UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES EN ECOSISTEMAS Y SUSTENTABILIDAD

ECOLOGÍA

Control biológico de enfermedades de la abeja *Apis mellifera*

TESIS

(**POR ARTÍCULO CIENTÍFICO**)

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

QUE PARA OPTAR POR EL GRADO DE:

MAESTRO EN CIENCIAS BIOLÓGICAS

PRESENTA:

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MORELIA, MICHOACÁN., JUNIO, 2018

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Directora General de Administración Escolar, UNAM
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Por medio de la presente me permito informar a usted, que el Subcomité de Ecología y Manejo Integral de Ecosistemas del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día **26 de febrero de 2018**, aprobó el siguiente jurado para la presentacion del examen de grado de **MAESTRO EN CIENCIAS BIOLÓGICAS (ECOLOGÍA)**, al alumno **DÍAZ GUERRERO JOSET TSIRI,** con número de cuenta **516012076**, por la modalidad de graduación de **tesis por articulo cientifico** titulado: "**Alterations in honey bee gut microorganisms caused by Nosema and pest control methods",** que es producto del proyecto realizado en la maestría que lleva por titulo "**Control biológico de enfermedades de la abeja *Apis mellifera*",** ambos realizados bajo la dirección de la **DRA. EK DEL VAL DE GORTARI,** quedando integrado de la siguiente manera:

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COORDINACIÓN

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Resumen en español

Los microorganismos asociados al intestino de las abejas son esenciales para su salud y nutrición. Estas comunidades microbianas pueden ser utilizadas como bioindicadores de la salud de las abejas, los cuales pueden ser caracterizados con el uso de ácidos grasos. Los microorganismos del intestino de las abejas están comúnmente expuestos a patógenos y a una serie de compuestos químicos y biológicos para el control de enfermedades. En este trabajo, examinamos como estos compuestos utilizados para el control de patógenos de abejas afectan las especies benéficas de *Lactobacillus spp.* y las comunidades microbianas, en términos de inhibición *in-vitro* y mediante ensayos de exposición oral aguda *in-vivo.* Evaluando la respuesta de los biomarcadores de ácidos grasos, encontramos marcados efectos negativos en la composición de la microbiota del intestino cuando estos fueron expuestos a compuestos para el control de enfermedades de las abejas, al antibiótico oxitetraciclina, al ácido oxálico, al patógeno Nosema spp. y al agente de biocontrol *L. plantarum,* a nivel de comunidades algunos grupos fueron más afectados dependiendo del tratamiento. Algunos compuestos considerados como seguros para las abejas mostraron marcados cambios en la estructura de las comunidades de microorganismos, lo cual podría tener serias consecuencias en la defensa ante patógenos, fisiología y en la salud de las abejas en general.

Abstract

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 fatty acid biomarkers. Commonly gut microorganisms are exposed to pathogens and to an array of chemical and biological pest control compounds. We examined how bee pest control compounds affect beneficial *Lactobacillus* spp. and the bee microbial communities in general, in terms of both *in vitro* inhibition and *in vivo* acute oral assays. Evaluating the fatty acid biomarker responses, 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 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 functional groups were more affected depending on treatment, but sharp microbial structure shifts were caused by compounds generally considered as bee safe. In conclusion our results show that bee gut microorganisms were altered by pest and pest control methods, which may have severe consequences for pathogen defence, physiology and general honey bee health.

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

Introducción

La abeja *Apis mellifera* es una especie clave para la producción agrícola mundial de gran relevancia social y económica, ya que genera 200 billones de dólares anuales en servicios de polinización (Meixner, 2010), lo que representa el 9.5% del valor de la producción agrícola mundial (Potts et al., 2010). El nivel de domesticación de *A. mellifera* permite el manejo de un gran número de individuos, asimismo la adaptabilidad de *A. mellifera* a una gran variedad de climas y cultivos las convierte en una especie de vital importancia para la seguridad alimentaria debido a que la polinización por animales y principalmente por abejas contribuye a un tercio de la producción mundial de alimentos (Klein et al., 2007).

El incremento en el cultivo de alimentos que requieren polinización con respecto a la cantidad de abejas manejadas (Aizen et al., 2009), la degradación en los servicios ecosistémicos proporcionados por polinizadores nativos, las grandes pérdidas de colmenas y la reducción en la producción de hasta un 90% de los cultivos que dejan de recibir estos servicios (Klein et al., 2007) ha incrementado el interés por las abejas llevando a la búsqueda de estrategias que nos permitan entender y manejar su salud.

La salud de las abejas se ve afectada por una diversidad de factores actuando individualmente o en sinergia, dentro de los principales tenemos: enfermedades emergentes, exposición a insecticidas, baja disponibilidad de recursos, falta de capacitación de los apicultores, entre otras (Potts et al., 2010, Jacques et al.,.2017). Los principales patógenos de las abejas se pueden clasificar en: ácaros, virus, bacterias y hongos (Evans y Schwarz, 2011).

La nosemosis es una de las enfermedades de las abejas de más amplia distribución (Klee et al., 2007), causada por los hongos microsporidios *Nosema ceranae* y *Nosema apis*. La

patogénesis ocurre por diversas vías como: ingesta de néctar, miel, polen o agua contaminados con esporas del patógeno, las cuales son diseminadas dentro de la colmena principalmente vía trofalaxis (Smith, 2012). Aunado a esto, la nosemosis es una enfermedad que se transmite sexualmente facilitando así su dispersión (Roberts et al., 2015). Los efectos negativos del parasito conocidos en las abejas son variados y pueden: afectar la fertilidad, disminuir la capacidad de forrajeo, dañar las células epiteliales del intestino medio afectando la capacidad de alimentación y almacenaje de nutrientes, debilitar el sistema inmune de las abejas provocando finalmente la muerte de la colonia (Antúnez et al., 2009, Dosselli et al., 2016, Naug y Gibbs, 2009, Peng et al., 2015). Durante años, se han utilizado compuestos sintéticos para el tratamiento de las enfermedades de las abejas. Sin embargo, debido a la toxicidad de estos y a la resistencia que han generado los patógenos (Milani et al 1999), se ha explorado la utilización de tratamientos alternativos como: barreras físicas, hongos entomopatógenos, ácidos orgánicos, aceites esenciales entre otros. Estos tratamientos generalmente son considerados como seguros para las abejas ya que muestran bajos o nulos efectos en mortalidad y bajos niveles de residualidad (Imdorf et al., 1999, Damiani et al., 2009). Además, son eficientes en el control de patógenos (Al Toufailia et al., 2015, Gashout et al., 2009). Sin embargo, el efecto de estos en las comunidades de microorganismos asociados a las abejas no ha sido evaluado, estos podrían modificar las complejas interacciones microbianas disminuyendo o incrementando ciertos grupos, produciendo cambios en la microbiota normal de las abejas. Los microorganismos asociados a las abejas son de vital importancia para la salud de estas, estas interacciones son complejas y sensibles a cambios ambientales y de manejo, la comunidad de microorganismos de *A. mellifea,* tiene diversas funciones dentro de las que destaca la defensa ante patógenos mediante exclusión por competencia, producción de

ácidos grasos de cadena corta, barreras físicas, acidificación del medio mediante la producción de ácido láctico y activación de respuestas humorales (Vasquez et al., 2012, Anderson y Ricigliano, 2017). Además, son vitales para la nutrición, participan en la síntesis de aminoácidos esenciales, metabolismo, transporte de azucares y digestión del polen principal fuente de proteínas de las abejas; liberando proteínas, aminoácidos, lípidos, carbohidratos, vitaminas y minerales (Gilliam et al., 1997, Kwong et al., 2016).

Las comunidades de microorganismos asociados a las abejas se clasifica en ocho filo-tipos de los cuales dos de los más abundantes son, Firm4 y Firm5 (Kwong et al., 2016), estos grupo están compuestos principalmente por bacterias acido lácticas las cuales tienen diversas funciones como; la estimulación del sistema inmune (Daisley et al., 2017) y promoción de la resistencia a dos de los patógenos más destructivos de las abejas; *Nosema* spp. y *Paenibacillus larvae* (Audisio et al., 2011, Mudroňová et al., 2011, Porrini et al., 2010).

Aunado a esto, existe una fuerte correlación entre la disminución de estos filotipos y el colapso de colmenas (Cox-Foster et al., 2007) resaltando así su importancia.

Los microorganismos asociados a las abejas comúnmente se ven expuestos a diferentes compuestos como; insecticidas, acaricidas, antibióticos y patógenos utilizados en la agricultura vía consumo de polen y néctar contaminado (Johnson et al., 2010) o en el tratamiento de las enfermedades de las abejas.

Para el tratamiento de enfermedades bacterianas y parásitos, las abejas son comúnmente alimentadas con antibióticos, acaricidas y ácidos orgánicos, estos se han utlizado durante de manera indiscriminada, generando resistencia de algunos grupos, con efectos sobre las comunidades de microorganismos asociados a las abejas en las cuales puede aumentar la presencia de microorganismos resistentes (Tian et al., 2012).

Uno de los compuestos más utilizados para el control de plagas es el insecticida neonicotinoide Imidacloprid (Simon-Delso et al., 2015), el cual se encuentran presentes en tres cuartos de la miel en el mundo exponiendo a la mayoría de las abejas del mundo a este vía ingestión (Mitchell et al., 2017). Imidacloprid es uno de los insecticidas más estudiados debido a que ha sido relacionados con una diversidad de efectos negativos para las abejas (Oldroyd et al., 2007). Causa alteraciones en el sistema neuronal afectando la memoria y capacidad de orientación (Decourtye et al., 2004) y debilita el sistema inmune disminuyendo su capacidad de respuesta ante patógenos (Brandt et al., 2016). Sin embargo, los efectos secundarios de este en la comunidad bacteriana de las abejas no ha sido previamente evaluado. Estudios previos han demostardo que el Imidacloprid causa alteraciones en la estructura de las comunidades bacterianas del suelo (Cycoń et al., 2010). Los objetivos de este trabajo fueron evaluar los efectos de diversos compuestos utilizados en la actividad apícola para el control de enfermedades, el patógeno *Nosema* spp. y el insecticida imidacloprid en los microrganismos asociados a las abejas. Además, probar el potencial de un lactobacilo aislado del intestino de las abejas para el control de las alteraciones causados por *Nosema* spp. Para esto, primero aislamos e identificamos a los lactobacilos asociados a las abejas mediante perfiles de ácidos grasos después, mediante aplicaciones orales y ensayos de inhibición *in-vitro* probamos el efecto antibiótico de estos compuestos y al final inoculamos como agente de control biológico a *L. plantarum* posterior a la infección por *Nosema* spp.

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

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Abstract

BACKGROUND

Honey bees are associated with gut microorganisms, which are essential for their nutrition and health. The microbial community composition can be used as a biological indicator of the bee health, and it can be characterized employing biomarker fatty acids . Gut microorganisms are commonly exposed to pathogens and to diverse chemical and biological pest control methods.

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 than others depending on the treatment, but pronounced changesin the microbial structure were caused by compounds generally considered as bee safe.

DISCUSSION

We examined how bee pest control compounds affect beneficial *Lactobacillus* spp. and the bee microbial communities in general, in terms of both *in vitro* inhibition and *in vivo* acute oral assays, our results show that bee gut microorganisms were altered

by pest and pest control methods, which may have severe consequences for pathogen defence, physiology and general honey bee health.

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].

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 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, these products are generally divided in to synthetic (*e.g.* miticides and antibiotics) and organic (*e.g*. plant extracts), which are generally considered safe for bees [11]. However, 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 been poorly examined. In addition, bee microorganisms are exposed to a set of pathogens and pesticides via food ingestion[13]. All these products represent stress factors, which may irreversibly alter the gut microbial composition leading to dysbiosis[14]. Alterations in gut bacterial communities in honey bees may represent an indicator of physiological stress. 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*, on 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 bees, collected in spring 2016 in Michoacán state, Mexico. From a previous experiment, we selected five localities in a range between 700 to 2100 m of altitude, with different mono-floral and multi-floral resources. Three 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 treatment in the last three months. 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. Catalase activity was tested by microscopy adding 3% H_2O_2 and isolated strains where 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. Ten strains were tested with five treatments 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 sub-lethal effects, concentrations were selected according to recommended field doses that had no effects on mortality: 3 % oxalic acid[15-17], thymol (1 mg L^{-1})[18,19], menthol (1 mg L^{-1})[20,21], oxytetracycline[22] and ecloprid (0.7 µg L^{-1} Sigma)[23-25]. 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.

2.3 Response of bee gut microorganisms to bee pest control compounds

Honey bees 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 bees from the same hive 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). Inoculation with *Nosema* was performed by feeding bees with fresh *Nosema* spores isolated from midguts of artificially infected honey bees, the spores were purified by maceration, filtration and successive centrifugations[26]. Before feeding the final *Nosema* concentration was adjusted in a haemocytometer to $1x10^6$ spores per bee, seven days after inoculation, guts were removed and microscopy analyzed 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* 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[27] 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[28]. 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, 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)[28-31].

2.6 Statistics

Variance of quantitative and qualitative measures of fatty acids was assessed by principal component analysis (PCA) in R software[32] with FactoMine package for analysis and factoextra for visualization[33,34]. Strain inhibition and fatty acid variance was analyzed by Generalized Linear Models (GLM) fitting the model with lowest AIC exhaustive analysis and Post-hoc Tukey´s test by least square means package (lsmeans)[35].

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 did not have a significant 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).

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 positively 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 showed a sharp shift caused by all the assessed compounds, with oxytetracycline, thymol and imidacloprid the most aggressive 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 biomarker. In PC1 (19.3% of the variance) there was a separation among *L. plantarum* and the subgroup *Nosema-L. plantarum* and control, whereas in this axis *Nosema* treatment was 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).

Finally, our results indicated that 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*[37-40]. These *Lactobacillus* species are linked with bee health due to their antimicrobial properties, high yield lactic acid production and biofilm formation, among others[41-44].

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 could be related to antibiotic resistance genes[37], *as reported for L. sanfranciscensis,* a strain closely related to *L. kunkeei, in which growth inhibition effect was not significantly. Thymol is well known for its antimicrobial properties*[45]*. However, thymolinhibitory effect was found only 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.[46] showed oxalic acid to have antibacterial activity at 500 mg/L, which was far lower from the common used dose 30 g/L[47] to treat

Varroa, supporting the findings that oral applications are detrimental for bee health[48].

*Among the bee pest control compounds included in the present study, m*enthol 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 leading to possible sub lethal effects, which may prime to poor bee colony development or pathogen susceptibility[49,50].

Detrimental effects of Imidacloprid on bees have been linked to suppression of the immune system and increased pathogen susceptibly[51]. 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[52] 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[18], also altered bee gut microbial communities in the present study, which 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[21]. 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[28,53,54], common in immune suppressed bees[55]. However, in quantitative terms the effects from *Nosema* inoculation on SF 5 (18:2ω6,9c/18:0a) were not significant. Our results showed that application of the probiotic bacterium *L. plantarum* after *Nosema* infection, mitigated alterations in bee gut microbial communities, 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 as previously reported[56-61]. 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*[28,62]. Strain adaptability, high yield lactic acid production and ability of altered gut recolonization make *L. plantarum* an ideal probiotic for pathogen control[63]. 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 variability in the response of *Lactobacillus* species and communities to the different bee pest control compounds, as well as the high dependence of honey bees on their endophytes, highlights the need of 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 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

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	Treatment						p
	Control	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 '***' 0.001 '**' 0.01 '*' 0.05.

Table 3. 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	Treatment				*p*
	Control	*L. plantarum*	*Nosema* sp *L. plantarum*	*Nosema* sp.	
15:0 a	4.31[a]	3.48[ab]	4.34[a]	2.89[b]	**
15:0 i	3.35[a]	2.64[a]	3.31[a]	1.54[b]	***
16:1 ω5c	5.38[a]	6.04[a]	4.93[a]	2.48[b]	***
16:0 i	3.95[a]	3.54[a]	3.81[a]	2.89[a]	0.09
17:1 ω8c	7.05[a]	11.3[a]	7.79[a]	4.11[b]	***
17:0 a	6.76[a]	6.86[a]	6.12[a]	2.20[b]	***
17:0 i	3.09[a]	2.69[ab]	3.53[a]	2.09[b]	*
SF 3	19.5[a]	24.4[a]	19.9[a]	17.5[a]	0.12
SF 5	49.8[a]	48.5[a]	55.4[a]	60.5[a]	0.68
SF 7	8.63[b]	31.8[a]	17.9[ab]	18.5[a]	***
SF 8	27.6[a]	30.7[a]	36.2[a]	14.5[b]	**

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.

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

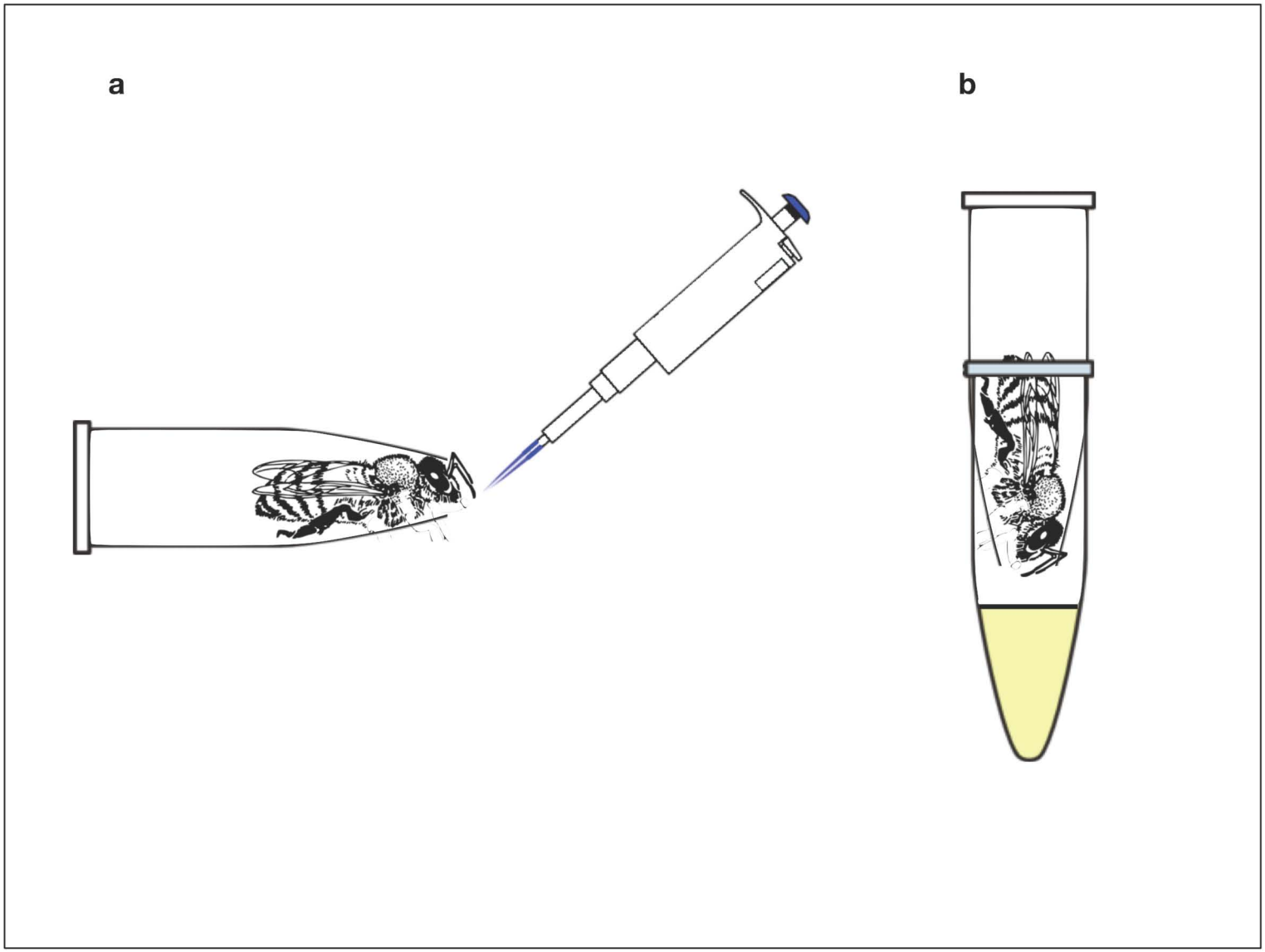


Figure 2 Growth response (mean growth ± SE) of *Lactobacillus* isolated strains with the different bee pest control compounds, same letters are not statistically different by GLM and post-hoc Tukey´s test (p<0.05).

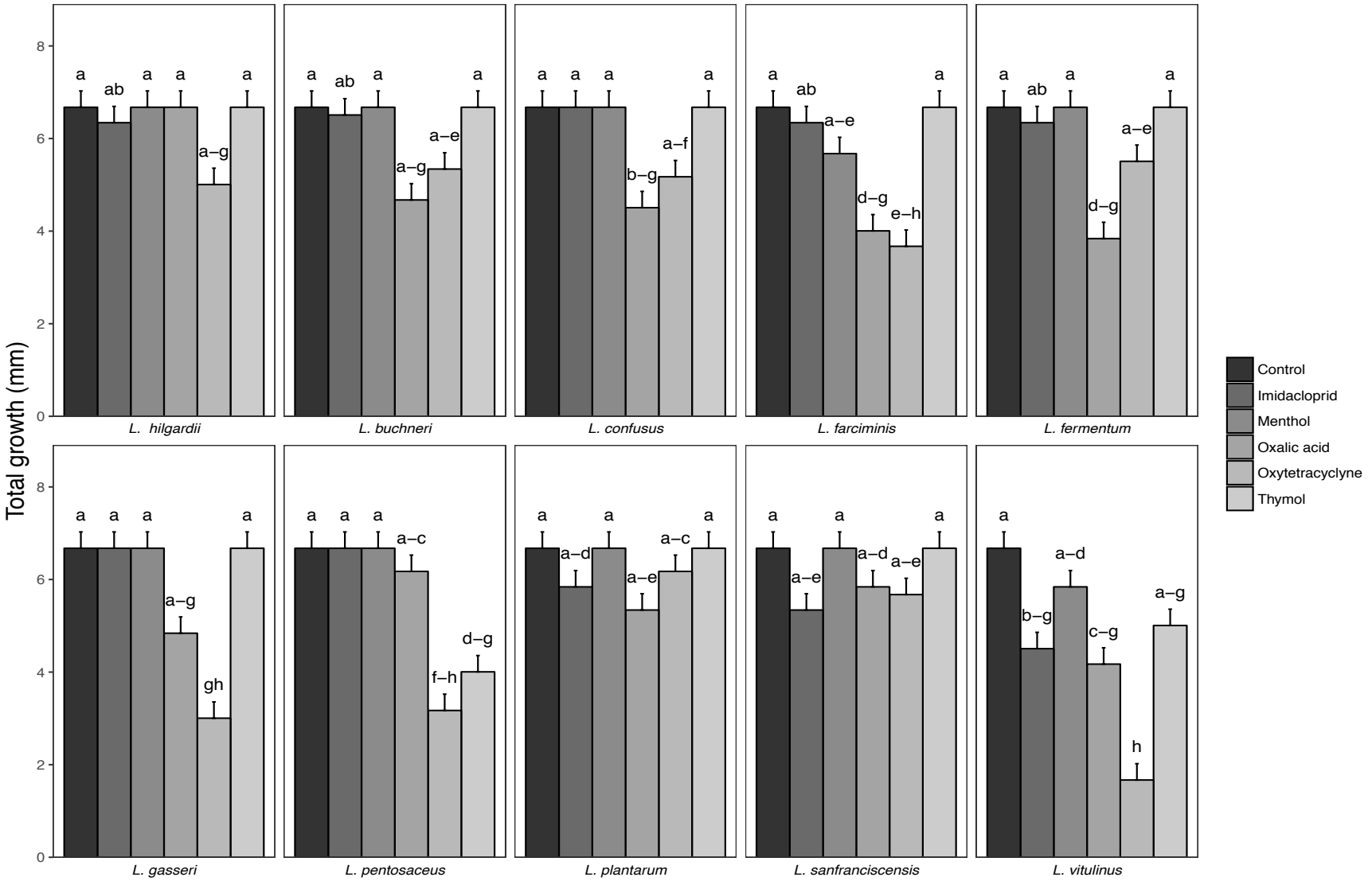


Figure 3. Principal component analysis of honey bee microbial community shifts caused by bee pest compounds.

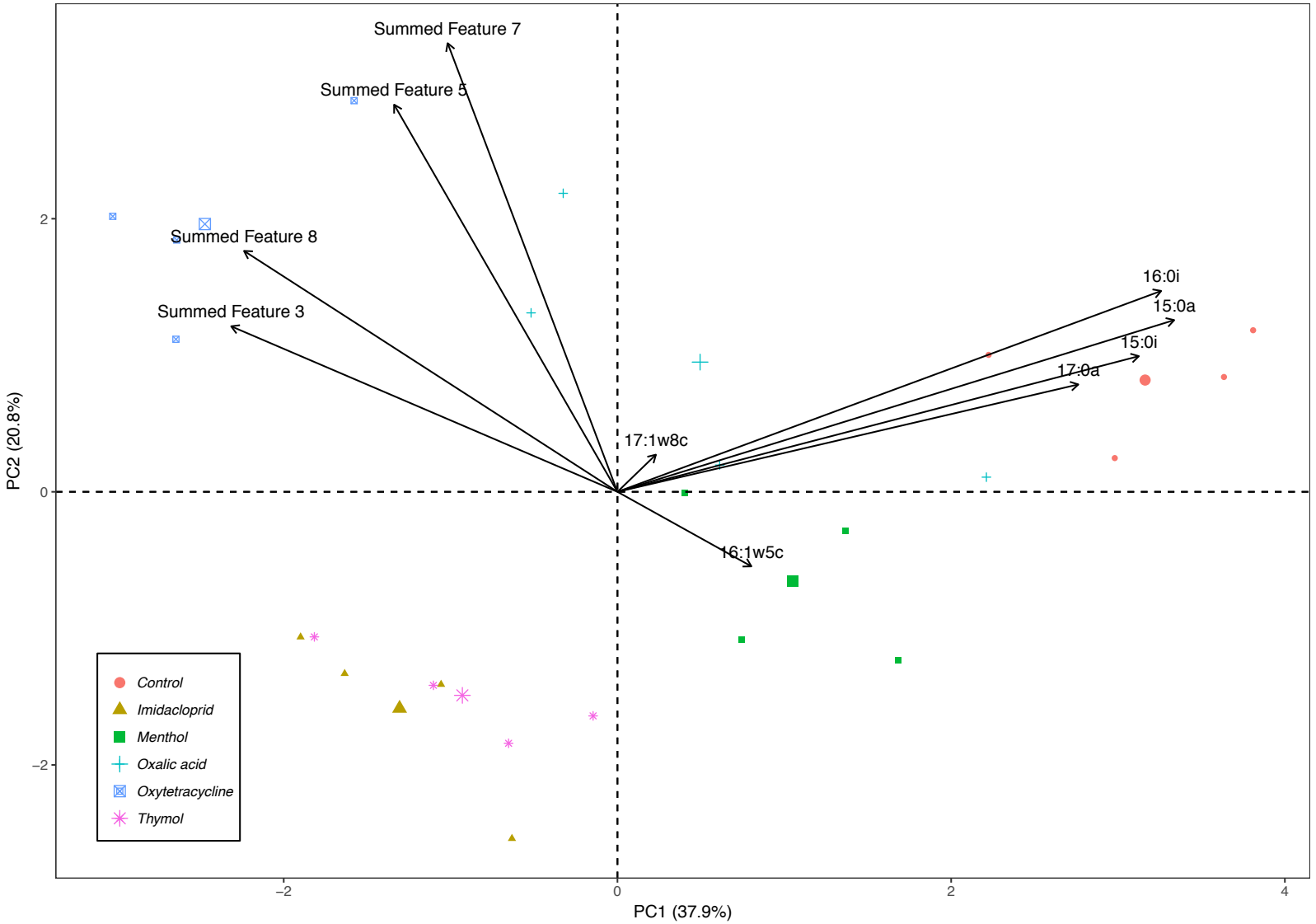
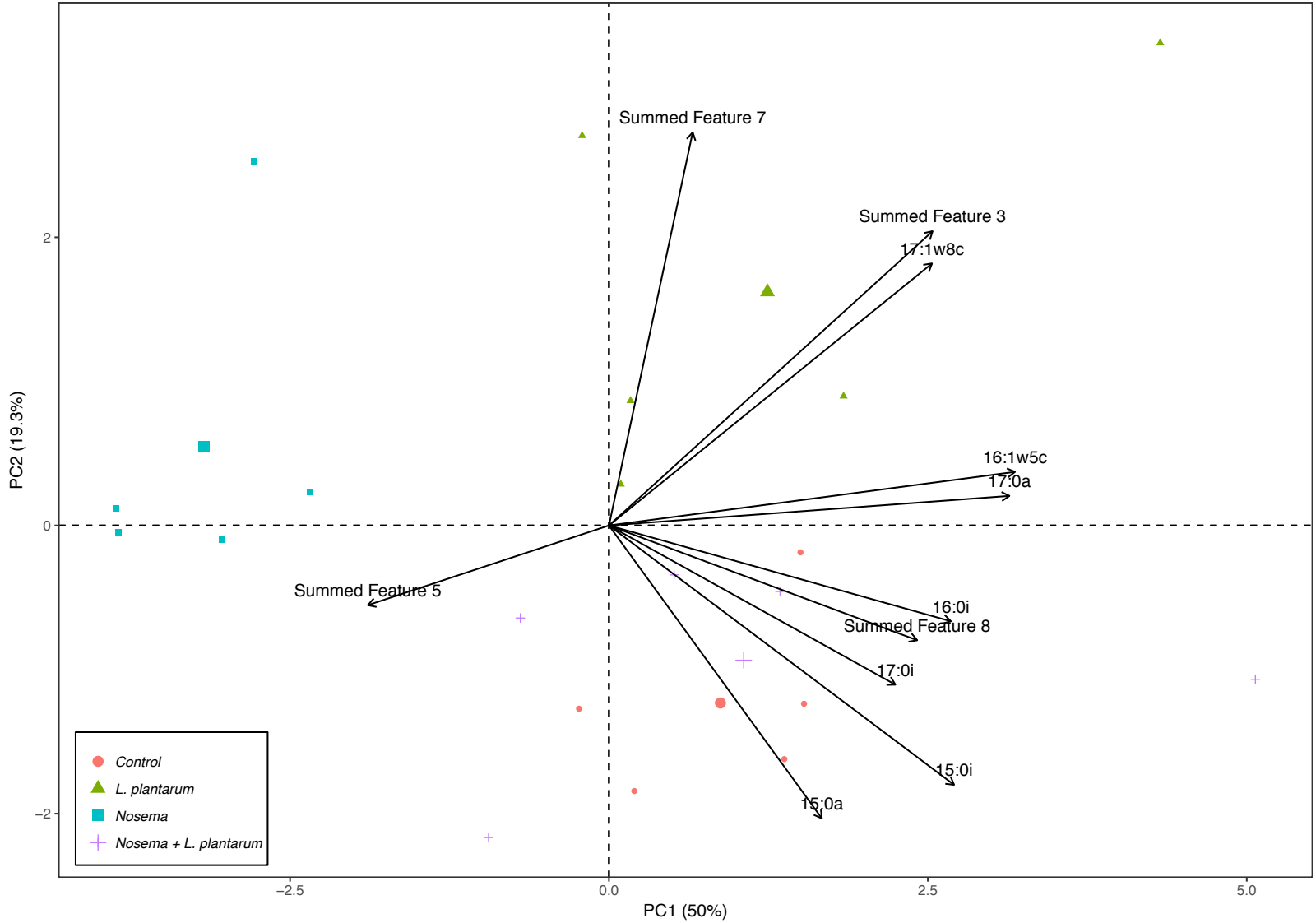


Figure 4 Principal component analysis of honey bee microbial community shifts caused by *Nosema* and *L. plantarum.*



DISCUSION GENERAL Y CONCLUSIONES

Los resultados de esta investigación mostraron la sensibilidad de los microorganismos asociados a las abejas a diversos compuestos utilizados por los apicultores en el tratamiento de enfermedades, al insecticida imidacloprid y a *Nosema* spp. Además, se mostró el potencial del lactobacilo *L. plantarum* como agente de control biológico para mitigar los efectos de la nosemosis.

Debido a la resistencia que han generado algunos patógenos de abejas a tratamientos sintéticos (González-Cabrera et al., 2018), se ha propuesto el uso en rotación de compuestos alternativos como; ácidos orgánicos y aceites esenciales, los cuales son considerados como seguros para las abejas y efectivos en el tratamiento de sus enfermedades (Imdorf et al., 1999, Calderone et al., 1997 y Charriére et al., 2002). Sin embargo, estos también han demostrado tener efectos bactericidas y fungicidas (Pattnaik et al., 1997, Didry et al., 1993 y Kwak et al. 2016). El ácido oxálico uno de los tratamientos más utilizados para el control de la varroa demostró ser uno de los compuestos con mayores efectos de inhibición sobre las cepas lactobacilos únicamente superado por el antibiótico oxitetraciclina, esto debido a que muestra actividad antimicrobiana a concentraciones de apenas 500 mg/L (Kwak et al. 2016) mucho menor a la concentración comúnmente utilizada para el tratamiento de la varroa 30 g/L (Higes et al., 1999), a pesar de esto a nivel de comunidades el ácido oxálico fue uno de los compuestos con menores efectos negativos. Como se esperaba, el antibiótico oxitetraciclina mostro los mayores efectos de inhibición tanto en las comunidades de microorganismos como en las cepas de lactobacilos, interesantemente algunos de estos grupos mostraron resistencia al antibiótico incluso

incrementando sus poblaciones, posiblemente debido a la resistencia que han generado estos por el uso indiscriminado para el tratamiento de enfermedades bacterianas en abejas (Elzen et al., 2002).

El timol, otro de los compuestos alternativos considerado como seguro para la salud de las abejas (Charpentier et al., 2014) causo alteraciones en las comunidades de microorganismos del intestino de las abejas, la aplicación de este es por vaporización. Sin embargo, cuando se utiliza de manera prolongado se acumula en el polen y la miel (Bogdanov, 2006), contaminándola y exponiendo a los microorganismos asociados a las abejas. El tercer compuesto alternativo para el tratamiento de enfermedades fue el mentol, el cual demostró ser el más seguro tanto para las comunidades como para las cepas de lactobacilos haciéndolo una opción viable de tratamiento. Sin embargo, el efecto en el control de parásitos es pobre (Gashout and Guzmán-Novoa, 2009) y requiere estar en solución con otros aceites esenciales .

Los tratamientos con imidacloprid causaron alteraciones cuantitativas y cualitativas en los biomarcadores para bacterias Gram positivas y Gram negativas mientras que los biomarcadores de hongos permanecieron estables, lo cual creemos puede causar alteraciones en las complejas interacciones microbianas actuando en sinergia con hongos patogénicos como Nosema aumentando la susceptibilidad de las abejas a estos (Alaux et al., 2010). Nosema uno de los parásitos más destructivos para las abejas también causo alteraciones en la microbiota, principalmente incrementando el biomarcador para hongos (18:2ω6,9c) posiblemente debido a la colonización de hongos oportunistas o por la misma colonización del patógeno (Moser et al., 1996; Grubss et al., 2015; Ptaszyńska et al., 2016). *Lactobacillus plantarum* es uno de los microorganismos más abundantes asociados a las abejas (Daisley et al., 2017), es conocido por promover la activación del sistema inmune

(Evans y Lopez, 2004) y por su capacidad de producción de ácido láctico, modificando el medio donde este se desarrolla, evitando así la colonización de patógenos (Wilson et al., 2005). También es conocido por la capacidad de recolonización del intestino de abejas que expuestas a antibióticos (Storelli et al., 2011). En este trabajo *L. plantarum* ayudo a mitigar los efectos causados por el patógeno Nosema en el intestino de las abejas, por lo cual los resultados indican un potencial como agente de control. Además, gracias a su capacidad de recolonización, este puede ser considerado como un agente de restauración de microbiota de abejas enfermas.

La mayor parte de los compuestos evaluados en este trabajo provocaron alteraciones en las comunidades de microrganismos asociados a las abejas, lo cual podría tener repercusiones en los mecanismos de defensa de las abejas ante patógenos y en la nutrición de estas, afectando la salud en general. El manejo de las comunidades de microorganismos asociadas a estas es vital para el correcto desempeño de las abejas, por lo que es necesario evaluar el efecto de los compuestos a los que estas se ven expuestas y seleccionar aquellos que sean menos agresivos para el tratamiento de sus enfermedades, sugerimos evitar el uso de estos compuestos en solución con azúcar para evitar que las abejas lo ingieran y entre en contacto con los microorganismos, haciendo otras formas de aplicación como la vaporización más seguras (Toufailia et al., 2015) para las comunidades de microorganismos. Aunado a esto, creemos es importante explorar alternativas para la restauración de la microbiota mediante cultivo e inoculación de bacterias benéficas aisladas de abejas sanas.

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