Alterations in honey bee gut microorganisms caused by *Nosema* spp. and pest control methods

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Abstract

BACKGROUND

Honey bees are associated with gut microorganisms essential for their nutrition and health. The microbial community composition can be used as a biological health indicator and characterized employing biomarker fatty acids. Commonly gut microorganisms are exposed to pathogens and to an array of chemical and biological pest control methods.

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RESULTS

We found a strong negative effect on the microbial gut community composition when exposed to the bee pest control chemicals oxytetracycline, oxalic acid, imidacloprid as well as when inoculated with the bee pest *Nosema* spp. and the potential bee pest biocontrol agent *L. plantarum*. Results from the *in vitro* test with bee pest chemicals showed a differential response of *Lactobacillus* spp. At the community level some taxonomic groups were more affected depending on treatment, but sharp shifts in the microbial structure were caused by compounds generally considered as bee safe.

CONCLUSION

Our results show that pest as *Nosema* spp. and pest control methods alter the composition of bee gut microorganisms, which may have severe consequences for pathogen defence, physiology and general honey bee health. In addition *L. plantarum* show potential as a biocontrol agent against *Nosema* spp.

Keywords: Honey bee; gut microorganisms; pest control methods; *Nosema*; biomarker fatty acids.

1 INTRODUCTION

Bees form complex social networks with apparent low variations in their associated microbial communities within and across species, seasonal and geographical regions[1,2]. Microbiome bee diversity is low and specialized suggesting high stable co-dependent symbioses crucial for honey bee health[3,4].

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Endophytes play a critical role in honey bee nutrition due to the production of essential amino acids, sugar metabolism and transformation of pollen through breakdown of the cellulose wall releasing proteins, amino acids and lipids[1].

Additionally, honey bee endophytes play an important role in pathogen defence by synthetizing antimicrobial peptides, organic acids, activation of humoral responses and by biofilm formation obstructing pathogen colonization among others[5].

Guts of newly emerged bees are relative free of microorganisms, but after emergence inoculation occur with nest-mates via trophallaxis, which is the principal acquisition route of pathogenic and non-pathogenic microorganisms[6]. Honey bees core gut microbiota community is composed by eight filo-types: Alpha1, Alpha2, Gilliamella, Gamma2, Snodgrassella, Firm4, Firm5 and Bifido[7]. In decreasing order of abundance gut microorganisms are represented by Gram negative, Gram positive and only one percent of yeast and fungi[8]. *Lactobacillus* spp. are keystone microorganisms in honey bees, highly abundant[9] and widely distributed across geographical regions and bee species[2].

Bees are exposed to pathogens and pests like virus, bacteria, fungi and varrora mites among others[10]. Beekeepers use a wide array of products to control bee diseases and pests, generally divided in to synthetic (*e.g.* miticides and antibiotics) and organic (*e.g.* plant extracts), which is mostly considered bee safe[11]. There is a trend to diversify the use of available products and promote their rotation to avoid pest resistance[12], but the effects of bee pest control compounds on honey bee gut microorganisms have not been fully examined. In addition, bee microorganisms are exposed to a set of pathogens and pesticides via food ingestion[13].

Imidacloprid is one of the most used insecticides to control pest, which can be found in ¾ of the honey in the world[14]. The plant oils menthol and thymol are used as alternative

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treatments to control varroa mites, which are normally applied by vaporization, but these compounds can also be found in honey and thereby potentially effect beneficial honey bee gut microorganisms[15]. Oxytetracycline is a commonly used antibiotic to treat bacterial diseases such as *Paenibacillus larvae*[16]. In general these compounds offer good honey bee pest control, but information on collateral non-target effects on beneficial organisms such as honey bee gut microorganisms is limited. All these products present stress factors, which may irreversibly alter the gut microbial composition leading to dysbiosis[17].

Nosemosis is a worldwide bee disease caused by three species of microsporidian intracellular obligate parasites *Nosema apis, N. ceranae* and the recently discovered *N. neumanni* [18]. In Mexico *N. apis and N.* ceranae have been reported infecting bees both individually and in combination[19-21], reducing hive performance and productivity[22] and increasing honey bee mortality[23]. When developing alternative biocontrol methods against *Nosema* spp. it is important to know how these fungal parasites interact with other gut microorganisms.

In order to understand the effects of bee pest and bee pest control measures on the gut microbiota of honey bees, we first assessed the *in vitro* response of *Lactobacillus* strains to pest control compounds, then we used biomarker fatty acids to evaluate the response at community level and finally we evaluated the effect of the pathogen *Nosema* spp. alone and in combination with the probiotic bacterium *L. plantarum*, in terms of possible alterations in bee gut microbial communities.

2 MATERIALS AND METHODS

2.1 Characterization of lactic acid bacteria (LAB) associated with honey bees

The LAB were isolated from midguts of seven days old adult worker honey

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bees, collected in spring 2016 in five localities in the state Michoacán in Mexico between 700 to 2100 m of altitude with different mono-floral and multi-floral resources. Bee collections were made in five localities including Uruapan (19.478084, -102.014749), Capacuaro (19.520948, -102.068856), Sabino (19.295525, -101.978231), Morelia (19.651560, -101.220107) and Copandaro (19.876717, -101.195495). Three apparently healthy hives per site and fifty bees per hive were collected. Hives were considered healthy when they had high population, healthy brood, low Varroa levels and no historical presence of *Nosema* spp. assessed by the Mexican Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) and additional visual examination for other pests and without any pest control treatments in the last three months. To reduce possible insecticide residues only new frames with new wax operculum foundation were used and bees were collected in hives relatively far from agricultural crops. Frames were individualized to avoid bee mixing and after seven days bees were caught and transported to the laboratory.

Midguts were aseptically dissected using sterile forceps from bees chilled at 4 ºC in the laboratory. Homogenized and filtered midguts were pooled by hive and plated in serial dilutions ($1x10^{-1}$ - $1x10^{-5}$), with three repetitions per dilution, in a semi selective media Man, Rogosa and Sharpe agar (MRS, Sigma, 0.1% L-Cysteine and 2.0% fructose) at 37 ºC until visible colony growth. Then, bacterial colonies per plate were randomly picked according to morphological characteristics (white, round) and sub-cultured. As a first Lactobacillus selection filter, isolates were subjected to a catalase test with 3% H_2O_2, which should be negative, as Lactobacillus isolated from bees do not produce catalase as indicated by the lack of bubbles, observed by microscopy[24]. Isolated strains were

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grown under standard conditions for fatty acid identification in MRS agar (48 hours at 35 ºC) according to the microbial identification system libraries (MIDI; Microbial ID, Inc., Newark, Delaware, U.S.A.)

2.2 LAB inhibition tests with bee pest compounds

Susceptibility of characterized LAB strains was tested by standard disc diffusion tests. A two-way factorial design was employed with Lactobacillus strains and bee pest compounds as factors. Ten Lactobacillus strains were tested with five bee pest compounds and four replicates, giving a total of two hundred experimental units. 0.2 g per strain were cultivated in MRS broth (37 ºC, 300 rpm, 48 hours) to 0.5 McFarland scale ≈1.5 x10 ^{8} cells/ml. 0.2 mL of each suspension were plated on MRS agar with a five mm disc in the centre impregnated with different pest control compounds. To evaluate effects of bee pest control compounds, concentrations were selected according to recommended field doses that had no effects on mortality: 3 % oxalic acid[25-27], thymol (1 mg L^{-1})[28,29], menthol (1 mg L^{-1})[30,31], oxytetracycline[32] and imidacloprid (0.7 µg L^{-1} Sigma)[33-35]. Each disc was impregnated with approximately 20 µL of the solutions (gravimetrically determined). Plates were incubated at 37 ºC for 72 hours and inhibition halos recorded in mm with a calliper. Imidacloprid was dissolved in acetone from an initial stock concentration of 100 mg L^{-1}. Through successive dilutions a final concentration of 0.7 µg L^{-1} was achieved. The final concentration of acetone was 0.1%, which was tested in a sucrose solution to ensure that possible effects of Imidacloprid on Lactobaccillus and/or gut microorganisms were not related to direct effects of the diluent. Feeding solution syrup was prepared by agitation with magnetic beads and avoiding exposing to direct sunlight.

2.3 Response of bee gut microorganisms to bee pest control compounds

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Honey bee pest control compounds were tested in a one-way factorial design with oxalic acid, menthol, thymol imidacloprid, oxytetracycline and a control treatment, giving a total of five treatments, with five replicates per treatment resulting in 25 experimental units.

Seven-day old honey bees obtained from the same hive located in Copandaro (19.876717, -101.195495) to reduce genetic variation and hence bee response, were individualized in sterile micro-centrifuge tubes. The tip of each tube was removed, leaving enough space for the head of the bee to be out and allowing them to feed. Food volume consumption per bee was daily recorded. Individualized bees were kept in a growth chamber with sufficient space among them to avoid trophallaxis at 30 º C, 70 % R.H. for seven days and fed with sterile sucrose solution (50% w/v) (Figure 1). Oral acute exposition was tested by feeding 20 µL per bee of each pest control compound from stock solutions, used in the inhibition test using a micropipette (Figure 1). Mortality was recorded daily for seven days. At the end of experiment bees were chilled, decapitated and guts removed using sterile forceps, and microorganism community was characterized by biomarker fatty acid analysis. Homogenized midguts were added to MRS broth enrichment cultures and incubated for 72 hours at 37 º C. Hereafter, 40 mg of cells from the exponential growth phase were harvested and characterized by biomarker fatty acid analysis.

2.4 Response of bee gut microorganisms to *Nosema* spp. and *L. plantarum* inoculations

A two-way factorial design was employed with the bee pest *Nosema* spp. (with and without) and the probiotic *L. plantarum* (with and without). Again, each treatment had five replicates giving a total of 20 experimental units. The experimental set-up was similar to that with bee pest control compounds (Figure 1).

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Nosema was originally isolated from bees showing infection symptoms (fecal marks in the front of the hive) in a tempered region of Michoacan, Madero (19.357171, -101.284292), which was confirmed by microscopy at 400x using a phase contrast microscope. After confirmation of Nosema spore morphology, spores were purified and concentrated by dissection of the midgut, which was macerated and spores obtained in the pellet after three successive centrifugations at 5000 G. Spores were re-suspended in phosphate buffered saline PBS. Isolated spores were 6 x 3 µm suggesting being *Nosema api*s spores, slightly bigger than *Nosema ceranae* 4,7 x 2.7 µm and *Nosema neumanni* 2.4 x 1.8 µm[36,18]. To further assure that the Nosema inoculum used was mainly *N. apis* the inoculum was chilled at 4 ºC for 4 days to reduce spore viability of *N. ceranae*[37] and *in-vivo* reactivated using 7 days old healthy bees. Hereafter, collected bees were individualized and artificially infected with *N. apis*. The infection was done by feeding bees with a 25 microliter pipette tubes containing spores to a final concentration of 1 x10^6 spores/dose in a sacarose solution at 50% (v/w).

Each bee was individualized using a 1.5 ml centrifuge tube (Figure 1). The feeding process was observed and bees which, did not eat the full dose were discarded. After inoculation bees were kept at 34 ºC and 70 % RH. Mortality was recorded daily at 14:00 hour. All bees inoculated with Nosema died after 8 to 15 days after inoculation. Nosema spores from the discolored midgut of the dead bees were extracted as mentioned above and used as inoculum in the main experiment. Inoculation with *Nosema* was then performed by feeding bees with fresh *Nosema* spores as explained above. Seven days after inoculation guts were removed and examined by microscopy to confirm infection. Inoculum of *L. plantarum* was produced in MRS broth for 48 hours, washed three times with sterile saline solution PBS and diluted in sterile sucrose solution 50% (w/v) to a final concentration 10^6 cells per bee. Treatments with *Nosema* spp. and *L. plantarum*

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were first inoculated with *Nosema* and after 48 with *L. plantarum.* At the end of experiment bees were chilled, decapitated and guts removed using sterile forceps, microorganism community was characterized by fatty acid biomarkers analysis. Similar to the previous experiment homogenized midguts were added to MRS broth enrichment cultures and incubated for 72 hours at 37 º C. Hereafter, 40 mg of cells from the exponential growth phase were harvested and characterized by biomarker fatty acid analysis.

2.5 Fatty acid analysis

Fatty acid methyl esters (FAMEs) were extracted according to standard procedures[38] with a three-step analysis: saponification with sodium hydroxide, methanol and water for 30 minutes at 100 ºC; methylation with chlorhydric acid and methanol for 10 minutes at 80 º C and extraction with hexane and methyl tert-butyl methyl ether. Fatty acids were detected in an Agilent GC 7890 equipped with a 25-m fused silica capillary column and analyzed by Sherlock software version 3.1 (MIDI Inc., Delaware, USA) using hydrogen as gas carrier. Quantification was made with an internal standard 19:0 (nonadecanoic methyl ester, Sigma) of known concentration and comparing peak areas. For standard calibration we used a combination of fatty acid methyl esters (10–20 carbon length) provided by MIDI (Inc., Newark, USA).

Honey bee microbial structure can be characterized by quantitative or qualitative analysis of fatty acid microbial biomarkers[39]. Biomarker fatty acids were used to examine possible alterations in communities of bee gut microorganisms. As biomarkers for Gram positive bacteria iso and anteiso branched fatty acids (14:0i, 15:0i, 15:0a, 16:0i, 16:0a, 17:0i and 17:0a), for Gram negative bacteria fatty acids (*cy*17:0, *cy*19:0, 18:1ω7c and 18:1ω9c), representing groups of unresolved Gram negative biomarkers,

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fatty acids; SF3(16:1ω7c/16:1ω6c), SF7(19:1ω6c/18:1ω9t and or 18:1ω12t), SF 8 (18:1 ω7c/18:1 ω6c) and fungi SF 5 (18:2ω6,9c/18:0a)[39-42].

2.6 Statistics

Variance of quantitative measures were performed with generalized linear models (GLM) and qualitative measures of fatty acid profiles was assessed by principal component analysis (PCA) in R software[43] with FactoMine package for analysis and factoextra for visualization[44,45]. The GLM fitted the model with lowest AIC exhaustive analysis and Post-hoc Tukey´s test by least square means package (lsmeans)[46].

3 RESULTS

3.1 Isolation and identification LAB from honey bee guts

Ten species of LAB were identified from honey bee guts (Table 1). Each strain had a particular fatty acid composition (Table 1).

3.2 *In-vitro* inhibition test

Oxytetracycline was the most growth depressive treatment, showing inhibition effects in all evaluated *Lactobacillus* species, however it was statistically significant only in four. In contrast, oxalic acid also showed inhibition effects, but only in four out of ten *Lactobacillus* species. Imidacloprid and thymol only significantly affected one and menthol had no effect. Under the assessed conditions *L. sanfranciscensis, L. hilgardii, L. buchneri* and *L. plantarum* were not significantly affected by any of the compounds tested. In contrast *L. vitilinus* was the most susceptible followed by *L. farciminis, L. pentosaceus, L. confusus, L. gasseri and L. fermentum.* Major inhibitory growth effect was recorded in *L. vitilinus* exposed to oxytetracycline (Figure 2).

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3.3 Effects of bee pest control compounds on bee gut microbial communities

Seven days after exposure to bee pest compounds, the PCA revealed significant shifts in fatty acid profiles (Figure 3). There is a strong association between fatty acid biomarkers SF 3, 5,7, 8 and oxytetracycline. In the same way, fatty acids 15:0 a, 15:0i, 17:0 a, 16:0i and control were positive correlated. Both axes of PCA revealed a imidacloprid-thymol clustering in the lower left corner of the plot, PC1 (37.9% of the data variation) showed a gradient of shifts starting with the antibiotic oxytetracycline then the cluster thymol-imidacloprid, oxalic acid, menthol and at the right of component the control. PC2 (20.85% of variance) reflects a clear separation among subset imidacloprid-thymol and oxytetracycline, control, menthol and oxalic acid in the center not well clustered. Fatty acids 17:1ω8c and 16:1ω5c have less influence on PCA. Most of the detected fatty acids were bacterial biomarkers, Gram-negative (SF 3, 7 and 8) and Gram-positive (15:0i, 15:0a, 16:0i, 17:0a), bacterial biomarkers grouped separately according to their functional groups.

Quantitatively fatty acid biomarkers clearly responded to all the assessed compounds, with oxytetracycline, thymol and imidacloprid the most inhibitory treatments, whereas the Varroa natural control compounds oxalic acid and menthol had limited effects. The Gram positive biomarker 15:0a was strongly suppressed in all treatments (Table 2).

3.4 Effect of Nosema and *L. plantarum* on bee gut microbial communities

Effects of *Nosema* on fatty acid profiles were more marked in PC 1 (50% of the data variance). Most of the variance was explained by two principal clusters, *Nosema* in the left side and control, *L. plantarum, Nosema-L. plantarum* at the right. PC1 was strongly influenced by 17:0a and 16:1ω5c Gram positive and Gram negative biomarkers respectively. In addition, *Nosema* was strongly correlated with fatty SF 5 a fungal

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biomarker. In PC1 (19.3% of the variance) there is a separation among *L. plantarum* and the subgroup *Nosema-L. plantarum* and control, in PC 1 *Nosema* treatment is not clearly separated. SF 3, 7 and 17:1ω8c are positively correlated with *L. plantarum* and 15:0a, 15:0i 17:0i, 16:0i, SF 8 with the control and *Nosema-L. plantarum.* Fatty acid clustering depending on microbial functional group was not evident (Figure 4). Quantitatively, inoculation with *Nosema* spp. reduced seven of the eleven microbial biomarkers, with 16:0i, SF3 and SF5 the only ones not affected, whereas in contrast for SF 7 a strong significant increase was observed. Treatments with *Nosema* spp. and *L. plantarum* as probiotic, were statistically equal to controls, despite this, a considerable increase in SF 7 was noticed, in *L. plantarum* treatments, and the SF7 biomarker was particularly increased by the probiotic (Table 3).

4 DISCUSSION

Results from the present study show high sensitivity of honey bee microorganisms to a set of pest control compounds commonly used by beekeepers. Also, infection with the bee pest *Nosema* spp. caused alterations in bee gut microbial communities, which was however mitigated by the probiotic bacterium *L. plantarum*.

Six of the ten isolated *Lactobacillus* species identified in the present study had been previously reported as honey bee endophytes including *L. plantarum, L. buchneri, L. gasseri, L. fermentum, L. pentosaceus* and *L. sanfranciscensis*[47-50]. These *Lactobacillus* species are linked with bee health due to their antimicrobial properties, high yield lactic acid production and biofilm formation, among others[51-54].

Strong and differential effect of bee pest control compounds on *Lactobacillus* spp. were observed in the present study. As expected, oxytetracycline was one of the most *in-vitro* inhibitory compounds, confirming the results of Vásquez et al.[5], who reported high sensitivity of the *Lactobacillus* isolated from bees to antibiotics. In tolerant strains this

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would be related to antibiotic resistance genes[47], as seen in *L. sanfranciscensis* closely related genetically to *L. kunkeei* in which growth inhibition effect was not significantly. Thymol is well known for its antimicrobial properties[55]. However, this inhibitory effect was only found in *L. pentosaceus*. Oxalic acid occurs naturally in honey and plants, however after oxytetracycline it was one of the most growth depressive treatments, possible due to the used concentration. Kwak et al.[56] showed oxalic acid to have antibacterial activity at 500 mg/L, which was far lower from the common used dose 30 g/L[57] to treat Varroa, supporting the findings that oral applications are detrimental for bee health[58]. Among the bee pest control compounds included in the present study, menthol seems to be the safest one, since none of the evaluated *Lactobacillus* species were affected by this compound. However, though not all *Lactobacillus* species were significantly affected by all compounds, each one has particular effect for honey bees health, which may result in poor bee colony development or increased pathogen susceptibility[59,60].

Detrimental effects of Imidacloprid on bees have been linked to suppression of the immune system and increased pathogen susceptibly[61]. In addition, dysbiosis observed in the present study may represent a strong detrimental repercussion to honey bee health as Gram-positive and Gram-negative biomarkers were affected, while fungal biomarkers were unaffected. On oxytetracycline treated bees, Gram-negative biomarkers (SF 3, 7 and 8) were clearly separated from Gram-positive biomarkers (15:0i, 15:0a, 16:0i, 17:0a), which coincides with Raymann[62] who reported sensitivity of the Gram-positive honey bee bacterial communities (*Lactobacillus* spp.) to antibiotics, whereas resistance was observed in the Gram negative core group.

Thymol a commonly used treatment for Varroa control, considered a natural bee safe treatment[28], also altered bee gut microbial communities in the present study, which

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suggest that sub-lethal effects of thymol should be taken into account when used in bee pest control. In contrast menthol seems to be one of the safest compounds with limited effects on bees gut microbial communities. However, it has a poor acaricide effect and serious side consequences like brood removal[31]. Oxalic acid the third evaluated Varroa control agent represent the safest method for gut microbial communities showing similar effects to menthol, however it has serious consequences on *in-vitro* strain growth inhibition. Since Varroa is one of the most detrimental widely distributed bee pests and the evaluated compounds are some of the most used by beekeepers for pest control, it is important to consider their non-target effects on the beneficial bee gut microbiota.

Nosema treatments coincided with an increase in the fatty acid SF 5 (18:2ω6,9c/18:0a), which is a fungal biomarker fatty acid either by pathogen growth or by gut colonization of opportunistic fungi[39,63,64], common in immune suppressed bees[65]. However, in quantitative terms the effects from *Nosema* inoculation on SF 5 (18:2ω6,9c/18:0a) were not significant.

Our results showing that application of the probiotic bacterium *L. plantarum* mitigated alterations in bee gut microbial communities, after *Nosema* infection, suggesting a potential use in biocontrol of bee pests. From the present study, the biocontrol mode of action cannot be revealed, but may be linked to production of antifungal compounds like antimicrobial peptides and short chain fatty acids[66-71]. Another possible mode of action of *L. plantarum* is the strong capacity to acidify its environment by high levels of lactic acid production, impeding efficiently the infection by bee pathogens like *Paenibacillus larve*[39,72]. Strain adaptability, high yield lactic acid production and ability of altered gut recolonization make *L. plantarum* an ideal probiotic for pathogen control[73].

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However, the use of *L. plantarum* in healthy bees might also lead to microbial perturbations principally in the Gram negative biomarker SF 7, which needs to be addressed before practical applications.

The observed high variable response of *Lactobacillus* species and communities to the different bee pest control compounds and high dependence of honey bees on their endophytes calls for further characterization of honey bee microbial alterations.

In conclusion, both *Nosema* and bee pest control compounds can cause strong alterations in bee gut microbial communities, leading in to adverse side effects in the complex honey bee microbial interactions. Also, the probiotic bacterium *L. plantarum* show potential to improve bee health, which however needs to be further addressed.

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Table 1. Fatty acid composition (%) of isolated *Lactobacillus* strains. Similarity Index (SI) according to the MIDI Sherlock libraries.

Strain	Fatty acid (%)											SI
	14:0	15:0i	16:0	18:1 ω9c	18:0	18:1 ω7c	19:0cy ωc	20:2 ω6,9c	SF 3	SF 5	SF 8	
L. plantarum	1.25		33.99		2.46	1.41	34.05	1.08	4.63		21.15	0.31
L. buchneri	1.24		27.91		1.99		39.16		5.55		24.16	0.49
L. confusus	1.72		34.63		1.85	1.31	38.15	1.03	5.49		15.82	0.45
L. farciminis		1.86	14.29	58.62	1.32				2.29		7.91	0.51
L. fermentum	1.35		47.95		3.2		33.31		3.83		10.36	0.36
L. gasseri	8.37		38.07	10.47	3.75		9.27		7.57	10.26	30.87	0.44
L. hilgardii			48.83		7.97						43.2	0.37
L. pentosaceus			30.08	34.04							27.51	0.64
L. sanfranciscensis			32.31	45.47							22.23	0.45
L. vitulinus	3.44		28.99	42.33							28.68	0.36

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Table 2. Average yield of individual fatty acids (nanogram g^{-1} pellet) after 48 hours of culture enrichment of honeybee guts (*n*=5). Different letters indicate significant differences between treatments for individual fatty acids.

Fatty acid	Pest control compounds						*p*
	Wihout	Menthol	Oxalic acid	Oxytetracycline	Thymol	Imidacloprid	
15:0 a	3.60^{a}	1.50^{b}	1.50^{b}	0.37^{c}	0.37^{c}	0.08^{d}	***
15:0 i	2.23^{a}	0.91^{ab}	0.53^{bc}	0.38^{bc}	0.36^{bc}	0.22^{c}	***
16:1ω5c	1.72^{a}	1.52^{a}	1.33^{a}	0.32^{a}	0.27^{a}	0.14^{a}	0.07
16:0 i	1.61^{a}	1.05^{a}	0.94^{a}	0.32^{b}	0.22^{b}	0.04^{c}	***
17:1 ω8c	4.62^{a}	3.03^{ab}	2.94^{ab}	2.15^{ab}	1.25^{ab}	0.83^{b}	*
17:0 a	2.09^{a}	1.75^{ab}	1.67^{ab}	0.85^{ab}	0.68^{ab}	0.53^{b}	**
SF 3	29.73^{a}	21.25^{ab}	6.95^{bc}	6.48^{bc}	5.95^{c}	5.19^{c}	***
SF 5	8.15^{a}	7.64^{a}	3.44^{ab}	2.71^{ab}	2.13^{ab}	1.47^{b}	***
SF 7	98.51^{a}	51.67^{ab}	44.00^{ab}	24.94^{bc}	22.25^{bc}	13.41^{c}	***
SF 8	53.59^{a}	39.48^{ab}	36.22^{ab}	15.43^{b}	14.83^{b}	12.86^{b}	**

Significant codes: ***, 0.001; **, 0.01; *, 0.05.

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Table 3. Average yield of individual fatty acids (nanogram g^{-1} pellet) after 48 hours of culture enrichment of honeybee guts (n=5) as affected by single and combined inoculation with the pathogen *Nosema* spp and *Lactobacillus plantarum*. Different letters indicate significant differences between treatments for individual fatty acids.

Fatty acid	Microbial inoculations				*p*
	Without	*Nosema* sp.	*L. plantarum*	*Nosema* sp and *L. plantarum*	
15:0 a	4.31[a]	2.89[b]	3.48[ab]	4.34[a]	**
15:0 i	3.35[a]	1.54[b]	2.64[a]	3.31[a]	***
16:1 ω5c	5.38[a]	2.48[b]	6.04[a]	4.93[a]	***
16:0 i	3.95[a]	2.89[a]	3.54[a]	3.81[a]	0.09
17:1 ω8c	7.05[a]	4.11[b]	11.3[a]	7.79[a]	***
17:0 a	6.76[a]	2.20[b]	6.86[a]	6.12[a]	***
17:0 i	3.09[a]	2.09[b]	2.69[ab]	3.53[a]	*
SF 3	19.5[a]	17.5[a]	24.4[a]	19.9[a]	0.12
SF 5	49.8[a]	60.5[a]	48.5[a]	55.4[a]	0.68
SF 7	8.63[b]	18.5[a]	31.8[a]	17.9[ab]	***
SF 8	27.6[a]	14.5[b]	30.7[a]	36.2[a]	**

Significant codes: ***, 0.001; **, 0.01; *, 0.05.

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Figure 1

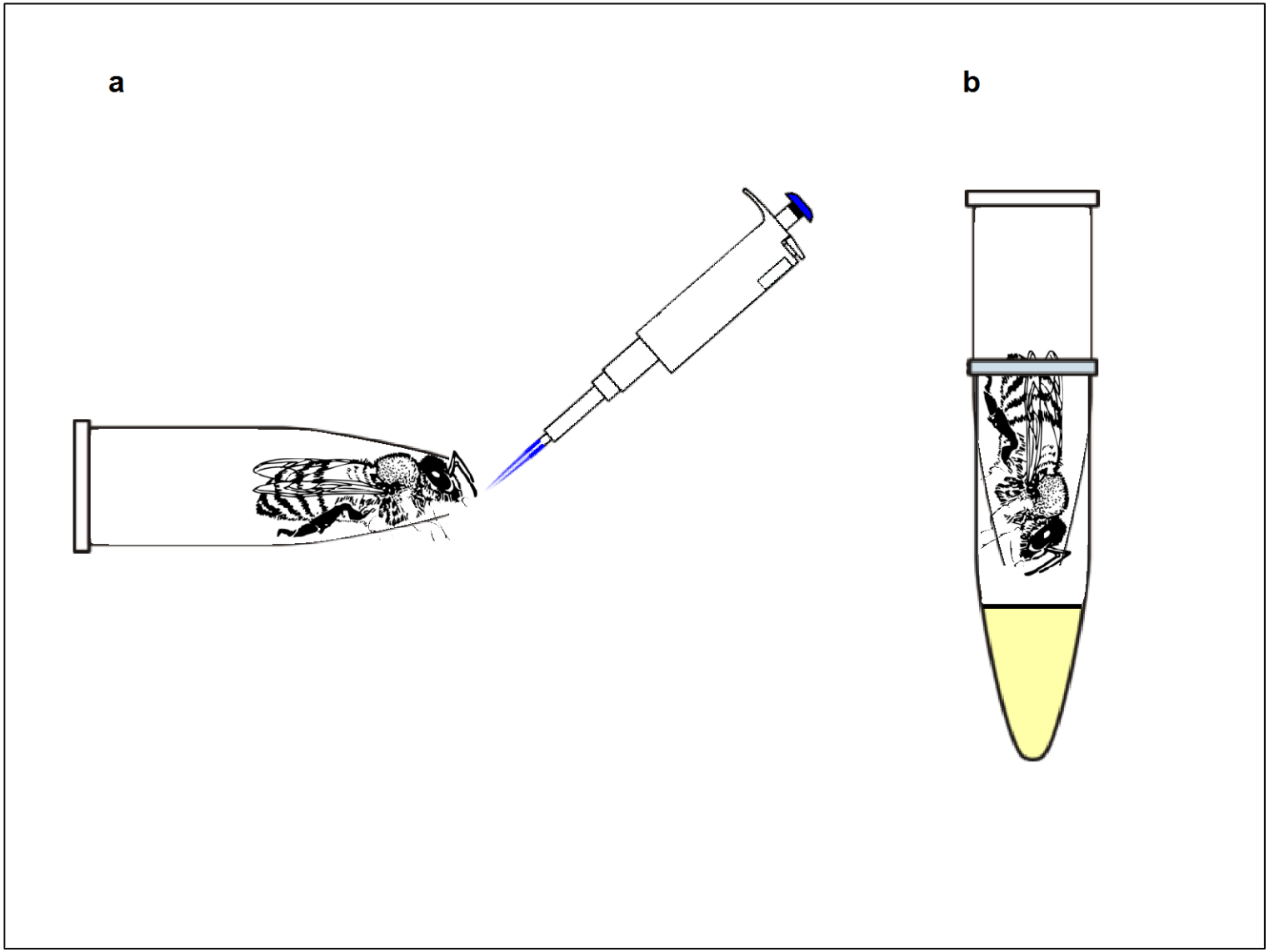

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Figure 2

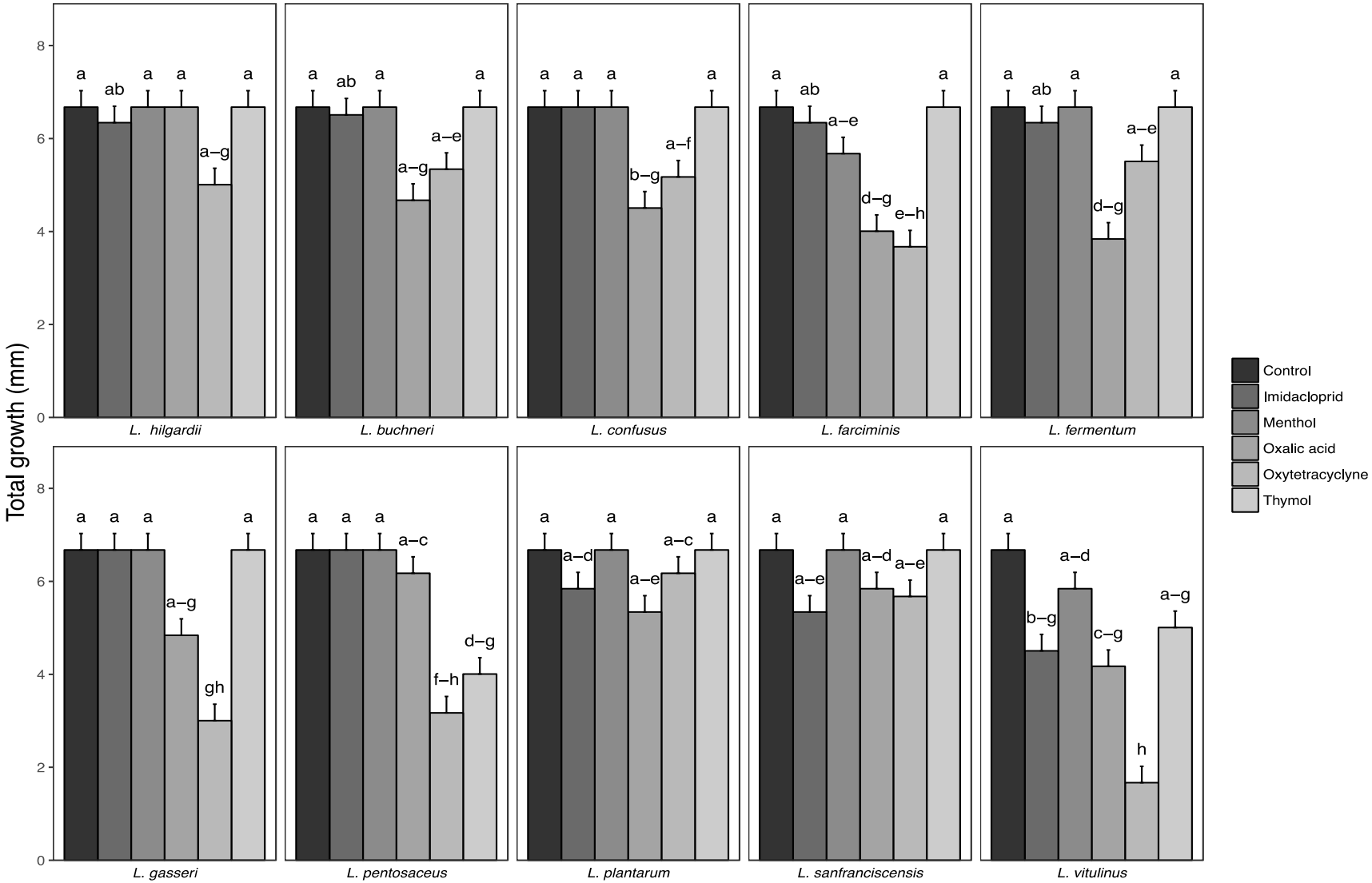

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Figure 3

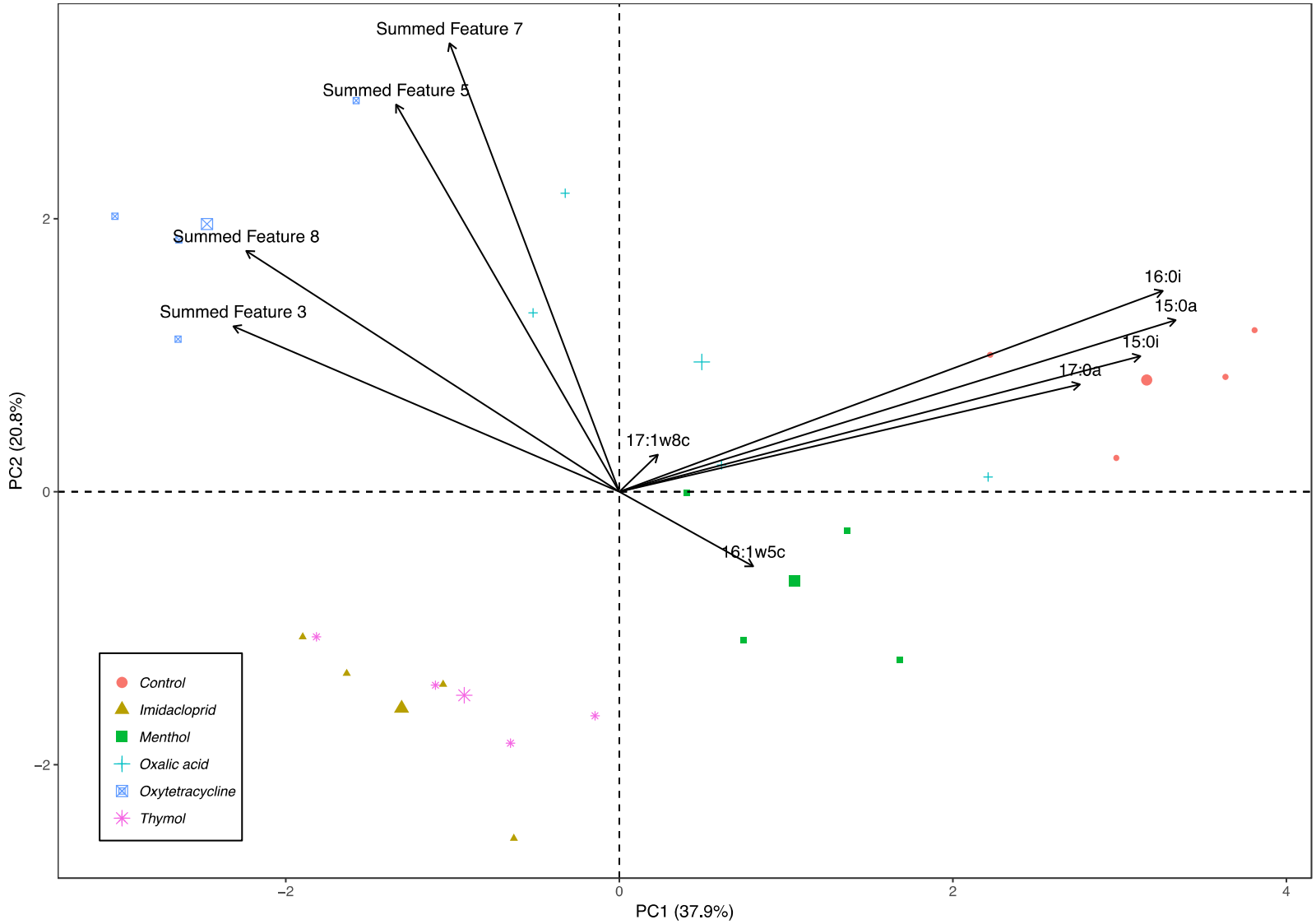

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Figure 4.

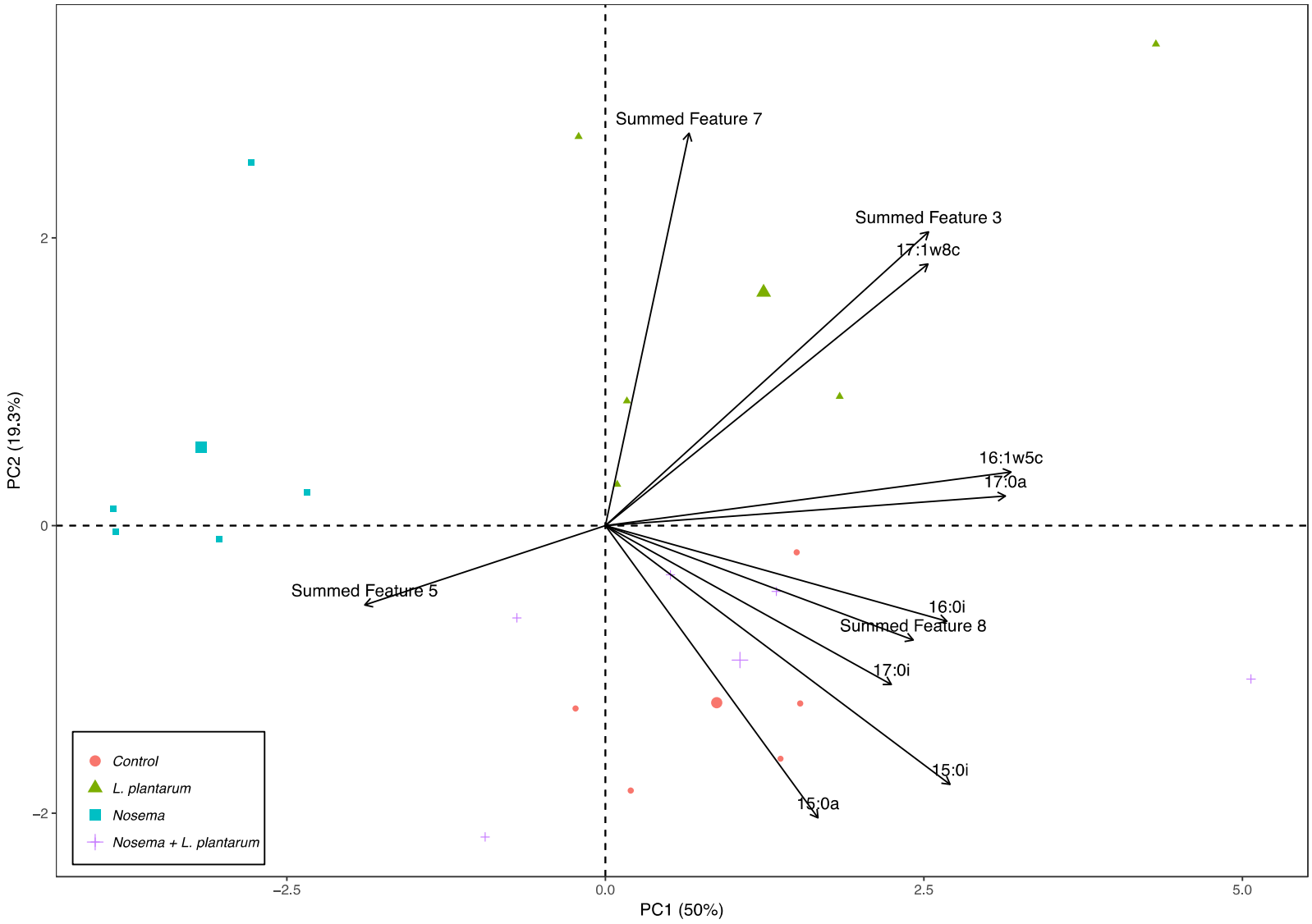

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FIGURE LEGENDS

Figure 1. Schematic presentation of experimental units: Inoculation (a) and feeding (b) method.

Figure 2. Growth response (mean growth ± SE) of *Lactobacillus* isolated strains with the different bee pest control compounds, same letters are not statistically different by GLM and post-hoc Tukey´s test ($p<0.05$).

Figure 3. Principal component analysis of honey bee microbial community shifts caused by bee pest compounds.

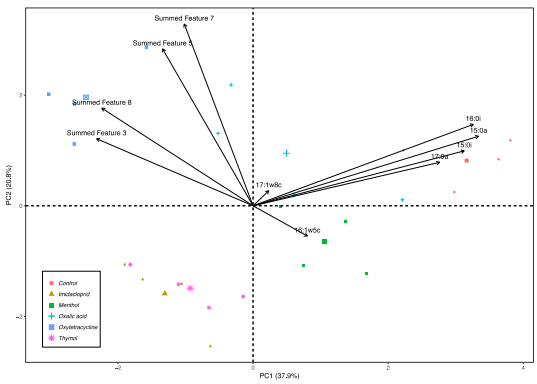
Figure 4. Principal component analysis of honey bee microbial community shifts caused by *Nosema* and *L. plantarum.*

GRAPHICAL ABSTRACT

Alterations in honey bee gut microorganisms caused by Nosema and pest control methods

Tsiri Diaz[a], Ek del-Val[a,c], Ricardo Ayala[b] and John Larsen[a,]*

Pest control compounds caused sharp shifts in the honey bee gut microbial structure which may have severe consequences for pathogen defence, physiology and general honey bee health.



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