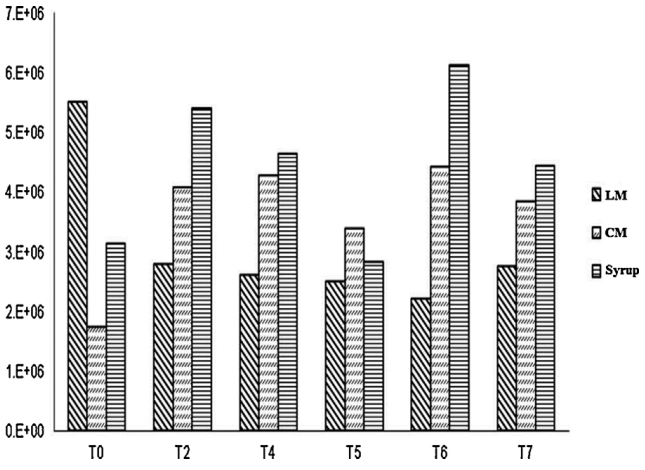

Fig. 6. Mean intensity of *Nosema ceranae* (spores/infected bee) over time for the three groups of colonies involved on the field trial. T_0: time before first treatment application (control). Basal status of the mean intensity of *N. ceranae* for each group of colonies. T_2 and T_4: status of the mean intensity of *N. ceranae* after two and four treatment applications respectively. T_5, T_6 and T_7: effects of the first, second and third fumagillin application against *N. ceranae* intensity. LM = group of colonies which received five applications of 50 ml of bacterial metabolite plus 200 ml of syrup 2:1 (sugar:water); CM = group of colonies which received five applications of 50 ml of culture media plus 200 ml of syrup 2:1; syrup = group of colonies which received five applications of 250 of syrup 2:1.

4. Discussion

Thus far, most studies on honey bee microbiota have focused on disease-causing microorganisms and colony declines while much less emphasis has been given to non-pathogenic microorganisms and their potential health benefits for individual bees or whole colonies. Intestinal microbiota of most organisms plays a crucial role in nutrient assimilation and immune function, and accumulating evidence for a worldwide association between *A. mellifera* and a core set of bacterial phylotypes does exist (Martinson et al., 2011). A growing awareness about the importance of the intestinal microbiota composition for honey bee health and growth has been reported (Martinson et al., 2011). Moreover, bees themselves are constantly exposed to pesticides, which in turn may impair beneficial in-hive microbes (Yoder et al., 2012). Because specific bacteria are consistently associated with *A. mellifera*, it has been suggested that these bacteria are beneficial mutualists (Martinson et al., 2011).

Honey bee gut microbiota appears to be relatively simple and consistent across individuals, compared to gut microbiota of other insects. Currently, knowledge about the benefits of *Apis* bacteria on bee health is scarce, with the exception of some lactic acid bacteria (LAB) effects. These bacteria might decrease the infection rate of *A. mellifera* larvae exposed to *Paenibacillus larvae* (Forsgren et al., 2010). In addition, LAB have been isolated from the crop of *A. mellifera*, freshly collected pollen and freshly fermented bee bread, which is the protein source for larval stages (Vásquez and Olofsson, 2009). Fermentative properties of LAB could aid in the conversion of nectar to honey as well as in the conversion of pollen to bee bread and its protection from spoilage (Vásquez and Olofsson, 2009). Bacteria associated with bumble bees can also benefit their hosts against parasites (Koch and Schmid-Hempel, 2012).

In this work, we have reported another type of LAB benefit in *A. mellifera* colonies. This benefit was measured indirectly by the administration of organic acids in bee hives produced by the strain *L. johnsonii* CRL1647. This bacterium was previously isolated from the bee gut and selected mainly by its organic acids synthesis (Audisio et al., 2011). The results of this study indicate firstly that feeding bees high doses of these metabolites is not lethal after 72 h of exposure. Control mortality did not exceed 10%, thus complying with the trials standards set forth by OECD (OECD, No. 213 and No. 214 (1998), OEPP/EPPO Guideline No. 170). Additionally, treating the colonies with the bacterial metabolite increased population levels. Similar results were found by Audisio and Benitez-Arhendts (2011), using *L. johnsonii* CRL1647 bacterial cells. In addition, those colonies fed with the bacterial metabolite survived better over winter than colonies in the control group (one colony fed only with syrup died during winter and the others showed decreased populations compared to the treatment group LM). Although it was known that particular strains of *L. johnsonii* can produce different antimicrobial peptides (as a result of its own metabolism), the strain CRL1647 used in this survey was selected on the basis of its acidificant power (138 mM of lactic acid) due to their synthesis of organics acids (Audisio et al., 2011).

Microbial diversity and by products may provide colonization resistance to pathogens (Dillon and Charnley, 2002) and may be of extraordinary relevance to honey bee health, given that they have a greatly reduced immune system relative to other model insects (Evans et al., 2006). Hence, colonies fed with LM could reach larger sizes than other treatment groups as a consequence of a better health status induced by the organic acids. Moreover, we have reported that fat bodies have also significantly increased after two applications of bacterial metabolites, where organic acids were the main components, when compared with control groups. Fat bodies play a major role in the life of insects, being involved in multiple metabolic functions. Among the most important functions are: (a) to store and release energy in response to energy demands of the insect and (b) to produce several antimicrobial peptides, acting similar to a vertebrate liver (Lavine and Strand, 2002). These two functions are closely related and could explain the better survival and the bigger population size of those colonies fed with the bacterial metabolite: a bee with more fat bodies implies a healthier bee. In *A. mellifera* colonies, nutritional deficiencies that affect the immune response can accelerate the spread of disease among nest mates, increasing pathogen levels and reducing adult longevity and survival (Mayack and Naug, 2009). What began as a nutritional deficiency could quickly develop into colony loss due to an infectious disease. By this pathway, nutrition is a key factor in resistance to pathogens (Rowley and Powell, 2007). A recent study on *A. mellifera* argues that poor nutrition depresses the immune system and consequently could drive colony loss (vanEngelsdorp et al., 2010). In the same way, Allaux et al. (2010) found that bees fed with diets rich in protein modify both individual and social immune competencies. These authors suggest a link between nutrition and immunity in bees, underlining the fundamental role of the availability of resources for pollinators' health. Proteins, carbohydrates, minerals, lipids, and vitamins are supplied primarily by nectar, pollen and water. When these resources are depleted, bees must use proteins and lipids from their own tissues to produce larval food and survive for a short period of time. Therefore, stronger colonies could enhance survival, moreover when poor nutrition is explicitly identified as a probable contributing factor in recent colony losses (vanEngelsdorp et al., 2010).

With regard to the bacterial metabolite and its impact on *Nosema* levels, two types of monitoring of the parasite were selected: prevalence (colony level) and intensity (individual level). Mean rate of infected honey bees (prevalence) seems to be the best reliable method to evaluate the health status of a colony (Higes et al., 2008; Meana et al., 2010). Our results showed that metabolite supplementation did not modify the dynamics of the disease at the colonial level. The initial prevalence of nosemosis in the colonies was comparable at the start of the experiment and, even with some minor variations in T_2, the colony infections were similar and did not show statistical differences in both pre- and post-fumagillin application. This fact could be related to the limited timeframe of the experiment. A long-term (*e.g.:* seasonal) metabolite supplementation may give appreciable results at this level. However, when nosemosis development was analyzed individually, differences between treatments were observed. A mean spore count *per* bee is used as an indicator of success of the pathogen in the individual host (Smart and Sheppard, 2011). A higher spore load affects the insect, causing a suite of metabolic changes (Bailey, 1981). Infected bees have lower levels of protein, resulting in a reduced hypopharengeal gland (Malone and Gatehouse, 1998), as well as altered fatty acid composition in the hemolymph (Roberts, 1968). Also, *N. ceranae* shortens bee life span causing greater colony mortality in winter (Nitschmann, 1957). Here, we have showed that application of our bacterial metabolite alone has strongly reduced the spore loads *per* bee. Additionally, when coupled with fumagillin, the amounts of spores *per* bee decreased during application of the metabolite, resulting in an improvement in the final efficiency of the drug: those colonies which did not receive the metabolite (groups CM and syrup) had regularly increased amounts of spores in the individuals examined. Hence, organic acids produced by a *L. johnsonii* strain could: (a) contribute as an organic tool to reduce individual loads of *Nosema* without fumagillin application and (b) keep controlled the reinfestation when antibiotic control is used, improving its efficacy. The significance of our findings is more evident considering that: (a) fumagillin is prohibited in some countries, (b) fumagillin has been the topic of controversy regarding its efficiency in *Nosema* control (Willians et al., 2011) and (c) fumagillin use could result in honey residues (Lopez et al., 2008). These results could be of high impact for IPM of nosemosis.

Our investigations were not sufficient to explain which mechanisms are involved on *Nosema* reduction when bacterial metabolite supplementation on colonies is applied. However, different hypotheses could be postulated: (a) Organic acids synthesized by *L. johnsonii* CRL1647

can modify or alter the microsporidium envelope (and/or the physiological midgut environment) affecting spore viability. Some of these acids were reported as chemical substances for the control of bee parasites. Porrini et al. (2010) have reported in their *in vitro* study that *N. ceranae* spores exposed to direct contact with surfactin (a particular bacterial metabolite) would decrease their infectivity. This surfactin, administered *ad libitum* from the individuals' emergence, led to a significant reduction in parasitosis development when bees were infected with untreated spores seven days post emergence. In the same way, modifications in osmotic, ionic or pH conditions, enzyme production and activity, or any other biochemical change into the gut would be reducing the number of extruded polar filaments, therefore decreasing the number of infected host cells (Malone and Gatehouse, 1998). (b) Organic acids induce thickening of the peritrophic membrane which is the first barrier that the microsporidium must overcome. (c) The reduction of *Nosema* sporulation registered in this study could be the result of the interactions among the above hypotheses. Whatever change is induced on the midgut, the final result should be a reduction in the sporoplasm penetration into digestive tissue. Another report supports our findings indirectly: Koch and Schmid-Hempel (2012) showed that the microbiota of *Bombus terrestris*, which resembles the *A. mellifera* microbiota, protects bee against the trypanosomatid *Crithidia bombi*. Their results stress the importance of considering the host microbiota as an "extended immune phenotype" in addition to the host immune system itself, and provide a unique perspective to understanding bees in health and disease. Furthermore, Forsgren et al. (2010) have demonstrated a protective effect of lactic acid bacteria against *P. larvae* secondary infections on honey bee breed. Vásquez et al. (2012) have also reported that prophylactic practices that enhance LAB, or supplementary feeding of LAB, might serve in integrated approaches to sustainable pollinator service provision.

We feel that the evidence presented here supports and encourages the potential of using metabolites isolated from nonpathogenic bacteria as diet supplements to improve healthier honey bee colonies. While more studies should be performed to test their effects against American foulbrood and Varroosis, these organic acids should be incorporated as an important tool for future IPM programs for Nosemosis.

Conflict of interest statement

The authors have no conflict of interest to declare.

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