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POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

MICROBIOME CHARACTERIZATION OF THE ESCAMOLERA ANTS UNVEILS MICROORGANISMS WITH HIGH BIOTECHNOLOGICAL POTENTIAL

Tesis que presenta

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Resumen

La caracterización del microbioma de la hormiga escamolera muestra que existen microorganismos con alto potencial biotecnológico

Los insectos se han adaptado a diversos ecosistemas a través de la versatilidad de sus hábitos de alimentación y su microbiota intestinal juega un papel clave en la canalización de los procesos biológicos contra las perturbaciones ambientales. La hormiga *Liometopum apiculatum* habita en ecosistemas áridos y semiáridos caracterizados por una escasez relativa de alimentos, en comparación con los ecosistemas tropicales. No obstante, esta hormiga ha logrado un equilibrio ecológico para sobrevivir, estableciendo diversas interacciones simbióticas con otros insectos y plantas. Sin embargo, la relación simbiótica con microrganismos aún se desconoce. Por lo tanto, el objetivo de esta investigación fue caracterizar los simbiontes microbianos asociados tanto a las larvas de *L. apiculatum* como a las hormigas reproductoras adultas usando herramientas como la metagenómica, culturómica y microscopia. El *Phylum* más abundante identificado por secuenciación en las larvas fue Firmicutes, mientras que en las hormigas adultas fue Proteobacteria. En ambas etapas se observó una gran población de bacterias fijadoras de nitrógeno. Por otro lado, el enfoque culturómico confirmó la existencia de 19 bacterias previamente identificadas por secuenciación, que junto con la caracterización funcional revelaron un papel importante de las bacterias intestinales en la descomposición de polisacáridos complejos y la liberación de nutrientes almacenados en materiales derivados de plantas. Además, se detectaron bacteriocitos con bacterias intracelulares, indicando un soporte nutricional a través de este sistema para el metabolismo de las hormigas. Estos resultados destacan la importante contribución de los simbiontes microbianos en la alimentación y la supervivencia de las hormigas en su ecosistema, y además, exponen una fuente prometedora de enzimas para posibles aplicaciones biotecnológicas.

PALABRAS CLAVE: MALDI-biotyping, Gen *16S rRNA*, Bacteriocitos, Enzimas lignocelulíticas

Abstract

Microbiome characterization of the escamolera ants unveils microorganisms with high biotechnological potential

Insects have adapted to several ecosystems through the versatility of their feeding habits, wherein their intestinal microbiota has played a key role in the biological processes canalization. *Liometopum apiculatum* ant is widely distributed in arid and semi-arid ecosystems where a relative shortage of food can be appreciated, compared to tropical ecosystems. *L. apiculatum* has achieved an ecological equilibrium for surviving notwithstanding environmental factors, establishing diverse symbiotic interactions with other insects and plants. However, the symbiotic relationship with microorganisms is still unknown. Therefore, the aim of this research was to characterize the microbial symbionts associated with *L. apiculatum* larvae and the reproductive adult ants using metagenomics, culturomics, and microscopic approaches. The most abundant *Phylum* identified by sequencing in the larvae was Firmicutes while in adult ants was Proteobacteria. Nonetheless, in both stage a large population of nitrogen-fixing bacteria was observed. On the other hand, the culturomics approach confirmed the existence of 19 bacteria previously identified by sequencing, which accompanied by functional characterization, revealed an important role of gut bacteria in the complex polysaccharides breakdown and releasing nutrients stored in plant-derived materials. Moreover, bacteriocytes with intracellular bacteria were detected, indicating a nutritional support through this system to ant's metabolism. This finding highlights the important contribution of the microbial symbionts in the feeding and surviving of ants. On the other hand, the metabolic versatility of microbial symbionts showed a promising source of enzymes for potential biotechnological applications.

KEYWORDS: MALDI-biotyping, *16S rRNA* gene, Bacteriocytes, Lignocellulolytic enzym

Background

Insects represent the largest animal biomass on the planet, it is estimated that from 1.4 million species of animals described in the world about one million are insects (van Huis et al., 2013). Insects carry out many key ecological functions such as pollination, disseminate seeds, and organic matter breakdown (Losey & Vaughn, 2006). Moreover, they form part of the human diet in many tropical countries. It has reported at least 1681 insect species are edible throughout the world (van Huis et al., 2013). They are an excellent nutrient source such as proteins, carbohydrates, fiber, minerals, and vitamins. Indeed, insects' consumption turns out to be better than the conventional diet regarding some nutritional components recommended in daily intake by FAO/WHO (van Huis et al., 2013).

In Mexico, insect consumption is a traditional food since prehispanic times, in fact, there exist around 549 edible insect species (van Huis et al., 2013). *Liometopum apiculatum* named as escamolera ant is one of the species of ecological, nutritional, and economic importance. This ant is consumed at their larvae stage of development (escamoles) and is known as the "Mexican Caviar" because of their exquisite flavor, so it has become a gourmet dish with a high price (200 US Dlls/Kg) (Ramos-Elorduy, 2006). Moreover, escamoles are an excellent nutrients source such as proteins (39.67%), fat (36.87%), carbohydrates (19.22%), and vitamins (37.3 mg) (Ramos-Elorduy, Costa Neto, Cuevas Correa, García-Figueroa, & Zentina, 2007).

L. apiculatum is distributed in the arid and semi-arid ecosystems, where it build their nests near *maguey* and *nopal* plants modifying the soil's biological, physical, and chemical properties to create advantageous microhabitats (Cruz-Labana et al., 2014). According to its feeding behavior, this ant has been described as an omnivorous insect. Its diet has been mainly associated with sugar solutions such as honeydew and floral nectar obtained by trophobiosis from insects and plants (Velasco et al., 2007), but it has also linked with seeds, pollen, fruits, plants, insect's pupae, crustaceans, annelids, mollusks, and animal droppings (García-

Herrera, Méndez-Gallegos, & Talavera-Magaña, 2010; Hoey-Chamberlain, Rust, & Klotz, 2013; Velasco et al., 2007).

L. apiculatum belongs to the Hymenoptera order, Formicidae family, and Dolichoderinae subfamily (Ward, Brady, Fisher, & Schultz, 2010). Ants' system of castes is formed by the workers and reproductives (drones and queens), whilst stages of their life cycle including egg, larva, pupa, and adult (**Figure 1.0**) (Ramos-Elorduy, Delage Darchen, Cuadriello Aguilar, Galindo Miranda, & Pino Moreno, 1984). The worker caste reproduction period is carried out the year around. By contrast, the reproductive caste only is carried out between March and April (Lara, Aguirre, Castillo, & Reyes, 2015). In this season local communities take advantage of this natural resource as an income source, collecting *escamoles* from the nests for selling. However, human-caused disturbance not only affects the ants' populations but also severely harm the ecosystems. Currently, there exists little knowledge of the managing and harvesting ants sustainably, taking into account manipulating forest vegetation or harvesting practices to increase, maximize or sustain populations of these ants. Therefore, knowledge regarding ecology and biology of *L. apiculatum* is a key point to develop strategies of conservation and management of these edible insects. Moreover, this information could contribute significantly not only in the food security but also in improving the nutritional features of these insects (Toledo & Burlingame, 2006).

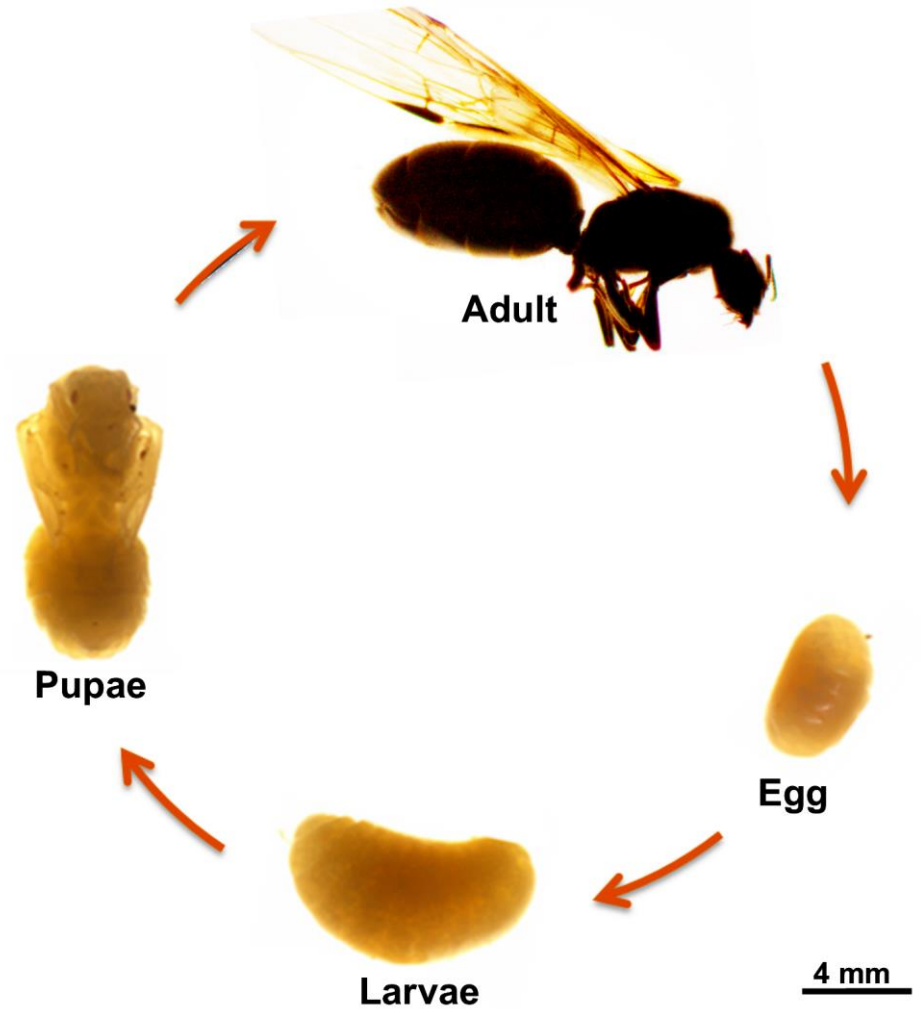

Figure 1.0 Reproductive caste life cycle from *Liometopum apiculatum*.

CHAPTER 1

Gut microbial characterization of the escamolera ant

1.1 Introduction

The world population will increase to 9 billion people by 2050, which will demand an increase of food production and currently innovative solutions to meet food security are needed. Insects are part of the diet of at least two billion people worldwide. However, from about one million of known insects only 1900 species are currently used as food (van Huis et al., 2013). Moreover, insects are ecologically sustainable because of their food conversion efficiency and growth rate. They use less natural resources, emit less green-house gases than conventional livestock, and produce protein more efficiently as compared with mammalians (van Huis, 2011). In many countries, insects are considered as delicacy and entomophagy, the practice of eating insects, has been extended in Western societies (Jansson & Berggren, 2015; van Huis, 2011). This suggest that insects are a good alternative to conventional livestock farming (Jansson & Berggren, 2015). However, little is known regarding the microbial relevance in the nutritional ecology of insects (Douglas, 2009).

Larvae of *L. apiculatum* ants, known in Mexico as "*escamoles*", are consumed since prehispanic times because of their nutritional and organoleptic characteristics, escamoles are recognized as a Mexican delicatessen (Ladrón de Guevara, Padilla, Garcia, Pino, & Ramos-Elorduy, 1995; Melo-Ruiz, Quirino-Barreda, Calvo-Carrillo, Sánchez-Herrera, & Sandoval-Trujillo, 2013). *Escamoles* have high protein content (39.67%) with a high ratio of essential amino acids; they are rich in essential lipids such as oleic (67.66%), linoleic (2.61%), and arachidonic (0.16%) acids. Vitamins (A and E), thiamin, riboflavin, and niacin are also present in these larvae (Melo-Ruiz, Quirino-Barreda, Díaz-García, & Gazga-Urioste, 2016). This nutrient richness present in *escamoles* is interesting because the ants live in arid and semiarid zones with relative food shortages. Thus it is possible that the *escamolera* ant is associated with diverse microorganisms to address specific imbalances of some nutrients as in other insects have been reported (López-

Sánchez et al., 2009; Warnecke et al., 2007. However, to our knowledge, there is no information regarding the microorganisms associated with *L. apiculatum*.

In other insects, the valuable nutritional properties have been attributed to a high efficiency of their endosymbionts (microorganisms living inside the host) that assist insects in the food management through the production of a broad array of enzymes responsible of plant lignocellulose degradation, reductive acetogenesis, and nitrogen recycling and fixation (Brune, 2014; Engel, Martinson, & Moran, 2012; López-Sánchez et al., 2009; Warnecke et al., 2007). The gut endosymbionts are mainly responsible for the production of metabolites such as essential amino acids, vitamins, and fatty acids that compensate the severe nutritional deficits of insects' diet (Brune, 2014; Burnum et al., 2010; Fan, Thompson, Dubois, Moseley, & Wernegreen, 2013; López-Sánchez et al., 2009; Sapountzis et al., 2015; Warnecke et al., 2007). While ectosymbionts, microorganisms living outside the host, mainly produce antimicrobial compounds as part of the host defense system (Barke et al., 2010; Santos, Dillon, Dillon, Reynolds, & Samuels, 2004).

Metagenomic approach has revolutionized the understanding of the microbiome of several organisms. Recent studies have used the *16S rRNA* gene sequencing to explore ants intestinal microbiota (Kautz, Rubin, Russell, & Moreaua, 2013). Results have shown an extensive relationship with nitrogen-fixing bacteria (Russell et al., 2009; Sapountzis et al., 2015). However, the advances in sequencing technologies have generated gaps corresponding to the unidentified sequences (Lagier et al., 2015). Therefore pure strains remain the key to identify the role of these bacterial endosymbionts and hence the traditional microbiology based on the growth of bacteria in culture medium has resurged as new research area named culturomics, which also is used to validate the metagenomic data (Hamad et al., 2017; Lagier et al., 2016). Culturomics uses the Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) for the identification of large number of isolated bacteria improving the microbiota characterization (Tandina et al., 2016). Other efforts have been carried out through microscopic and proteomic approaches, which have helped to understand the functional contribution of *Blochmannia*, an intracellular endosymbiotic bacteria of

Camponotus or carpenter ant (Fan et al., 2013; Stoll, Feldhaar, Fraunholz, & Gross, 2010). All those works have hypothesized that microorganisms are a system of nutrient supply for ants, which are linked to the essential amino acids biosynthesis, beginning with the recycling of waste nitrogen or the atmospheric nitrogen fixation. The microbial importance in insect nutritional ecology is a key tool to preserve their nutritional quality by the development of appropriate conservation and management strategies. However, most of the works with insects are focused in a narrow range of model organisms, such as bees and termites (Engel et al., 2012; Warnecke et al., 2007).

Hence, the aim of this research was to characterize the gut endosymbionts associated with *L. apiculatum* larvae and reproductive adult ants through amplicon-based metagenomics and culturomics approaches. The work was complemented with microscopic analyses to gather the knowledge about the location and possible function of the escamolera ant endosymbionts.

1.2 Material and methods

1.2.1 Collection site and conditions for obtaining bacterial samples

L. apiculatum larvae and adult ants were collected at Pocitos-Charcas, San Luis Potosí, Mexico (latitude 23°09′41.60″ N and longitude 100°58′08.94″ W). Ecosystem is characterized by the scarcity of flora, being cacti and agave the predominant species (**Figure 1.1**). Biological triplicates of larvae and adult ants were collected from three different colonies at distances of approximately 5 km from each other. Each replicate was prepared from a representative sample (pool) with at least 50 larvae (18–25 days of age) and 50 adult ants (60–70 days of age). Samples were collected directly from their nest, placed into plastic bags and transported in ice to the laboratory (Suen et al., 2010).

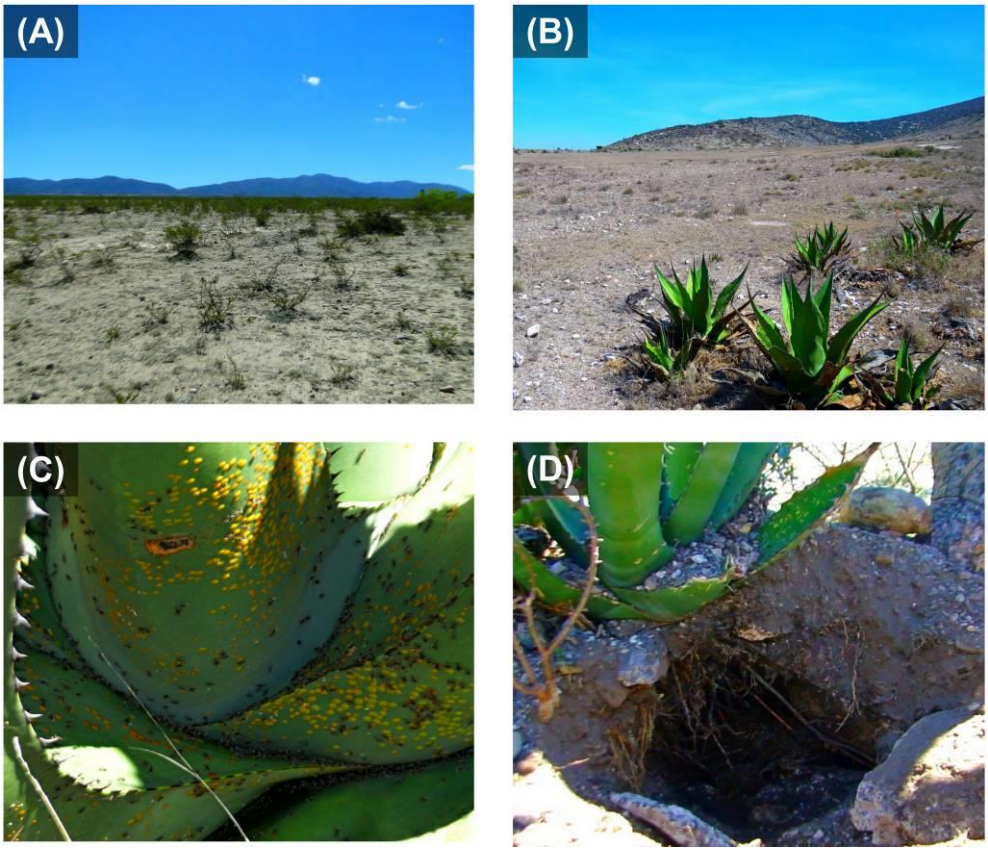

Figure 1.1 Images of *L. apiculatum* ant ecosystem. A) and B) general images of the *L. apiculatum* niche. C) Agave plant where adult ant interacts with other insects. D) Ants' nest located in the roots of the agave plants.

Samples were surface sterilized through continuous washes with 70% ethanol solution, 15% sodium hypochlorite (5.25% solution), sterile water, and sterile phosphate-buffered saline (PBS) pH=7.4. The final PBS wash was plated on the Brain Heart Infusion (BHI) medium as a negative control. The complete larvae and the guts of adult ants, aseptically dissected, were macerated and crushed with a pestle and mortar using PBS. Samples were divided in two fractions: one was used for microbial DNA extraction and the other for bacteria isolation in different culture media.

1.2.2 Total DNA extraction, *16S rRNA* gene sequencing, and analysis

Microbial cells were extracted by differential centrifugation, as described in (Engel et al., 2012) with some modifications. Crushed larvae and ant samples were passed through a succession of filters and centrifuged at 1,935×g for 15 min at 4 °C. Samples were washed to remove proteins and fat using a solution of 1% Triton X-100 and subsequently eluted in TE buffer (10 mM Tris, 1mM EDTA, pH=8.0). Differential centrifugation was carried out by discontinuous Percoll gradient. Two Percoll concentrations (40% and 80%) were prepared in 10 mM $MgSO_4$, 0.01% bovine serum albumin, 0.01% Ficoll, 0.1% polyethylene glycol 4000, and 0.086% sucrose, pH=7.5. Density gradients were made in 50 mL tubes with a bottom layer of 80% Percoll solution (10 mL) and a top layer of 40% Percoll solution (10 mL). The cell suspension (5 mL) was placed at the top and centrifuged (39,191×g, 30 min, 4 °C). Microbial cells were collected from the Percoll interface and washed with a solution containing 0.3 M mannitol, 1mM EDTA, and 10 mM phosphate buffer, pH 7.5. Microbial cell pellets (200 mg) were processed to obtain total genomic DNA using the UltraClean Microbial DNA isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA) following the manufacture's protocol. Extracted DNA was visualized using agarose gels (1%) and quantified with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA sequencing (larvae and ants triplicates) was carried out through paired-end tags assay (2×250) using the Illumina MiSeq platform at Research and Testing Laboratory (Lubbock, TX, USA). Microbial libraries were constructed amplifying the V3-V4 variable region

of the *16S rRNA* genes with primers 357wF (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC) (Dannemiller, Weschler, & Peccia, 2017). Raw sequences were quality-filtered and merged with PEAR software v0.9.6 (Zhang, Kobert, Flouri, & Stamatakis, 2014) using a cut-off of 0.01 (*P*-value). A minimum sequence length of 200 bp and a minimum average quality of 25 (Phred score) were taken as the quality criteria (Kautz et al., 2013). The redundant sequences were removed in USEARCH v7.0 to yielding data set with only unique representative sequences and chimeras were excluded using UCHIME v4.2 (Edgar, 2013; Edgar, Haas, Clemente, Quince, & Knight, 2011). Clustering into operational taxonomic units (OTUs) of sequences was performed with a 3% divergence threshold in USEARCH v7.0 (Edgar, 2013). The representative OTUs were taxonomically assigned using RDP I (RDP Classifier) and RDP II (MG-RAST) (Lan, Wang, Cole, & Rosen, 2012). Non-bacterial and unknown sequences were removed.

1.2.3 Culturomics approach

Bacterial samples were serially diluted (10^{-3} to 10^{-6}) before plating on different growth media (Difco Laboratories, Detroit, Mich): Blood Agar Base (BAB), Man Rogosa Sharpe (MRS), Brain Heart Infusion (BHI) and Sabouraud Dextrose Agar (SDA). The solid cultures media were incubated at 35 °C under aerobic, anaerobic, and microaerophilic conditions using GasPak EZ systems (Becton, Dickinson and Company, Sparks, MD, USA). Triplicates of each growth media/condition/ collection site for each larvae and adult ants were carried out. Bacteria were isolated until the colonies were visible however plates were incubated up to one month to isolate those bacteria with slow growth. Visible colonies were individually spread onto growth media and eventually transferred on new plates until pure cultures were obtained. Bacterial isolates were characterized and screened by their colony morphology and cell morphology using Gram staining. Subsequently, individual colonies were stored at −20 °C in 30% glycerol.

The selected strains were taxonomically identified by MALDI-TOF MS coupled to Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). All

samples were prepared as recommended by the manufacturer Standard Operating Procedure (Gekenidis, Studer, Wüthrich, Brunisholz, & Drissner, 2014). A single colony was inoculated onto a 1 mL fresh BHI medium for 18–24 h at 35 °C until OD_{600} of 1 was reached. After centrifugation at 16,200×g, pellet was resuspended in 1.2 mL of 75% ethanol (Sigma-Aldrich) by mixing thoroughly using a vortex device. Sample was centrifuged for 2 min as above and pellet was resuspended in 50 µL of 70% formic acid and 50 µL of acetonitrile (Fisher Scientific) and mixed thoroughly using a vortex device. Another centrifugation (16,200×g for 2 min) was done and 1 µL of the protein containing supernatant was collected, applied on a metal plate, and allowed to dry at room temperature. The dried mixture was overlain with 1 µL of matrix solution (α-cyano-4-hydroxycinnamic acid [HCCA]; Bruker Daltonics) dissolved in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid (Fisher Scientific) and allowed to dry prior to analysis. A bacterial test standard (BTS; Bruker Daltonics), was used for instrument calibration. The metal plate with the samples was placed in a MALDI chamber for analysis. Automatic measurements of the spectrum and a comparative analysis with reference spectra of bacteria were performed using the AutoFlex III mass spectrometer and MALDI-Biotyper 3.0 software with library version 3.3.1.0 (4613 entries). Manufacturer- recommended cut-off scores were used for identification, with scores ≥2.0 indicating identification to the species level, scores between 1.7 and 1.9 indicating identification to the genus level, and scores <1.7 indicating no identification.

1.2.4 Microscopic analysis

Four males and four queens were used for the microscopic characterization. The guts were carefully removed from the ant's abdomen under aseptic conditions using a Zeiss SteREO Discovery V8 microscope (Carl Zeiss, Inc., Oberkochen, Germany), fixed with glutaraldehyde (4%), and stored at 4 °C. For Transmission Electron Microscopy (TEM) analysis, guts were washed three times with phosphate buffered saline (PBS) pH=7.4, immersed in a 5% sucrose solution, and frozen under liquid nitrogen. Ultrathin sections were obtained by cryo-freezing using a

RMC PowerTome PT-PC cryo-ultramicrotome (RMC, Tucson, AZ, USA), contrasted with uranyl acetate and observed on a TEM JEOL 200CX (JEOL USA Inc., Peabody, MA, USA). For Scanning Electron Microscopy (SEM) analysis, guts were fractured to expose the internal structures, washed (3×) with PBS pH=7.4 and fixed in osmium tetroxide solution (2%) for 1 h at 4 °C. Samples were dehydrated through increasing ethanol concentrations and dried with CO_2 using Samdri PVT-3B critical-point dryer (Tousimis Research Co, Rockville, MA, USA). Samples were coated with gold particles (8 nm) and observed on a SEM FEI Quanta 200 (FEI Co., Eindhoven, Netherlands). For Transmitted Light Microscope (TLM) analysis, a total of six whole-larvae were fixed with paraformaldehyde (10%) in PBS (pH=6.4), dehydrated through a series of ascending ethanol concentrations and embedded in paraffin. Sections of 7 µm were cut using a SLEE microtome (LIS Ltd., SLEE MAINZ, Germany), de-waxed overnight at 58 °C and rehydrated through an ethanol series to water. Samples were stained with hematoxylin and eosin and observed on a TLM Zeiss Axio Imager M2 (Carl Zeiss, Inc., Oberkochen, Germany).

1.2.5. Statistical analyses

Rarefaction curves and first order jack-knife method were applied to estimate the diversity of species in each sample by taking into account a bootstrap with 200 replications. Statistical analysis consisted of a normality assessment of data by Shapiro-Wilk test and a two-tailed *t*-test was employed to calculate the total diversity of species on larvae and adult ant, expressed as the mean ± standard error (SE), and considered as significant if $p < 0.05$. A Venn diagram was generated with the OTUs identified at the genus level and the Sorensen similarity index was estimated through multiple-community measures to assess the proportion of genera shared in each sample. To investigate the bacterial-orders dynamics between larvae and adult ants, a Principal Component Analysis (PCA) using MatLab Software (MathWorks Inc., Natick, MA) was carried out. Normalized data was used to calculate matrices using Euclidean distances as a measure of diversity and Ward's minimum variance approach for data similarity clustering. A

multinomial statistical model was used to classify the specific affinity and bacteria distribution between the larvae and adult ant. All analyses were done in R using iNEXT (Hsieh & Chao, 2016), Spade (Chao & Walther, 2003) and CLAM packages (Chazdon et al., 2011). Phylogenetic analyses were generated by multiple alignments of representative sequences with >10 OTUs contained in the total dataset of each stage of the ant. Maximum likelihood tree was built using the Kimura 2-parameter model in Mega 6.0. Circular phylogenetic tree was done using the online Interactive Tree of Life resource (Letunic & Bork, 2007).

1.2.6. GenBank accession numbers

Nucleotide sequences for *16S rRNA* gene libraries from microbial communities by assembling paired-end Illumina reads can be found under Accession Numbers: MG041386-MG041488, MG051725-MG052102, MG049505-MG049666, MG052351-MG052446, MG052447-MG052590 and MG052103-MG052350.

1.3 Results

The escamolera ants have adapted to arid and semi-arid environments using strategies that until now are not well known, such strategies have allowed them to survive under persistent stress caused by high temperatures or even food shortages. One of the escamolera ant's survival mechanisms could be attributed to their microbial symbionts. For this reason, the microorganisms associated with *L. apiculatum* larvae and adult ants were analyzed by using the *16S rRNA* gene sequencing. High-quality sequences (53,191 for larvae and 54,716 for adult ants) in all samples were recovered. Through a taxonomic classification, 26,394 and 24,867 sequences were obtained, for larvae and ants, respectively, which resulted in 646 and 493 OTUs corresponding for each data set. A Venn diagram was constructed to visualize the homogeneity and shared genera of these two groups, where a convergence of 416 OTUs in larval stage and 386 OTUs in the adult stage were found (**Figure 1.2 and Table 1.1**), that means that all replicas shared a high number of OTUs. The Sorensen similarity index was estimated for the composition of these bacterial communities, showing an average proportion of bacteria shared through all samples of 63% for larvae and 68% in the adult ants (**Figure 1.3**).

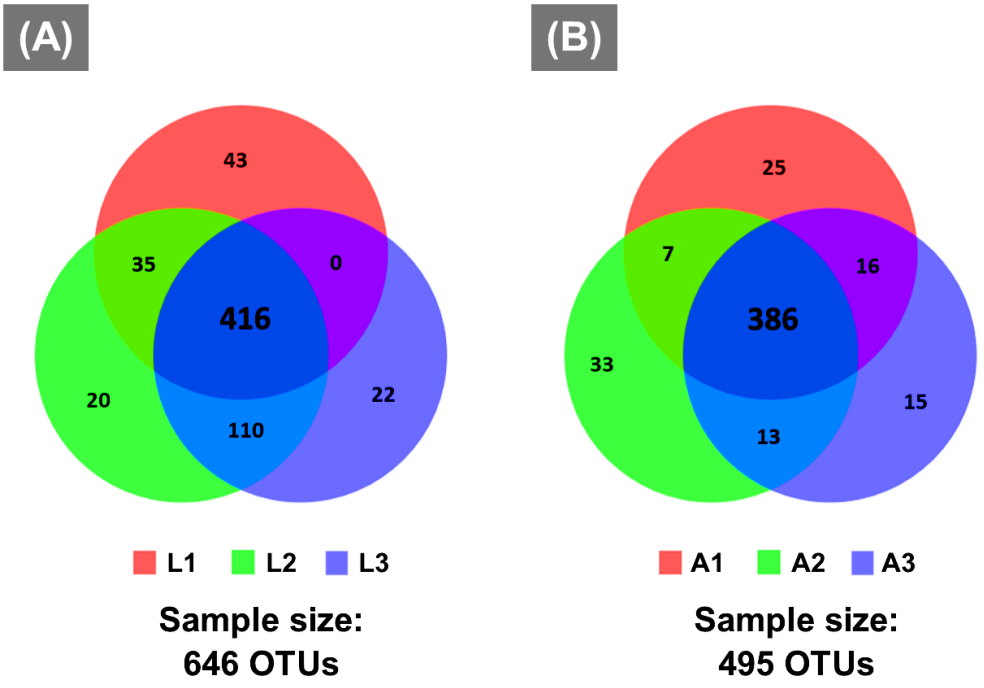

Figure 1.2 Venn diagram showing the overlapping of the OTUs at genus level of the taxa identified in the different biological samples of *L. apiculatum*. A) larvae; B) adult ant

Table 1.1 Subsets of overlapping genera found in different samples of *L. apiculatum*

Larvae							
Shared in L_1-L_2-L_3	Total OTUs	Shared in L_1-L_2	Total OTUs	Shared in L_1-L_3	Total OTUs	Shared in L_2-L_3	Total OTUs
Weissella	283	*Aeromonas*	16	0	0	*Lactococcus*	88
Leuconostoc	25	*Burkholderia*	5			*Caulobacter*	14
Stenotrophomonas	23	*Escherichia*	2			*Actinobaculum*	3
Macrococcus	16	*Micrococcus*	4			*Lactobacillus*	3
Serratia	16	*Propionibacterium*	5			*Herbaspirillum*	2
Enterococcus	13	*Serpens*	3				
Streptococcus	10						
Bacillus	9						
Pseudomonas	9						
Acinetobacter	6						
Adult ants							
Shared in L_1-L_2-L_3	Total OTUs	Shared in L_1-L_2	Total Otus	Shared in L_2-L_3	Total OTUs	Shared in L_2-L_3	Total OTUs
Arsenophonus	198	*Bacteroides*	5	*Barnesiella*	14	*Rhizobium*	3
Brevundimonas	55	*Lachnospiracea*	2	*Solirubrobacter*	2	*Geodermatophilus*	2
Pseudomonas	32					*Gp16*	2
Acinetobacter	12					*Methylobacterium*	2
Stenotrophomonas	12					*Micrococcus*	2
Exiguobacterium	10					*Steroidobacter*	2
Lactobacillus	9						
Sphingomonas	7						
Azomonas	6						
Flavobacterium	6						
Ralstonia	6						
Achromobacter	4						
Blastococcus	4						
Gp6	4						
Lactococcus	4						
Mesorhizobium	4						
Serratia	4						
Burkholderia	3						
Pelomonas	3						

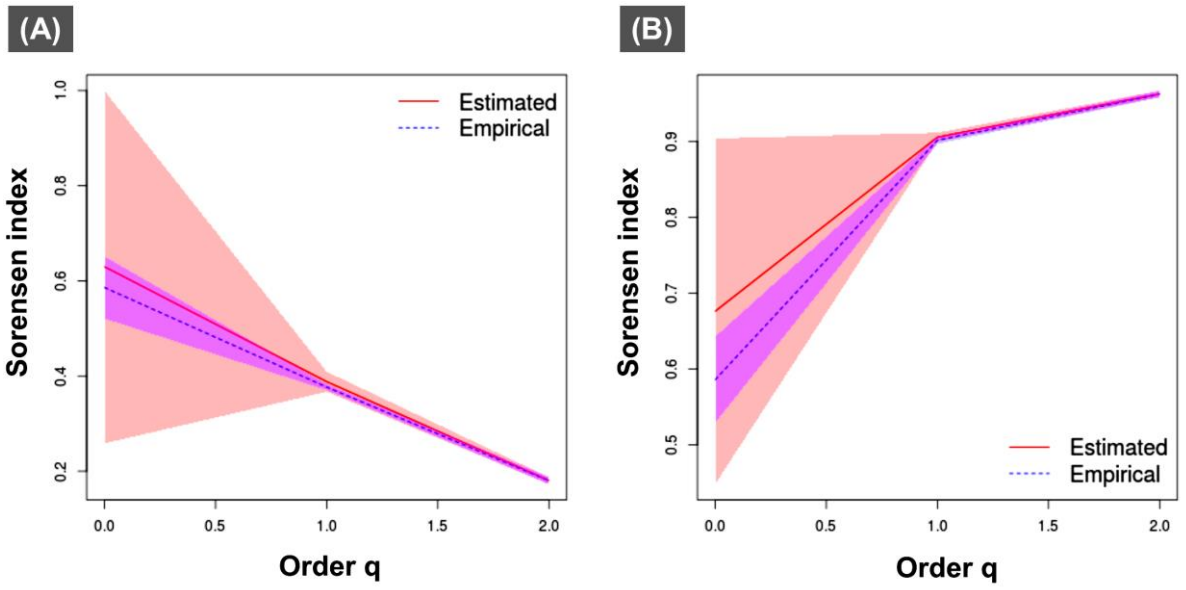

Figure 1.3. Sorensen index for compare the similarity and dissimilarity on the composition of bacterial communities at level of the genus among biological replicates of *L. apiculatum*. A) Larvae, B) Adult ants. The x-axis is the diversity order q in the Hill number, for q=0 (species richness), 1 (Shannon diversity), 2 (Simpson diversity). The Sorensen similarity index is equals the percentage of overlap in each assemblage.

These analyses showed the core of microorganism's composition for each stage of the *L. apiculatum* observed through their biological replicates. The approach to determine the total bacterial diversity present in *L. apiculatum* larvae and adult ants was based on modeling and extrapolation from the rarefaction curve prolonged to coverage of the highest size of 15,000 sequences to a fair comparison between samples (Chao & Chiu, 2016). Estimations of microorganism were 299 and 285 species in larvae and adult ant, respectively (*t*-test; t=0.321; p=0.764) (**Figure 1.4**). Comparatively, the first order jack-knife estimator revealed similar values (**Table 1.2**). However, both estimators did not show any significant difference with respect to the number of species between both larvae and adult ant.

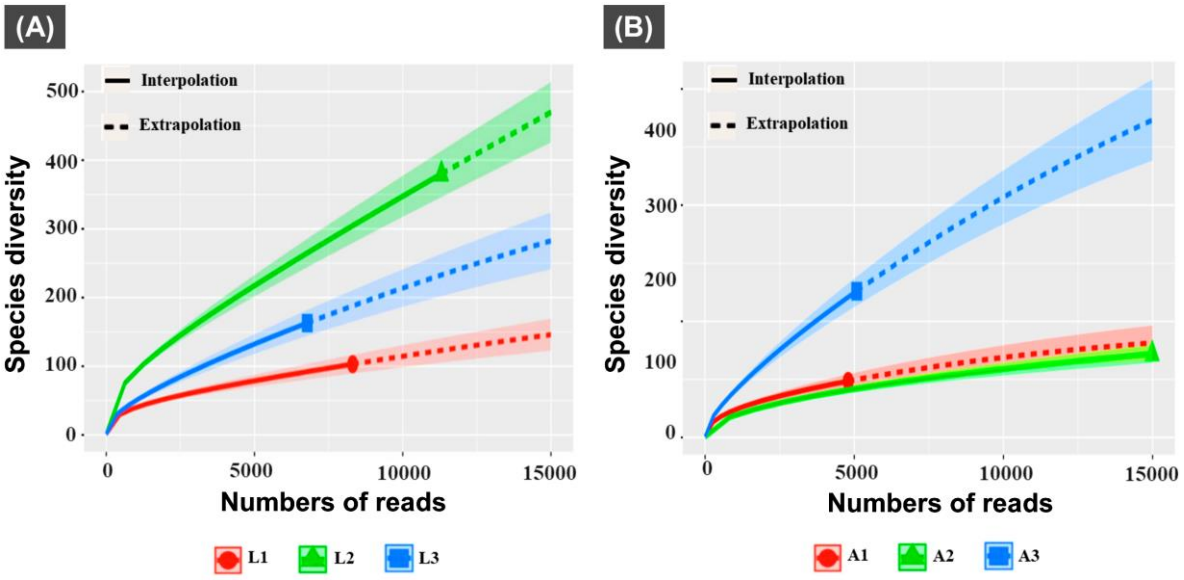

Figure 1.4 Species richness estimation through simple-size-based rarefaction and extrapolation sampling curves. A) Larvae, B) Adult ants. Based on a Bootstrap method with 200 replicates and an extrapolation increase to a maximum sample size of 15000 sequences for achieve a fair comparison of species richness between larvae and adult of *L. apiculatum*.

Table 1.2. Single diversity indices of larvae and adult ants from *Liometopum apiculatum*.

Sample		Richness estimators	
		Rarefaction*	1st order jackk-nife
Larvae	L_1	146	161
	L_2	469	660
	L_3	282	276
Adult	A_1	163	144
	A_2	144	218
	A_3	547	437

Two richness estimators were used to compare richness of the samples.*Sample-size-based rarefaction was computed with an extrapolation extends up to a maximum sample size of 15,000.

Taxonomic classification of the nucleotide sequences showed that at Phylum level Firmicutes (60%) were mainly presented in larvae, followed by Proteobacteria and Actinobacteria, while in adult ants, the 80% of sequences corresponded to Proteobacteria, the rest was composed by Actinobacteria, Firmicutes, and Bacteroidetes (**Figure 1.5A**). However, the most remarkable feature was observed through taxonomic level of order, where a distinctive bacterial pattern between samples was observed (**Figure 1.5B**). Significant differences in distribution of bacterial orders between larvae and adult ants were supported by principal component analysis (PCA). Two main components (PC1=45.88% and PC2=25.2%) described the total variance, showed that for larvae the main Order was Lactobacillales followed by Enterobacteriales, Aeromonadales, and Bacillales, while adult ants were characterized by Pseudomonadales, Sphingomonadales, Caulobacterales, Sphingobacteriales, and Flavobacteriales (**Figure 1.6**).

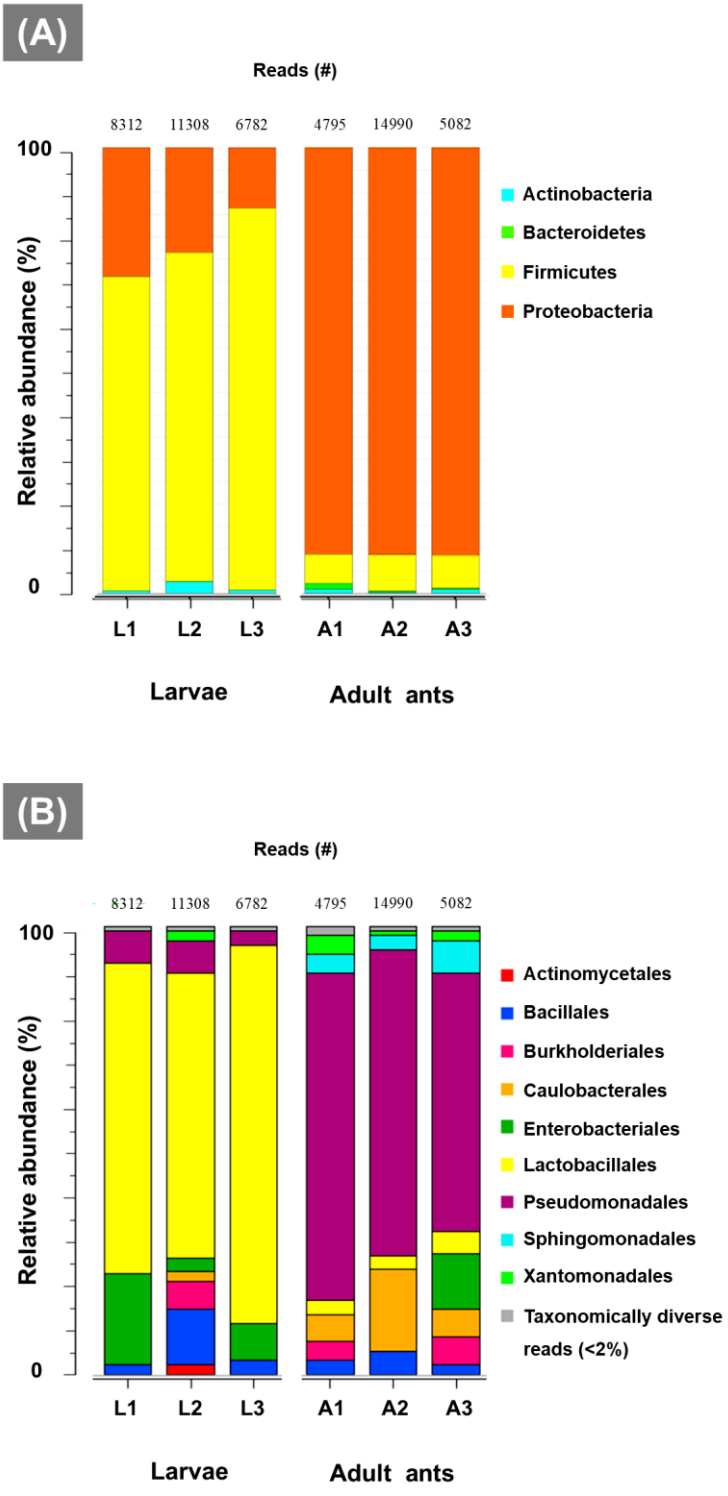

Figure 1.5 The microbial community identified in *L. apiculatum* larvae and adult ant A) at the taxonomic level of phylum B) at the taxonomic level of order. The relative abundance represents the operational taxonomic units recorded in each sample with a divergence of 3%.

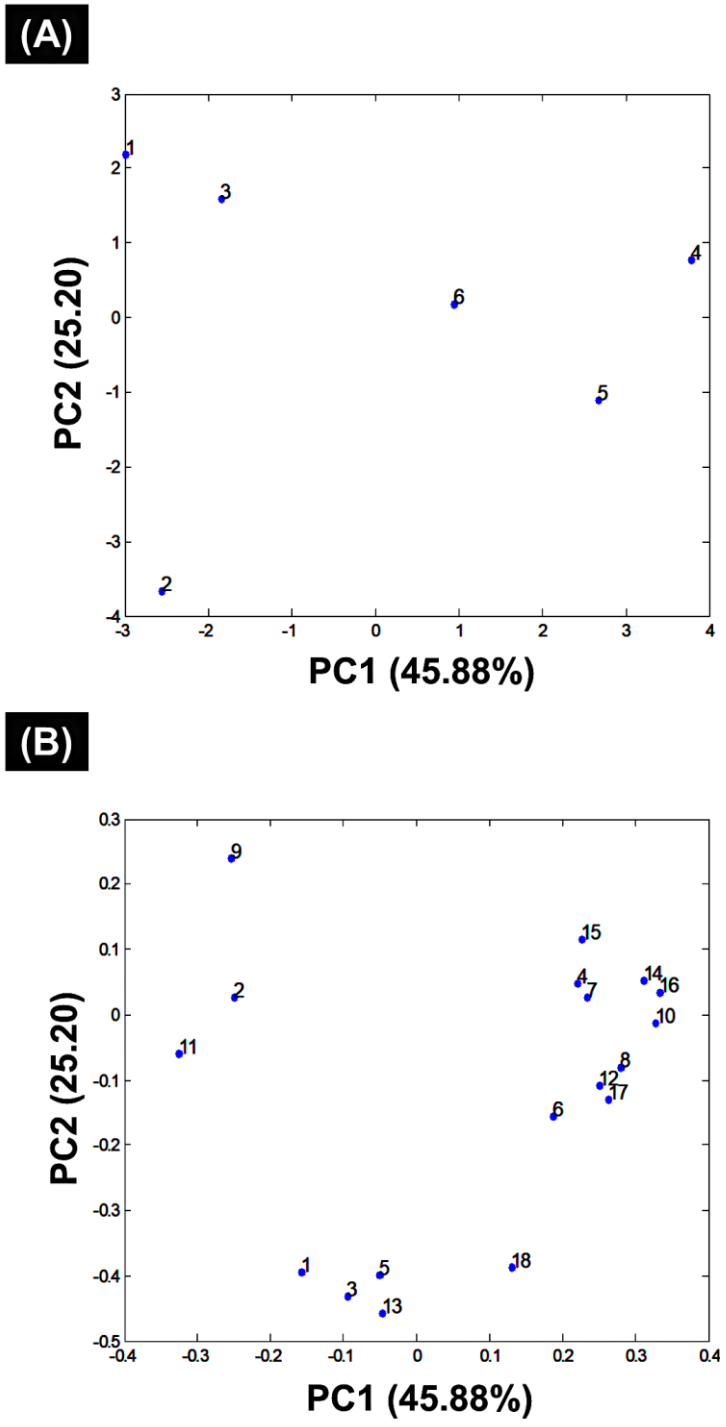

Figure 1.6 Principal component analysis (PCA) plot comparing bacterial orders associated with *L. apiculatum* larvae and adults ants. Score plots A) and loading plots larvae stage (objects 1-3) and adult ants (objects 4-6). B) Result of PCA for centered and standardized data, which rows represent studied the ant microbiome: Parameters: 1=Actinomycetales, 2=Aeromonadales, 3=Bacillales, 4=Bacteroidales, 5=Burkholderiales, 6=Caulobacterales, 7= Clostridiales, 8=Acidobacteria, 9=Enterobacteriales, 10=Flavobacteriales, 11= Lactobacillales, 12= Pseudomonadales, 13= Rhizobiales, 14= Rhodospirillales, 15= Solirubrobacterales, 16= Sphingobacteriales, 17= Sphingomonadales, 18= Xanthomonadales.

On the other hand, a molecular phylogenetic analysis based on a Maximum likelihood of the most abundant genera showed that larval stage is mainly dominated by a substantial number of lactic acid bacteria. By contrast, the adult ant is mainly dominated by a group of bacteria that has been widely related to nitrogen-fixing function (**Figure 1.7**).

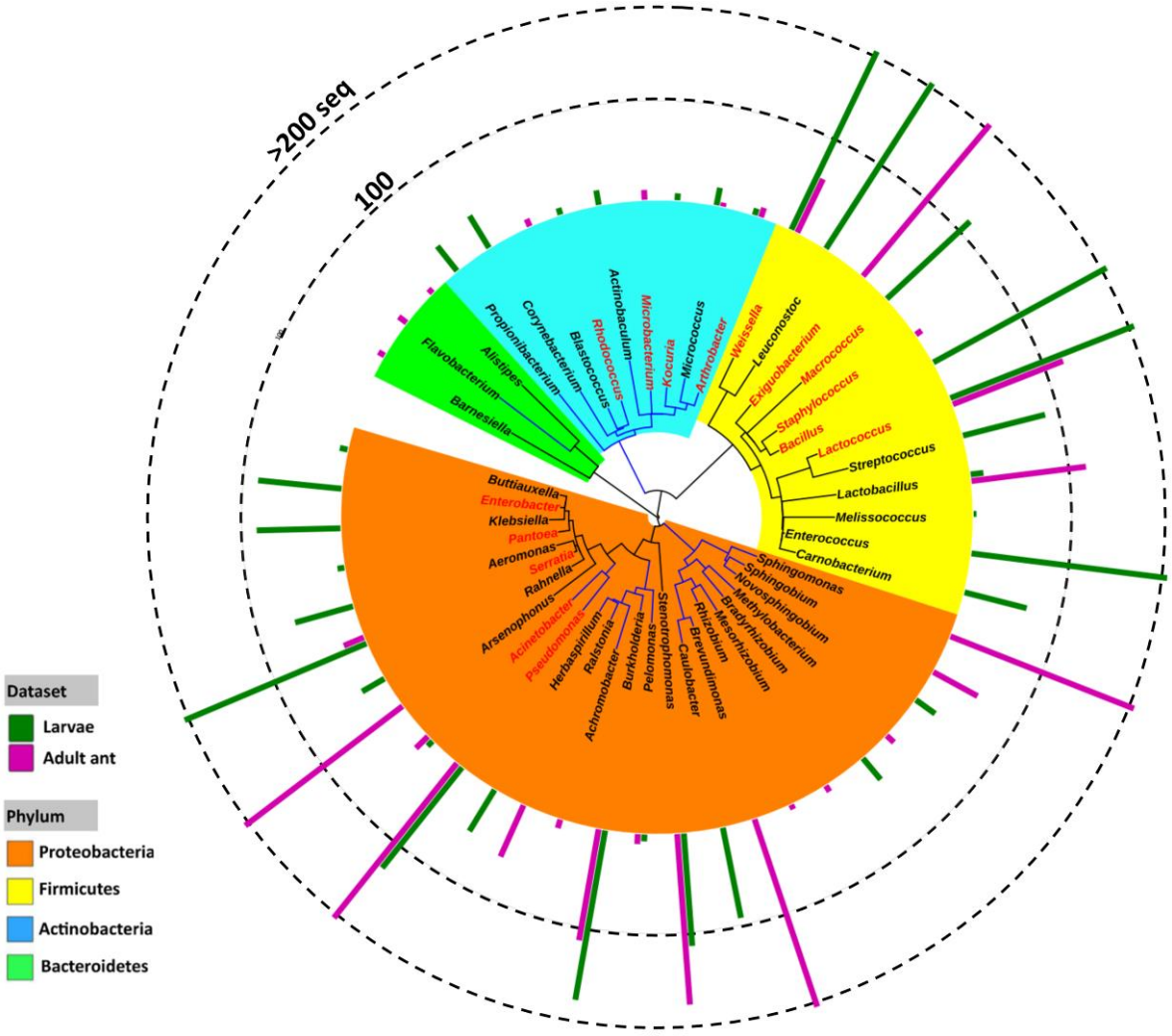

Figure 1.7 Phylogenetic tree of the bacteria found in the metagenomic analysis and bacteria isolated by culturomics. ML analysis was constructed using the alignment of *16S rRNA* gene sequences, based on a bootstrap method with 1000 replications. Distances were estimated using the Kimura 2-parameter model in Mega 6.0. The bacteria identified by Maldi-Biotiping are in red letters. Relative abundances of the microbial sequences can be observed through dashed black line. The blue branches shows the bacteria that have been previously reported with the nitrogen-fixing function.

Using a multinomial statistical model for bacterial classification, groups of generalist, specialist, and rare bacteria were identified in both, larvae and adult ants (**Figure 1.8**). Shared bacteria between larvae and adult ant were *Burkholderia*, *Pelomonas*, and *Stenotrophomonas*. In larvae mainly specialist bacteria such as *Enterococcus*, *Weissella*, *Lactococcus*, *Bacillus*, *Leuconostoc*, *Macrococcus* and *Serratia* were detected. In adult ants, the specialist bacteria were *Pseudomonas*, *Exiguobacterium*, *Sphingomonas*, *Sphingobium*, and *Brevundimonas*, *Arsenophonus*. The rare genera group was consistently classified by its low abundance (singletons and doubletons). These analyses allowed us to identify the exclusive and most abundant bacterial taxa in larvae and adult ant of *L. apiculatum.*

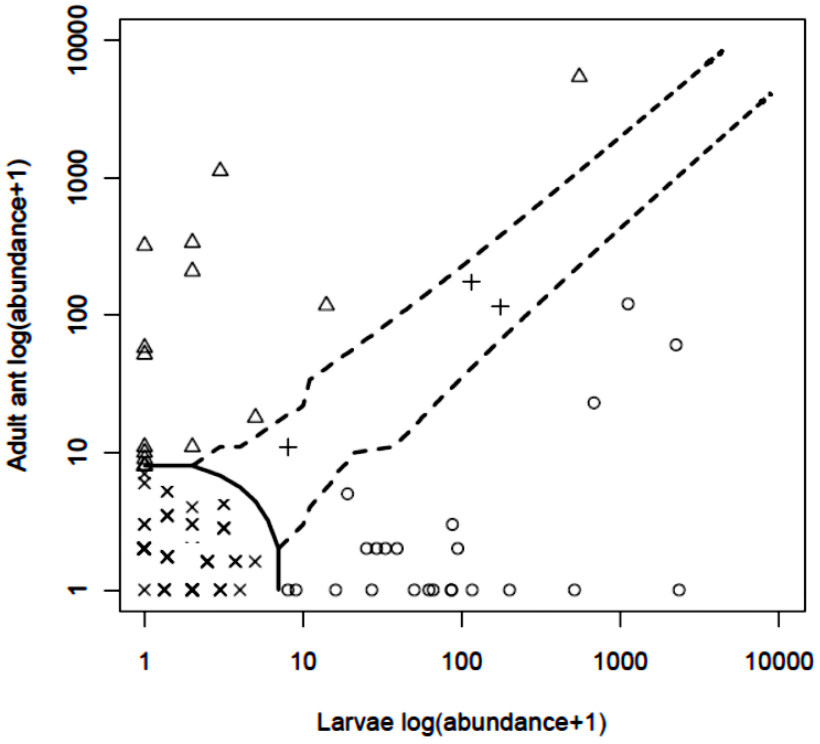

Figure 1.8 Analysis of bacterial genera classification in larvae and adult ants of *L. apiculatum*. Plot was based in the super majority specialization threshold (K=2/3, P=0.005). Sign of symbols: +=Generalist (*Burkholderia, Stenotrophomonas and Pelomonas*); o=specialist in larvae stage (*Enterococcus, Weissella, Lactococcus, Bacillus, Serratia, Leuconostoc* and *Macrococcus*); ∆=specialist in adult stage (*Pseudomonas, Sphingomonas, Sphingobium, Exiguobacterium Brevundimonas*, and *Arsenophonus*); x=too rare to classify.

Interestingly, through taxonomic analysis of *16S rRNA* gene sequences against the RDPII, a match against the primary endosymbiont of *Sitophilus zeamais* belonging to Enterobacteriaceae family with a minimum identity cutoff of 90% and a minimum alignment length cut-off of 290 pb was detected. The phylogenetic tree containing the *16S rRNA* gene sequences from primary endosymbionts found in different insects (including sequences of the primary endosymbiont from *L. apiculatum*) was constructed (**Figure 1.9**). Regardless of the presence of primary endosymbionts found in ants, the analysis showed a closer relationship with the primary endosymbionts of *Sitophilus* sharing a 99% homology.

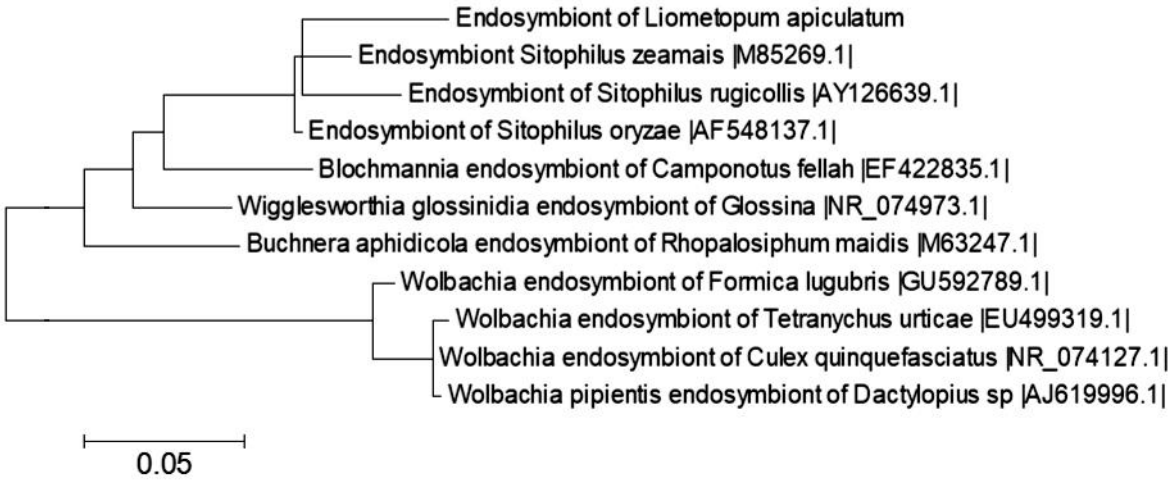

Figure 1.9. Phylogenetic analysis of primary symbiont associated to *L. apiculatum***.** NJ analysis was constructed using the alignment of *16S rRNA* gene sequences from different insects deposited in GenBank and sequence generated in this study. It based on a bootstrap method with 1000 replications. Distances were estimated using the Kimura 2-parameter model in Mega 6.0.

L. apiculatum metagenomic analyses showed high diversity of microbial communities that are not yet cultivated and their functions are unknown. Through microorganism's isolation by culturomics approach, allows us the validation of some genera detected by sequencing. A total of 113 bacterial colonies were isolated from both larvae and adult ants (67 and 46, respectively). Unique colonies (36 and 23) were selected and were successfully identified by MALDI-Biotyping

with spectra score ≥1.8 both abundant and non-abundant genera found in the *16S rRNA* gene sequencing (**Table 1.3, Figure 1.10, and Table 1.4**).

Table 1.3 Number of colonies isolated in the culture media

	BAB		MRS		BHI		SDA	
	Larvae	Adults ants	Larvae	Adults ants	Larvae	Adults ants	Larvae	Adults ants
Number of isola-	24	17	20	9	19	16	4	4
Number of colonies selected for MALDI-TOF	13	9	10	4	11	8	2	2

BAB: Blood Agar Base; (MRS) Man Rogosa Sharpe; (BHI) Brain Heart Infusion; (SDA) Sabouraud Dextrose Agar

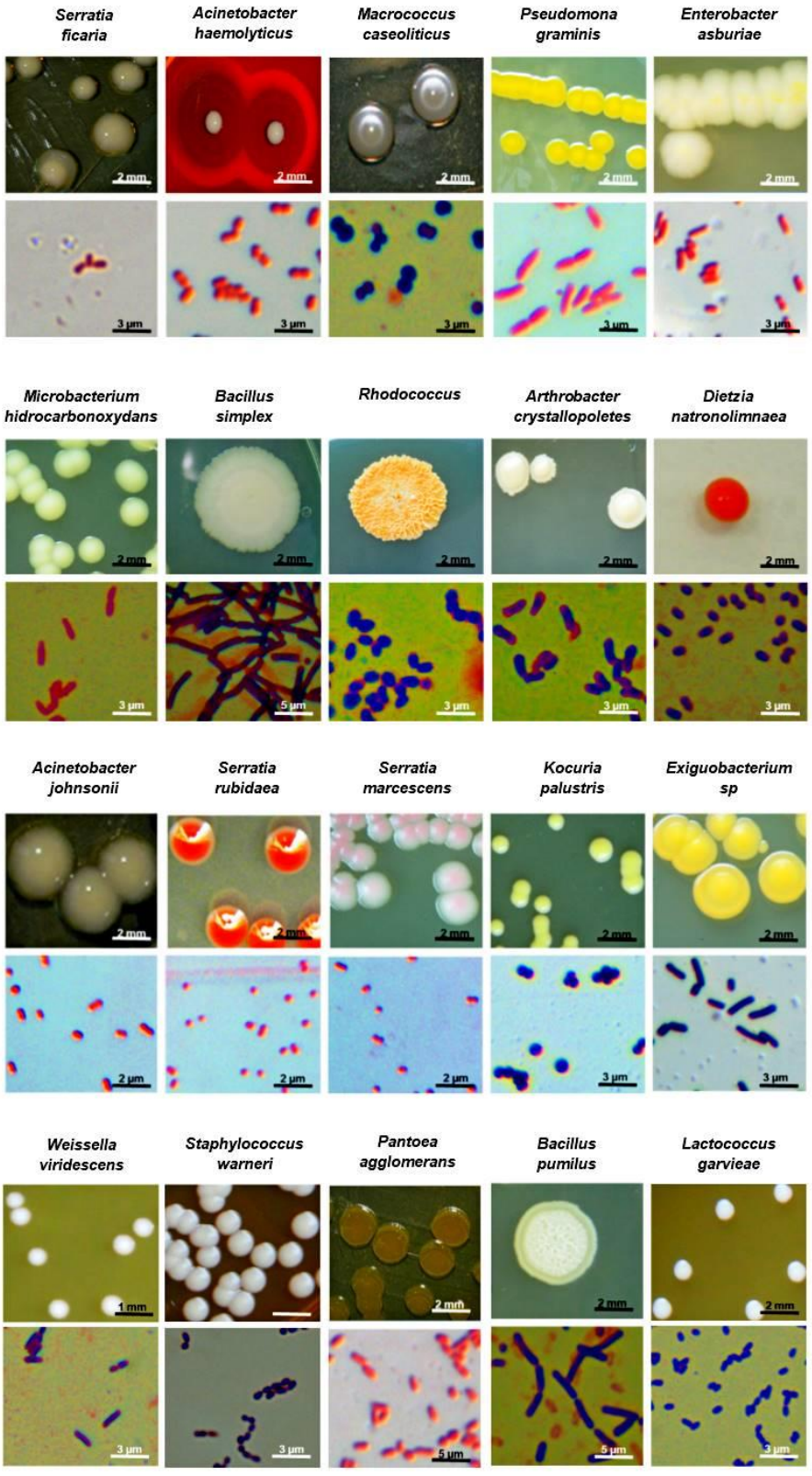

Figure 1.10 Macroscopic and microscopic morphology of bacteria isolates of this study. Names are based on Maldi-Biotyping.

Table 1.4 Bacteria isolated and identified in *L. apiculatum* larvae and adult ant

Bacteria	BioTyper (score)[a]	Description	Identification characteristics[b]	No. of isolates		16S sequencing result[c]
				Larvae	Adult ant	
Serratia ficaria	2.40	Colonies: white to grayish, circular, regular, wet, and raised. Bacterium: Gram-negative bacilli	BAB and BHI culture media under aerobic conditions	3	1	Identified
Acinetobacter haemolyticus	1.82	Colonies: milk white, circular, regular, wet, raised with beta hemolysis. Bacterium: Gram-negative bacilli	BAB and BHI culture media under aerobic conditions	3	1	Identified
Macrococcus caseolyticus	1.93	Colonies: pale yellow, circular, regular, slightly wrinkled, and smooth. Bacterium: Gram-positive cocci	BAB, BHI, and MRS culture media under aerobic conditions	3	ND	Identified
Pseudomonas graminis	1.80	Colonies: greenish-yellow, circular, slightly convex, regular, and wet. Bacterium: Gram-negative bacilli	BAB culture medium under aerobic conditions	1	3	Identified
Weissella viridescens	2.25	Colonies: small, greyish white, round, smooth, entire, glistening, and convex. Bacterium: Gram-positive bacilli	MRS culture medium under aerobic and microaerophilic conditions	3	1	Identified
Enterobacter asburiae	2.20	Colonies: white to pale yellow, smooth, irregularly round to rough. Bacterium: Gram-negative bacilli	BAB culture medium under aerobic conditions	2	ND	Identified
Pantoea agglomerans	2.34	Colonies: translucent yellow, smooth, irregularly round, and mucoid. Bacterium: Gram-negative bacilli	BHI and BAB culture media under aerobic conditions	2	ND	Identified
Lactococcus garvieae	2.17	Colonies: small, gray, circular, smooth, entire and glistening. Bacterium: Gram-positive cocci	MRS and BAB culture media under aerobic and microaerophilic conditions	3	2	Identified
Acinetobacter johnsonii	2.40	Colonies: greyish white, convex, rounded, and mucoid. Bacterium: Gram-negative bacilli	BHI and BAB culture media under aerobic conditions	2	2	Identified
Serratia rubidaea	2.17	Colonies: brilliant red, circular, convex, regular, and mucoid. Bacterium: Gram-negative bacilli	BAB and BHI culture media under aerobic conditions	2	1	Identified
Serratia marcescens	3.36	Colonies: pink to pale red, irregular, convex, and mucoid. Bacterium: Gram-negative bacilli	BAB and BHI culture media under aerobic conditions	2	1	Identified
Kocuria palustris	2.18	Colonies: greenish-yellow, opaque, irregular, dry, and convex. Bacterium: Gram-positive cocci	BHI and SDA culture media under microaerophilic conditions	2	ND	Identified
Exiguobacterium sp	1.95	Colonies: pale orange, circular, slightly raised, irregular, and mucoid. Bacterium: Gram-positive bacilli	BHI culture medium under anaerobic conditions	ND	2	Identified
Staphylococcus warneri	1.83	Colonies: white, circular, smooth, circular, and mucoid. Bacterium: Gram-positive cocci	MRS culture medium under microaerophilic conditions	1	1	Identified

T**able 1.4** (*Continued*)

Bacteria	BioTyper (score)[a]	Description	Identification characteristics[b]	No. of isolates		16S sequencing result[c]
				Larvae	Adult ant	
Rhodococcus	1.8	Colonies: orange, irregular, dye, rough, velvety and smooth,	SDA culture medium under aerobic conditions	ND	1	Identified
Microbacterium hydrocarbonoxy-dans	2.17	Colonies: opaque yellow, smooth, sticky, circular and convex. Bacterium: Gram-positive bacilli	BHI culture medium under aerobic conditions	ND	2	Identified
Bacillus simplex	2.05	Colonies: cream, glossy, irregular, slightly raised and umbonate. Bacterium: Gram-positive bacilli	BHI culture medium under aerobic conditions	3	1	Identified
Arthrobacter crystallopoietes	1.80	Colonies: whiteish-grayish, round, glistening, dry and convex. Bacterium: Gram-positive bacilli	BHI and SDA culture media under aerobic conditions	1	2	Identified
Dietzia natronolimnaea	1.93	Colonies: orange, circular, regular, convex and slightly mucoid. Bacterium: Gram-positive cocci	BHI culture medium under anaerobic conditions	ND	2	Not Identified
Bacillus pumilus	1.98	Colonies: white, wrinkled, irregular, smooth with the undulate margin. Bacterium: Gram-positive bacilli	MRS culture medium under aerobic conditions	3	1	Identified

[a]Score value of identification results after comparison of the generated spectrum with the BioTyper software. Identification with values ≥ 1.8 were considered as correct genus identification. [b]Based on aerobic-, anaerobic- and microaerophilic growth of the bacteria on the plaque.
[c]Based on genera found in *16S rRNA* sequencing analysis. ND: Not detected

In larvae, the main identified bacteria were *Weissella viridescens*, *Macrococcus caseolyticus*, *Lactococcus garvieae*, *Bacillus pumilus, Bacillus simplex, Serratia marcescens, Serratia ficaria* and *Serratia rubidaea*. In adult ants the main identified bacteria were *Pseudomonas graminis, Exiguobacterium* sp., and *Lactococcus garvieae*. On the other hand, bacteria detected exclusively in larvae were *Macrococcus caseolyticus, Enterobacter asburiae, Pantoea agglomerans*, and *Kocuria palustris,* while in adult ants *Exiguobacterium spp., Rhodococcus*, and *Microbacterium hydrocarbonoxydans* were only detected. *Dietzia natronolimnaea* was detected in adult ants by MALDI-biotyping, but this bacterium was not identified by gene sequencing. Possibly this fact was due to the sequences quality filter process because *Dietzia natronolimnaea* belongs to Actinobacteria which constitute one of the non-abundant Phylum in the ants microbiome (**Figure 1.7**). The culturomics strategy confirmed the existence of some specialist bacteria in the escamolera ants as observed previously with the statistical classification model (**Figure 1.8**). In general, the culturomics approach confirmed 15 bacterial genera identified by *16S rRNA* gene sequencing.

As in other ants, *L. apiculatum* has a gut constituted by three primary regions: foregut, midgut, and hindgut, which in turn are subdivided into distinct functional sections. The crop and proventriculus are attached to the foregut; the Malpighi tubules and bacterial pouch are coupled at the bottom of the midgut; while the ileum and rectum are connected to the hindgut (**Figure 1.11**).

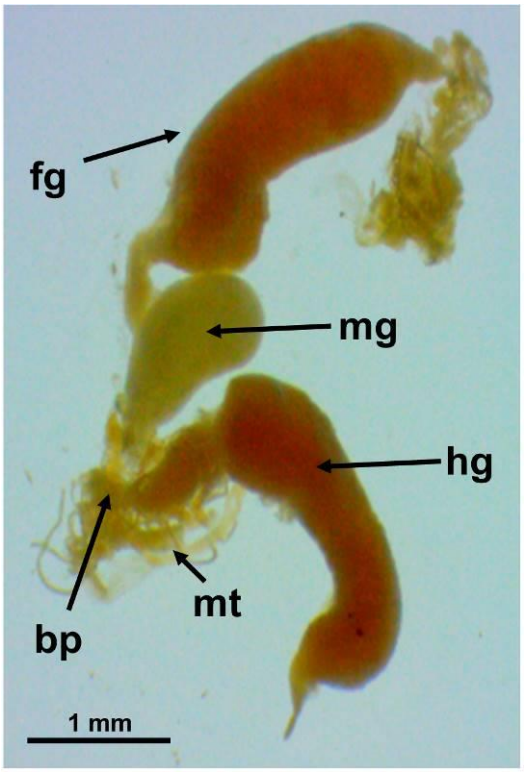

Figure 1.11 Gut organization of adult ant of *L. apiculatum* observed by optical microscopy; fg, foregut; mg, midgut; hg, hindgut; mt, Malpighian tubules; bp, bacterial pouch.

The SEM examination along the ant intestine revealed microorganisms with different morphologies attached to food fibers and apical surfaces, these cells ranged from coccoid to curved and straight rods (**Figure 1.12**). TEM examination of the bacterial pouch content showed numerous rod-shaped bacteria of approximately 1 µm length and 0.5 µm diameter (**Figure 1.13**). Moreover, along the larvae body and in the adult ants midgut, bacteriocytes with intracellular bacteria in the cytoplasm were observed, which clearly displayed changes in size and number from larvae to adult ants. A high number of bacteriocytes with a size of approximately 40 µm were observed in the larvae body (**Figure 1.14A**), while fewer bacteriocytes with minor size (20 µm) were detected in adult ants (**Figure 1.14B**). In addition, three different intracellular symbiotic bacteria harbored in bacteriocytes with coccoid- (0.4 µm), rod- (0.8 µm×0.4 µm) and ovoidshaped (2 µm) were detected by TEM (**Figure 1.4C and D**) This remarkable feature could implicate a key function in the developmental phases of the escamolera ant.

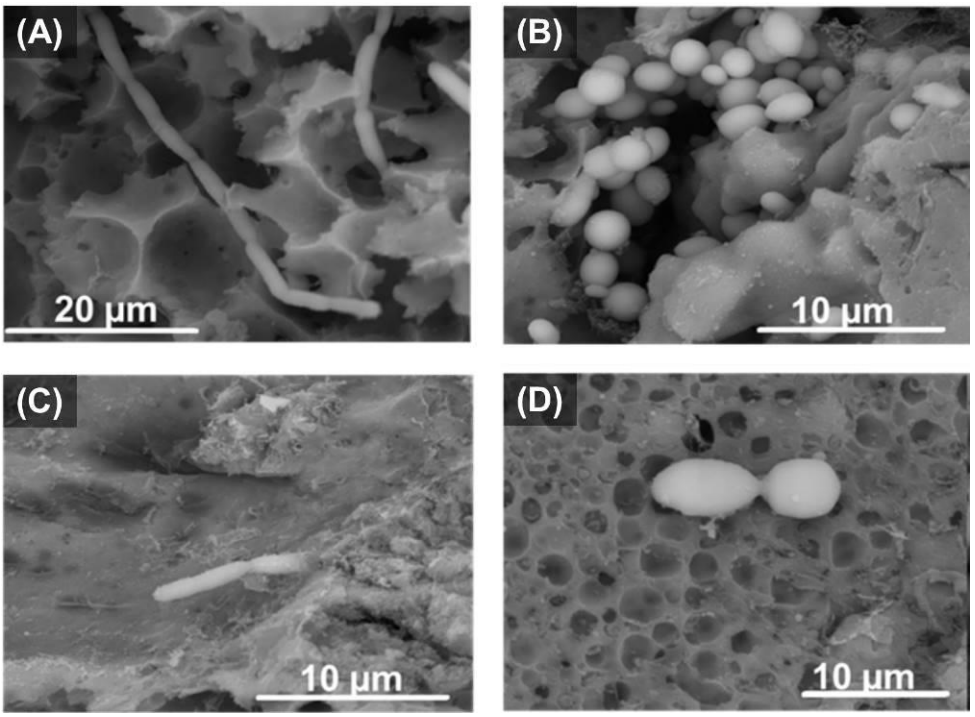

Figure 1.12 Morphology of facultative symbionts examined by SEM. A) bacillary bacteria in the form of chains, B) coccoid bacteria, C) bacillary bacteria. D) yeast.

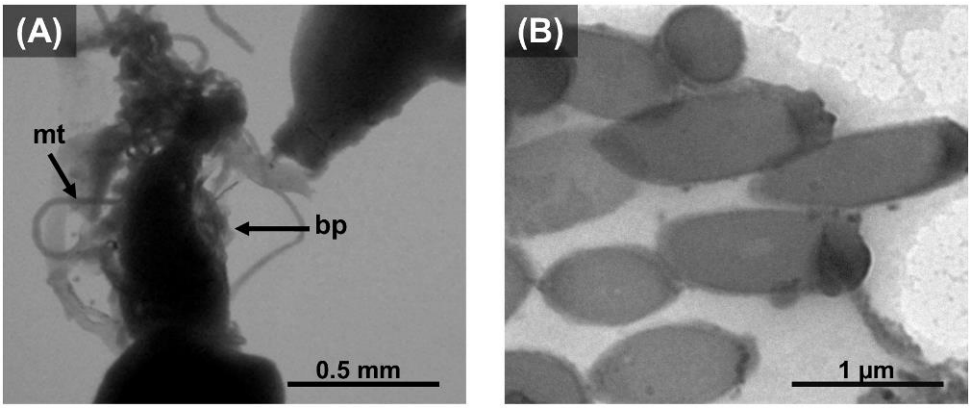

Figure 1.13 Bacterial symbionts harbored in gut pouch of adult ant of *L. apiculatum.* A) Micrograph obtained by optical microscopy of the bacterial pouch. B) Transmission electron micrograph of the semithin section through bacterial pouch displaying bacterial contents. bp, bacterial pouch; mt, Malpighian tubules.

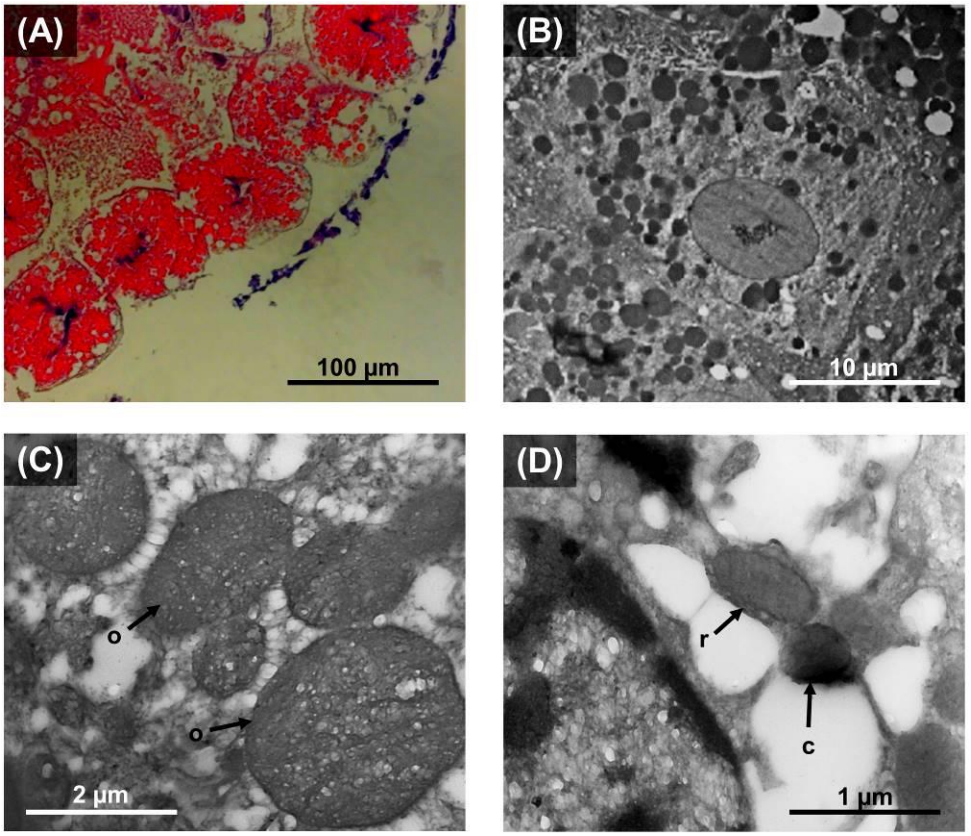

Figure 1.14. System of primary symbionts harbored in bacteriocytes of *L. apiculatum*; A) structure of bacteriocyte in larvae observed by TLM. B) Structure of bacteriocyte in adult ant observed by TEM. C) and D) morphology of primary symbionts found inside of bacteriocytes from larvae and adult ants; the coccoid- (c), the rod- (r) and ovoid- (o) shaped microorganisms.

1.4 Discussion

Bacterial symbionts in the *escamolera* ants were analyzed using metagenomic, culturomics, and microscopic approaches. A high diversity of microorganisms in both larvae and adult ants (299 and 285 genera, respectively) was detected (**Table 1.2**), which was higher than the number of microorganisms reported in other ant species, such as *Cephalotes varians* (150 species) and *Acromyrmex octospinosus* (70 species) (Kautz et al., 2013; Sapountzis et al., 2015). This dynamics of microorganisms has also been observed in other insects such as *Spodoptera littoralis*, *Acyrthosiphon pisum*, and *Cephalotes rohweri* (Chen et al., 2016; Lanan, Rodrigues, Agellon, Jansma, & Wheeler, 2016; Simonet et al., 2016). According to the evolutionary diversification hypothesis, microorganisms that co-exist in the same ecosystem have a substantial contribution to their functioning (Bell, Newman, Silverman, Turner, & Lilley, 2005; Soen, 2014). Because *L. apiculatum* lives in arid and semiarid zones with extreme temperature variations and shortage of flora and fauna, the abundance of the detected microorganisms could be related to the need of obtaining the essential food supplies as well as protection mechanisms to help them to survive under these conditions (Shao, Arias-Cordero, Guo, Bartram, & Boland, 2014; Soen, 2014).

In *L. apiculatum* larvae, a high relative abundance of bacteria belonging to the order Lactobacillales was detected (**Figure 1.5 and 1.6**). In addition, specialist bacteria such as *Weissella*, *Lactococcus, Bacillus*, and *Macrococcus* were identified using both metagenomic and culturomic approaches (**Figure 1.7, 1.8, and Table 1.4**). This microbial profile could be linked with their feeding habits, because the larvae remain in the nest and usually they are fed through oral regurgitations (trophallaxis) from their nestmates which usually feed from nectar produced by the plants (Hoey-Chamberlain & Rust, 2014). Similar profiles have been reported in some insects, which have the same food preference such as bees and bumblebees (Engel et al., 2012; Praet et al., 2015). The functional role of these bacteria could be involved in the breaking of carbohydrates for the energy supply for their metabolic demand during the larval developmental process. Furthermore, these bacteria are known for their antimicrobial activity by the

production of organic acids and bacteriocins, which are used as protective agents since larvae (in this stage) are very susceptible to the pathogen infections (Audisio, Torres, Sabaté, Ibarguren, & Apella, 2011; Currie, 2006; Engel et al., 2012; Santos et al., 2004).

In *L. apiculatum* adult ants the microbial composition was mainly dominated by the order Pseudomonadales followed by Flavobacteriales, Sphingobacteriales, and Sphingomonadales (**Figure 1.5 and 1.6**). These bacterial orders have been reported within the core microbiome of agave species (Coleman-Derr et al., 2016) and have been also found in insects such as termites, beetles, and others ants (Adams et al., 2013; Aylward et al., 2013; Kautz et al., 2013). This microbial profile may reflect the stability of cooperative interactions in their ecosystem since the ants usually make their nest in the agave roots and interact with others insects along the leaves (Anderson et al., 2012; Lucas, Bill, Stevenson, & Kaspari, 2017; Mason & Raffa, 2014).

Pseudomonas was detected in the adult ants of *L. apiculatum* by sequencing and culturomics approaches. This bacteria has also been identified in *Cephalotes varians* ants (Kautz et al., 2013), as well in other ants (Anderson et al., 2012; Hu, Lukasik, Moreau, & Russell, 2014; Ishak et al., 2011; Meirelles et al., 2016), suggesting *Pseudomonas* is a ubiquitous symbiont. It has been shown that *Pseudomonas* as *Sphingomonas* and *Sphingobium* play an important role in the insects' nutrition due that these bacteria have the ability to metabolize polysaccharides and several recalcitrant aromatic compounds (Adams et al., 2013; Aylward et al., 2013; Hinteregger, Leitner, Loidl, Ferschl, & Streichsbier, 1992).

The closest ants-microorganisms associations have been found in the primary or obligate endosymbionts that are harbored in specialized cells known as bacteriocytes. Bacteriocyte-associated endosymbionts have been detected in very few ants, such as *Camponotus* and *Formica* (Heddi et al., 2005; Stoll et al., 2010). These endosymbionts usually have important biological functions such as reproduction, growth, survival, and adaptation processes due to biosynthetic capabilities (Bourtzis & Miller, 2003; Lugtenberg, 2015). Interestingly, a high number of bacteriocytes were identified in the escamolera ant's larvae, by contrast,

in the adult ant it was observed less number and smaller size of this specialized organs (**Figure 1.14**). This trend has been associated with the drop-off adult ant's metabolism (Simonet et al., 2016; Stoll et al., 2010). The obligate endosymbiont detected in *L. apiculatum* showed a 99% homology with the primary endosymbiont of *Sitophilus zeamais* (**Figure 1.9**). This microorganism is involved in the supply of essential metabolites such as vitamins and amino acids necessary for larvae growth, which require a vast amount of metabolites to complete their metamorphosis (Heddi et al., 2005).

Another important finding was the identification of bacteria such as *Pseudomonas, Bradyrhizobium, Flavobacterium, Burkholderia, Methylobacterium, Corynebacterium, Brevundimonas Arsenophonus, Sphingomonas, Rhizobium*, and *Sphingobium* in both larvae and adult ants (**Figure 1.7**). These bacteria have been identified in other insects and have been related to nitrogen-fixing function (Morales-Jiménez et al., 2013; van Borm, Buschinger, Boomsma, & Billen, 2002). Additionally, it was also identified a structural organization of organs that are linked with fixing and recycling of nitrogen, which include the bacterial pouch, the Malpighi tubules (**Fig. 1.11**). The bacterial pouch is interconnected through a network of tracheolar and Malpighi tubules has been described as a system that reintegrates metabolic nitrogen waste derived from the insect metabolism and also uptake dinitrogen gas from the air (van Borm et al., 2002). However, the main players are bacteria associated with this system, since they have enzymatic machinery for the production of amino acids (Billen & Buschinger, 2000; López-Sánchez et al., 2009; Potrikus & Breznak, 1981; van Borm et al., 2002). These suggest that the escamolera ant symbionts possess a mechanism for amino acid production, which is possibly integrated with the host metabolism. On the other hand, recently studies have suggested that the insects gut microbiota are also responsible for producing vitamins and volatile and non-volatile fatty acids, which promote social behavior and weight gain (Tinker & Ottesen, 2016; Zheng, Powell, Steele, Dietrich, & Moran, 2017). In this context, we have identified Actinobacteria such as *Rhodococcus*, *Propiobacterium*, and *Corynebacterium* that have been associated with the unsaturated fatty acid biosynthesis (Gago, Diacovich, Arabolaza, Tsai, & Gramajo,

2011). Therefore, the nutritional composition of escamolera ants can be derivate of several associations of microorganisms that synthesize essential nutrients.

1.5 Conclusion

Our results have shown a change of the microbial endosymbionts in the stages of *L. apiculatum*. We have also observed a large population of nitrogen-fixing bacteria in both stages; which could be linked with the high protein content in escamoles. The escamolera ant endosymbionts likely also help in the digestion of carbohydrates or cellulose-rich plant material for providing essential biosynthetic precursors such as acetate, butyrate, and propionate, which in turn might be used in the synthesis of high nutritional value compounds. Bacterial associated with unsaturated fatty acid biosynthesis were also identified. Moreover, bacteroycites-associated endosymbionts potentially contribute to vitamins and amino acids biosynthesis that are accumulated in escamoles. By other hand, the exploring of microbiota of *L. apiculatum* ant also opens a deep insight into the escamoles food safety, discarding potentially pathogenic microorganisms. Finally, the present results could help to a better understanding of biological and ecological roles of microbial endosymbionts in *L. apiculatum*, an insect of high nutritional and economic value.

CHAPTER 2

Functional screening of isolated bacteria

2.1 Introduction

Biotechnological processes with a main contribution to sustainable development are currently based on the utilization of renewable raw materials, contaminated ecosystems bioremediation, and harnessing beneficial microorganism in agriculture (Busby et al., 2017; Kothari, Tyagi, & Pathak, 2010; J. Pandey, Chauhan, & Jain, 2008). These bioprocesses are basically dependent on metabolically versatile bacteria which carry out biodegradation on various compounds including some highly recalcitrant and toxic (Díaz, 2004; J. Pandey et al., 2008). Therefore, there is a constant interest for new microbial resources, mainly those thriving in adverse environments, since they developed diverse strategies to obtain energy (Diaz, 2004).

Recent culture-independent investigations have demonstrated that symbiotic bacteria contribute to insect's responses to environmental stress and evolutionary adaptations (Engel et al., 2012; Shi et al., 2013; Warnecke et al., 2007; Xia et al., 2017). These studies have suggested that one of the main bacterial metabolic roles is linked to the decomposition of complex polymers or pollutants by the production of various enzymes. For instance, in honey-bee *Apis mellifera,* pectin-degrading enzymes from microorganism which participate in the breakdown of pollen walls, were found in guts (Engel et al., 2012). In termite's species it have been identified bacterial genes for cellulose and xylan hydrolysis, indicating their cooperation in degradation of wood (Warnecke et al., 2007). Moreover, microorganisms from the mountain pine beetle (*Dendroctonus ponderosae*), have been associated with catabolism of aromatic-, sulfonate- and nitroaromatic compounds (Adams et al., 2013). In the diamondback moth (*Plutella xylostella*) some bacteria have been linked with catabolism of xenobiotic and terpenoid compounds (Xia et al., 2017). On the other hand, through culturomics studies, bacterial enzymatic activities have been reported for a small number of insects. Although, this approach is essential for elucidating the microorganisms

functional role, as well for validation of data obtained by high-throughput methods (Lagier et al., 2016). In isolated bacterial from aquatic crane fly (*Tipula abdominalis*) cellulolytic and hemicellulolytic activity have been observed (Rogers & Doran-peterson, 2010) and in diamondback moth (*Plutella xylostella*) chitinolytic activity has been reported (Indiragandhi et al., 2007). Another important finding was reported in symbionts from the bean bug (*Riptortus pedestris*), where organophosphorous compounds-degrading bacteria were identified, establishing an important function to its host for insecticide resistance (Kikuchi et al., 2012). All these findings suggest that microorganisms are involved in detoxification functions from plant compounds and chemical contaminants, contributing in many insects to thrive in disadvantageous environments. Therefore, insect microbial consortia have become of major interest, since it represents a potential source of valuable enzymes for biotechnology applications. However, exploration of the microbial consortium of many insects is still lacking.

The *Liometopum apiculatum* ant, called escamolera ant is an important species of edible insect that contributes to the food supply for humans (Ramos-Elorduy, 2006). *L. apiculatum* is distributed in arid and semi-arid ecosystems where a relative shortage of food can be appreciated, compared to tropical ecosystems. However, the escamolera ant has reach an ecological balance through diverse symbiotic interactions with other insects, plants, and microorganisms to survive (González-Escobar et al., 2018; Velasco, Corona-Vargas, & Peña-Martinez, 2007). *L. apiculatum* ant has been described as an omnivorous insect because of the versatility of its feeding habits. Its diet has been mainly associated with sugar solutions such as honeydew and floral nectar obtained by trophobiosis from insects and plants (Velasco et al., 2007), but it has also linked with seeds, pollen, fruits, plants, insect's pupae, crustaceans, annelids, mollusks, and animal droppings (García-Herrera, Méndez-Gallegos, & Talavera-Magaña, 2010; Hoey-Chamberlain, Rust, & Klotz, 2013; Velasco et al., 2007). Nonetheless, there is a limited information regarding the *L. apiculatum* capacity to process a wide range of organic matters. Therefore, we explored functional contribution of escamolera ant gut microbiota, regarding the microbial importance in insect nutritional ecology and in potential biotechnological applications.

2.2 Materials and methods

2.2.1 Bacteria description

Bacteria were isolated from the *L. apiculatum* gut microbiome previously (González-Escobar et al., 2018). Twenty bacteria identified by MALDI-Biotyping and characterized by Gram staining were used in this study (**Table 2.1)**.

Table 2.1. Bacteria used in the functional screening.

Bacteria	BioTyper (score)	Gram stain
Serratia ficaria	2.40	-
Acinetobacter haemolyticus	1.82	-
Macrococcus caseolyticus	1.93	+
Pseudomonas graminis	1.80	-
Weissella viridescens	2.25	+
Enterobacter asburiae	2.20	-
Pantoea agglomerans	2.34	-
Lactococcus garvieae	2.17	+
Acinetobacter johnsonii	2.40	-
Serratia rubidaea	2.17	-
Serratia marcescens	2.36	-
Kocuria palustris	2.18	+
Exiguobacterium sp	1.95	+
Staphylococcus warneri	1.83	+
Rhodococcus	1.80	+
Microbacterium hydrocarbonoxydans	2.17	+
Bacillus simplex	2.05	+
Arthrobacter crystallopoietes	1.80	+
Dietzia natronolimnaea	1.93	+
Bacillus pumilus	1.98	+

[a]Score value of identification results after comparison of the generated spectrum with the BioTyper software. Identification with values ≥ 1.8 were considered as correct genus identification.

2.2.2 Screening of enzymatic activities using API® microsystems

Bacterial enzyme activity was assayed with API® 20NE and API® ZYM systems (BioMérieux, Marcy l'Etoile, France). The API® ZYM system contains dehydrated chromogenic substrates for 20 enzymatic reactions involved in the breakdown of peptides, phosphomonoesters, lipids, and polysaccharides, including chitin, cellulose, starch, and galactans. On the other hand, the API® 20NE is used to evaluate NO_3 reduction, indole production from tryptophan, L-arginine dihydrolase

activity, fermentation of glucose, urease activity, hydrolysis of gelatin, and growth on 12 different organic carbon compounds. We used a total of 20 unique bacterial isolates from *L. apiculaum* ant to investigate their enzymatic activities through API® microsystems. Pure cultures were grown on Brain Heart Infusion (BHI) broth at 37 °C for 24 h (bacteria) and 72 h (actinobacteria). Cultures (10 mL) were harvested by centrifugation at 1,935×g for 15 min at 4 °C. Pellet was washed twice and re-suspended in 3 mL of sterile saline solution (0.85%). This bacterial solution was used to get two concentration of bacterial cell suspension, one with OD_{600}=0.4 for API® ZYM and the second with OD_{600}=0.07 for API® 20NE. These tests were performed and examined according to the manufacturer's instructions. The bacterial isolates were examined at least three times with independent subcultures (Frette, Johnsen, Jørgensen, Nybroe, & Kroer, 2004; Tamang, Tamang, Schillinger, Guigas, & Holzapfel, 2009).

2.2.3 Amylolytic- and cellulolytic- activity assay on plates

Amylase and carboxymethylcellulase (CMCase) activity were evaluated by corresponding polysaccharide hydrolysis in solid medium containing: 10 g/L polysaccharide (starch or carboxymethylcellulose); 15 g/L agar; 2.0 g/L peptone; 2.0 g/L NH_4Cl; 1.0 g/L NaCl; 1.0 g/L $CaCl_2.2H_2O$; 1.0 g/L $MgSO_4.7H_2O$. Pure bacterial cultures (20 µL) were inoculated on solid medium and incubated for 3 days at 37 °C. Plates were stained with 4% iodine solution to detect starch hydrolysis and 1 % Congo red solution to detect carboxymethylcellulose hydrolysis (Afrisham, Badoei-Dalfard, Namaki-shoushtari, & Karami, 2016; Pinheiro et al., 2015).

2.3. Results

Results of bacterial enzymatic activities analyzed with the API® ZYM system are reported in **Figure 2.1**. Proteolytic activity associated with leucine aminopeptidase (LAP), valine aminopeptidase (VAP), and cystine aminopeptidase (CAP) was observed mainly in bacteria belonging to Proteobacteria and Actinobacteria. Leucine aminopeptidase (LAP) showed the highest catalytic activity with 40 nanomoles of hydrolyzed substrate. While α-chymotrypsin (CTR) showed an activity of 20 nanomoles and it was observed in Firmicutes, *Macrococcus caseolyticus* and *Lactococcus garvieae.*

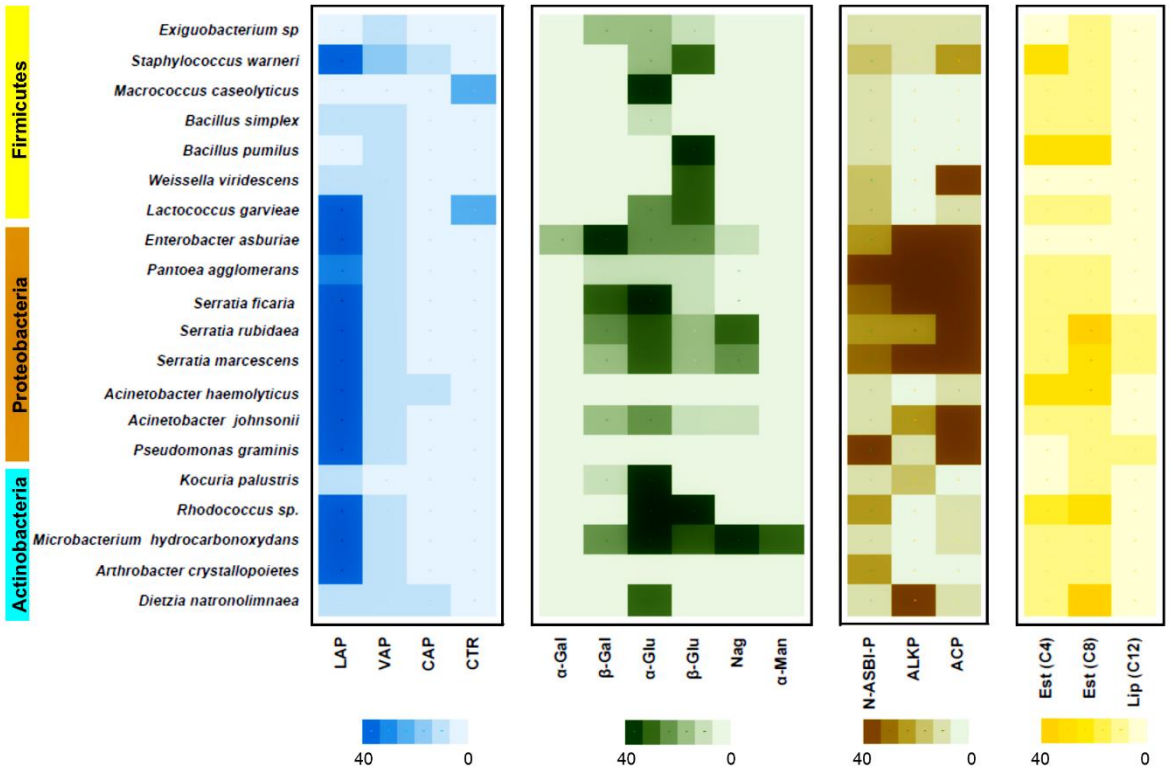

Figure 2.1 Bacterial enzymatic activities using the API® ZYM system. Color indicates the proportion of the catalytic activities corresponding to a number value rank from 0 to 40 nanomoles of hydrolyzed substrate. Leucine aminopeptidase (LAP), valine aminopeptidase (VAP), cystine aminopeptidase (CAP), α-chymotrypsin (CTR), α-galactosidases (α-Gal), β-galactosidases (β-Gal), α-glucosidases, (α-Glu), β-glucosidases (β-Glu), β-N-acetylglucosaminidase (β-Nag), α-mannosidases (α-Man), phosphohydrolase (N-ASBI-P), acid phosphatase (ACP), and alkaline phosphatase (ALKP), esterase C4 (Est C4), esterase C8 (Est C8) and lipase (C12).

Throughout bacterial community of *L. apiculatum,* polysaccharide-degrading enzymes such as α-glucosidases (α-Glu), β-glucosidases (β-Glu), α-galactosidases (α-Gal), β-galactosidases (β-Gal), β-N-acetylglucosaminidase (β-Nag), and α-mannosidases (α-Man) were detected. However, α-Glu linked with starch breakdown and β-Glu linked with cellulose breakdown displayed a high enzymatic hydrolysis above 30 nanomoles. *Macrococcus caseolyticus*, *Serratia ficaria*, *Serratia rubidaea*, *Serratia marcescens*, *Kocuria palustris*, *Rhodococcus* sp, *Microbacterium hydrocarbonoxydans, and Dietzia natronolimnaea* were mostly characterized by high α-Glu activity. While *Staphylococcus warneri, Bacillus pumillus, Weissella viridescens, Lactococcus garvieae, Rhodococcus sp, and Microbacterium hydrocarbonoxydans* were mostly characterized by high β-Glu activity. Moreover, β-Gal enzymatic activity associated with xiloglucan breakdown was mainly observed in *Serratia ficaria* and *Enterobacter asburiae*, and β-NAG enzymatic activity associated with chitin breakdown, was mainly observed in *Microbacterium hydrocarbonoxydans* and *Serratia rubidaea*.

In regard to phosphatases, we detected an abundant catalytic activity linked with phosphohydrolase (N-ASBI-P), acid phosphatase (ACP), and alkaline phosphatase (ALKP) in bacteria belonging to Proteobacteria. *Enterobacter asburiae*, *Pantoea agglomerans*, *Serratia ficaria*, *Serratia rubidaea*, *Serratia marcescens* showed a high enzymatic hydrolysis above 20 nanomoles in the three phosphatases. Nonetheless, most bacteria displayed enzymatic activity at least with two phosphatases. On the other hand, lipases such as esterase C4 (Est C4) and esterase C8 (Est C8) were considerably observed in a low level. Bacteria such *Bacillus pumilus*, *Acinetobacter haemolyticus*, *Rhodococcus sp* showed catalytic activity above 10 nanomoles with those lipases. *Dietzia natronolimnaea* and *Serratia rubidaea* showed the highest Est C8 activity with 20 nanomoles. The Lipase C12 (Lip C12) was observed at a low level only in three bacteria.

Results of bacterial enzymatic activities studied with the API® 20NE system are reported in **Figure 2.2.** It was observed the hydrolysis of nitrogenous molecules such as gelatin (GEL), urea (URE), arginine (ADH), and tryptophan (TRP). Microorganisms with aminopeptidase activities (**Figure 2.1**) such as

Serratia ficaria, *Serratia rubidaea*, *Serratia marcescens*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Microbacterium hydrocarbonoxydans*, *Exiguobacterium sp*, *Bacillus pumillus,* and *Kocuria palustris* were capable to degrade the GEL. But also this ability was detected in *Micrococcus caseolyticus* with α-chymotrypsin activity. Hydrolysis of ADH, TRP, and URE were observed in few microorganisms. On the other hand, nitrate reduction (NR) was widely identified in the most bacteria.

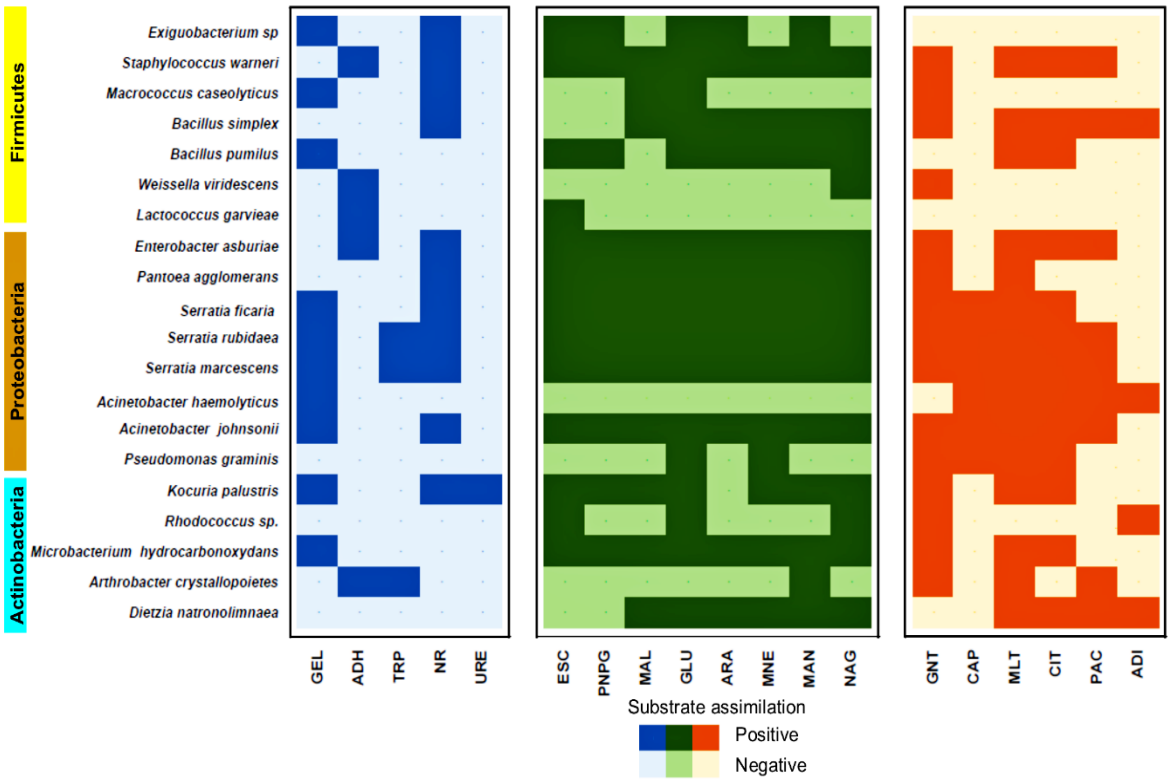

Figure 2.2 Bacterial enzymatic activities using the API® 20NE system. Color indicates substrate assimilation. Gelatin (GEL), arginine (ADH), tryptophane (TRP), nitrate reduction (NR), urea (URE), esculin (ESC), 4-Nitrophenyl β-glucopyranoside (PNPG), malate (MAL), glucose (GLU), arabinose (ARA), mannose (MNE), mannitol (MAN), N-acetyl glucosamine (NAG), gluconate(GNT), capric acid (CAP), malate (MLT), citrate (CIT), phenylacetic acid (PAC), and adipic acid (ADI).

Carbohydrate breakdown was broadly observed in the bacterial population using the API® 20NE systems (**Figure 2.2**). Several bacteria assimilated sugar monomers such as maltose (Mal), glucose (GLU), arabinose (ARA), mannose (MNE), and mannitol (MAN). Moreover, this analysis confirmed the catalytic activities of β-Gal and β-Gluc. Hydrolysis of esculin (7-hydroxycoumarin 6-β-

glucopyranoside) (ESC) evidenced the β-Gluc activity in 12 bacteria previously reported with API® ZYM (**Figure 2.1**), only *Weissella viridescens* was not able to hydrolyze this sustrate. Hydrolysis of the 4-Nitrophenyl β-glucopyranoside (PNPG) confirmed the β-Glu activity in all bacteria observed in API® ZYM. However, *Bacillus pumilus* and *Staphylococcus warneri* with previously unidentified activity were also able to hydrolyze PNPG. We also observed that all bacteria with β-N-acetylglucosaminidase activity were able to assimilate N-acetylglucosamine monomers. Nonetheless, nine bacteria that do not have the ability to degrade the complex polysaccharide were able to use its monosaccharides.

Assimilation of organic acids such as gluconate (GNT), malate (MLT), and citrate (CIT) was detected in most of the microorganisms (**Figure 2.2**). By the contrary, few bacteria assimilated capric acid (CAP), phenylacetic acid (PAC), and adipic acid (ADI). Six bacteria degraded capric acid with lipolytic activities previously detected (**Figure 2.1**). Nine bacteria assimilated phenylacetic acid and four bacteria assimilated adipic acid.

Plate assays confirmed the enzymatic activity of some bacteria on starch and carboxymethylcellulose breakdown. *Exiguobacterium sp*, *Acinetobacter johnsonii*, *Serratia marcescens*, *Pantoea agglomerans*, showed clear halos on starch containing plates, indicating high amylolytic activity. By the contrary, *Enterobacter asburiae*, *Bacillus simplex*, *Serratia ficaria*, and *Serratia rubidaea* were characterized by slight clear zone around the growth, displaying a limited amylolytic activity (**Figure 2.3**). Nonetheless, five bacteria with amylolytic activity observed in API® ZYM assays did not show starch hydrolysis. On the other hand, *Bacillus pumillus*, *Enterobacter asburiae*, *Pantoea agglomerans*, *Acinetobacter johnsonii* showed a clear zone on carboxymethylcellulose-containing plates. The clear zone around the colony was considerably greater for *Bacillus pumilus*, indicating higher cellulolytic activity (**Figure 2.4**). However, *Acinetobacter johnsonii, Pantoea agglomerans* and *Enterobacter asburiae* were associated with cellulose and starch degradation.

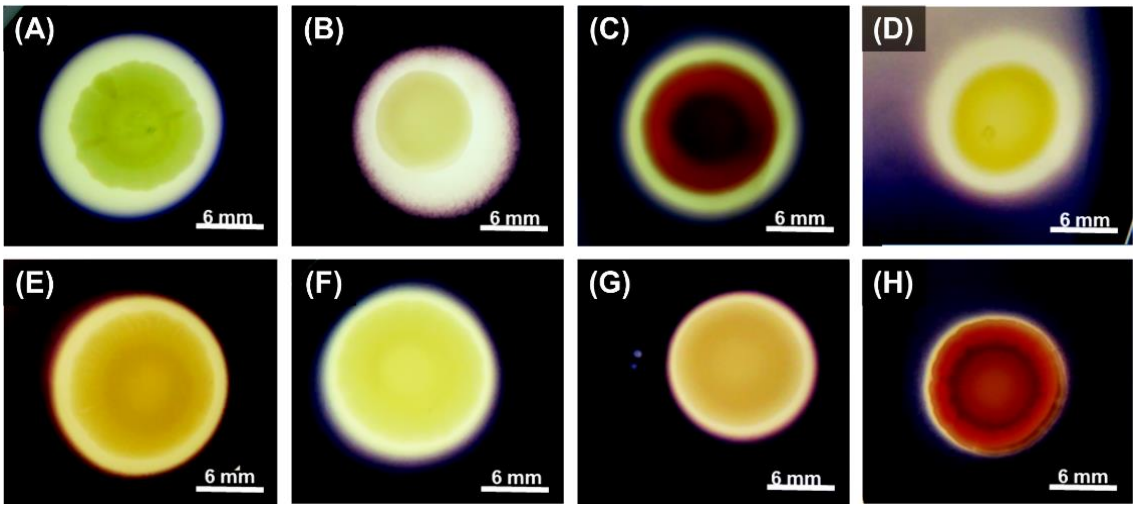

Figure 2.3 Bacterial amylolytic activity on plate assays. (A) *Exiguobacterium* sp, (B) *Acinetobacter johnsonii,* (C) *Serratia marcescens*, (D) *Pantoea agglomerans*, (E) *Enterobacter asburiae*, (F) *Bacillus simplex*, (G) *Serratia ficaria*, and (H) *Serratia rubidaea*.

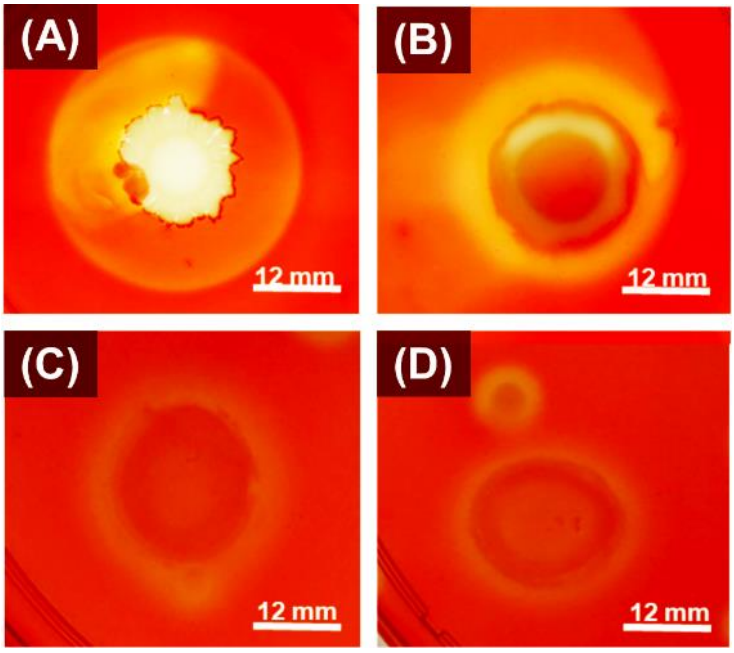

Figure 2.4. Bacterial cellulolytic activity on plate assays (A) *Bacillus pumilus*, (B) *Acinetobacter johnsonii*, (C) *Pantoea agglomerans*, (D) *Enterobacter asburiae*.

2.4 Discussion

Cooperative activities of microbial symbionts associated with metabolism and energy supply in *L. apiculatum* have not been established. Therefore, we studied the functional diversity of the cultivable bacterial community of escamolera ant gut microbiota. Bacterial isolates showed a capacity to solubilize phosphate and to degrade proteins, complex polysaccharides, lipids and organic acids, suggesting that bacterial enzymatic activities are involved in food digestion in the ants gut. Our results highlight the important contribution of the microbial symbionts in the feeding and surviving of ants, and addition, expose a promising source of enzymes for potential biotechnological applications.

Insects gut microbiota is strongly influenced by its host diet. In fact, some studies have argued that through the composition of the diet is possible to determine the nature and activity of the gut microbial community (Colman, Toolson, & Takacs-Vesbach, 2012; Engel & Moran, 2013; Vital, Gao, Rizzo, Harrison, & Tiedje, 2014). Moreover, the gut bacteria diversity seems to be higher in omnivorous insects than in stenophagous (carnivorous and herbivorous) insects, indicating that feeding behavior is greatly linked with the microbial diversity's functional contribution for the different types of food consumed (González-Escobar et al., 2018; Yun et al., 2014). Therein context microorganism plays a critical role in regulating the insect's metabolism beginning with efficient digestion through diverse enzymes for energy extraction from ingested food (Engel et al., 2012; Warnecke et al., 2007).

In this work, isolated bacteria from escamolera ant displayed a high proteolytic activity associated with leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, and α-chymotrypsin (**Figure 2.1**). Proteases are central enzymes that participate in food digestion, breaking-down proteins into amino acids that insects uses for growth and development (Terra & Ferreira, 2012). However, recent studies have observed proteolytic enzymes from the insect gut bacteria to contribute beyond of basic biological processes of its host. In the velvet bean caterpillar (*Anticarsia gemmatalis*) several microbial symbionts synthesize serine- and cysteine aminopeptidase involved in protection not only against

proteases inhibitors (PI) of plants, but also against synthetic PI (insecticides) (Pilon, Visotto, Guedes, & Oliveira, 2013). Indeed, proteases produced by *Bacillus* sp. YP1 from cotton bollworm (*Helicoverpa armigera*) degrade the PI present in seeds (Shinde, Shaikh, Padul, & Kachole, 2012). On the other hand, proteolytic enzymes have been linked with multifunctional activities such as lipases, arylesterase, thioesterase, and phospholipases (Akoh, Lee, Liaw, Huang, & Shaw, 2004). Therein context, we have also observed enzymatic activity associated with hydrolysis of triacylglycerols, saturated fatty acids, and organic phosphorus (**Figure 2.1**). Many omnivorous insects like escamolera ant satisfy their nutritional requirements through its diets since they are unable to biosynthesize them (Canavoso, Jouni, Karnas, Pennington, & Wells, 2001). Therefore, these results could indicate that several enzymes or multifunctional enzymes from bacteria are involved in releasing nutrients stored in plant-derived materials. But also, it has been reported that some esterases play a key role in the organophosphorus compounds detoxification (insecticides) (Dang, Doggett, Singham, & Lee, 2017; G. Li, Wang, & Liu, 2008). Then, the enzymatic activities of the escamolera ants gut microbiota could suggest a potential mechanism for plants components digestion as well as for coping with plant defenses and other toxic compounds.

Fermentation of the cellulose-, xylan-, starch-, and chitin- compounds was observed through trials with the bacterial community of *L. apiculatum* (**Figure 2.1-2.4**). As a result of this microbial fermentation, the gut microbiota has a wide effect on ant nutrition and health by metabolizing recalcitrant dietary polymers, which cannot be degraded by the host. These results seem to be directly linked with feeding behavior of the escamolera ant, which requires several enzymes for the digestibility of different components of its diet. In many xylophagous- and detritophages- insects several enzymes from symbiotic bacteria such as cellulases, xylanases, and amylases have been reported to assist in the degradation of the plant-derived materials (Fabryová et al., 2018; Rogers & Doran-peterson, 2010; Warnecke et al., 2007). However, in few insects bacterial chitinolytic enzymes have been observed, as in diamondback moth (*Plutella xylostella*) and termite (*Macrotermes barneyi*) (Indiragandhi et al., 2007; Sun, Li, Du, Xiao, & Ni, 2017). Chitinases are important not only for chitin containing compounds digestion but

also for functional maintaining the peritrophic matrix of insects' midgut (Shen & Jacobs-lorena, 1997). Another important finding was the phenylacetic acid assimilation, which has a central role in the pathway of aromatic compounds degradation. Certain insects' symbionts have developed an enzymatic system to catabolize lignin and other secondary metabolites of plants (Adams et al., 2013; Aylward et al., 2013; Kuhnigk et al., 1994; Xia et al., 2017). Interestingly, in this study nine bacteria were able to assimilate phenylacetic acid (**Figure 2.2**), suggesting a participation of key enzymes for its biodegradation. Moreover, in previous studies, some bacterial consortium members from *L. apiculatum* such as *Acinetobacter, Arthrobacter, Rhodococcus*, *Bacillus, Staphylococcus*, *Serratia*, *Enterobacter,* and *Dietzia* have already been reported their abilities to degrade aromatic compounds (Bisagni et al., 2017; Bugg, Ahmad, Hardiman, & Rahmanpour, 2011; de Gonzalo, Colpa, Habib, & Fraaije, 2016; DeAngelis et al., 2013; Wang et al., 2016). Therefore, bacterial lignocellulolytic enzymes seem to play a key role not only in the monosaccharides production from the vegetal matters, but also sugars fermentation to organic acids for energy supply in the escamolera ant.

From a biotechnological perspective, the metabolic versatility of microbial symbionts from *L. apiculatum* ant constitutes promising enzymes sources for potential applications in fermentation-, food- and pharmaceutical- industry, but also in processes associated with agriculture and bioremediation. Currently, proteolytic enzymes are used in the food, dairy, detergent, and medicine industries (Q. Li, Yi, Marek, & Iverson, 2013). For instance, cysteine- and leucine aminopeptidase have been used as meat tenderizers as well in baking and production of protein hydrolysates. Chymotrypsin has been mainly used in diagnostic and analytical methods (Dhillon, Sharma, Rajulapati, & Goyal, 2017). On the other hand, lignocellulolytic enzymes have a high demand for biofuel production using renewable raw materials. But also, amylases and cellulases have a large number of applications in textile, paper, cotton, baking, clinical chemistry and detergent industries (Berasategui, Shukla, Salem, & Kaltenpoth, 2016). Chitinases have currently gained interest in agriculture as antimicrobial or insecticidal agents because of their ability to degrade chitin in insect exoskeleton and the fungal cell

wall (Karthik, Binod, & Pandey, 2017). With regard to monooxygenases, lipases, esterases, and other enzymes associated with catabolism of aromatic compounds, could be used strategically in processes of bioremediation and wastewater treatment (de Gonzalo et al., 2016; Wang et al., 2016). Moreover, lipases and proteases have acquired an important interest in the biodiesel production from vegetable oils (Berasategui et al., 2016).

2.4 Conclusion

Results have provided insights regarding the metabolic capabilities of intestinal bacteria associated with escamolera ant. We have identified bacterial enzymatic activities not only linked with the food digestion and nutrition but also with plant defenses and toxic compounds detoxification. These results suggest bacterial community in the *L. apiculatum* gut is functionally complex since bacterial community could be work synergistically to supply energy to its host. Therefore, *L.apiculatum* feeding behavior is strongly influenced by the bacterial functional contribution, suggesting microbial symbiosis in the escamolera ant has been a potential mechanism for its survival in a semiarid ecosystem. On the other hand, isolated bacteria exhibited interesting enzymatic activities, revealing that escamolera ants represent an important source of bacteria with the biotechnological potential. Therefore, our study provides a large set of bacterial candidates worthy for further investigation.

3. Future work

The results described in this work revealed a wide range of research possibilities that would be interesting explore in the future.

1. A metaproteomics analysis should be included to identify microbial proteins involved in the ant metabolism.

2. A deeper analysis should be carried out to analyze the functioning of mutualism between these symbionts and *escamolera* ant.

3. A bacterial growth study of Glucosidases-producing bacteria, which allow degradation of variety of polysaccharides, should be carried out on different substrates. The total sugar assay and reducing sugar assay should be performed in order to determine the enzyme activity.

4. The effect of co-cultures could be examined to determine possible synergy between bacteria during degradation of different plant-derived polysaccharides.

5. The candidate enzymes worthy of research could be cloned, over-expressed and purified for their biochemical characterization, including activity assays with different xenobiotic substrates

Annexes

Paper published

1. Jorge L. González-Escobar, Alicia Grajales-Lagunes, Adam Smoliński, Alicia Chagolla-López, Antonio De Léon-Rodríguez, Ana P. Barba de la Rosa. ¨Microbiota of edible *Liometopum apiculatum* ant larvae reveals potential functions related to their nutritional value¨. 2018. **Food Research International** 109, 497-505, doi: 10.1016/j.foodres.2018.04.049

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2. José A. Huerta-Ocampo, María S. García-Muñoz, Aida J. Velarde-Salcedo, Eric E. Hernández-Domíngueza, Jorge L. González-Escobar, Alberto Barrera-Pacheco, Alicia Grajales-Lagunes, Ana P. Barba de la Rosa. ¨The proteome map of the escamolera ant (*Liometopum apiculatum Mayr*) larvae reveals immunogenic proteins and several hexamerin proteoforms. 2018. **Comparative Biochemistry and Physiology - Part D** 28, 107-1211, doi: 10.1016/j.cbd.2018.07.004

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