Effect of Lactic Bacteria on *Ascosphaera apis* and *A. atra*

Marcos Raul Tejerina, Maria José Cabana, Leonor Carrillo and Marcelo Rafael Benitez-Ahrendts*

Microbiology, Faculty of Agricultural Sciences, National University of Jujuy (UNJu)
Alberdi47, 4600 S.S. of Jujuy, Argentina.

**Corresponding author's email: mrba71 [AT] yahoo.com.ar*

ABSTRACT—*The lactic bacteria contribute to fermentation of corbiculae pollen and beebread production. Three species of Lactobacillus, isolated from beebread for the first time in Jujuy (Argentine), formed a monophyletic clade separate from others bee intestinal strains. Lactic bacteria isolated in this work affected the growth and sporulation of Ascosphaera apis and Ascosphaera atra, and they can provide to beehive a protection against ascospores.*

Keywords— *Lactobacillus, Ascosphaera*, antifungal action

1. INTRODUCTION

The production of apples, pears, melons, strawberries, as well as cotton, alfalfa, onions, peas, asparagus, celery, among other crops, depends on bee populations, either domesticated or native bees, wild solitary or social species because they pollinate these crops [14].

Ascosphaera (Ascosphaeraceae, Pezizomycotina, Ascomycota) is an entomopathogenic fungus affecting bees. It has 28 species. *A. apis* affect the social bees *Apis mellifera*. In mixed infections, combining *A. atra* and *A. apis* ascospores, increased larval death rate significantly compared to *A. apis* alone [19]. *A. apis* only produces sexual spores and is heterothallic, but *A. atra* is homothallic. While adult bees are not susceptible, they can transmit the disease within and between beehives. Spores can be accumulated on all parts of the beehive and in all beehive products (e.g., wax, stored pollen, honey). Any hive material infected will serve as a source of infection to pollinator species [3].

There are common problems associated with synthetic pesticides and antimicrobials use. These agents are ineffective against the infectious spores or are adverse effects on the vitality of larvae or bee longevity. Some phenolic and terpenoid compounds from plants and propolis are antimicrobial activity [2].

Microbes have profound effects on insect health as pathogens, nutritional or facultative symbionts. Some bacteria compete in the bee gut environment, inhibiting the growth of others microbes. The heterogeneous genus Lactobacillus has been found in insects such as termites, bumblebees and honey bees, and some species. It confers a health benefit to host [13].

The aim of this work was to determine the effect of Lactobacillus strains isolated from beebread on two Ascosphaera species.

2. MATERIALS AND METHODS

2.1 Isolation of Lactobacillus strains

The strains of *Lactobacillus* were isolated from beebread collected from three apiaries located at El Ceibal, 24°18'22.4"S 65°16'50.8"W (San Antonio Department), El Carmen 24°23′48,98″S 65°15′49,75″W, (El Carmen Department) y Nogales 24°07'22.8"S 65°27'11.3"W (Dr. Manuel Belgrano Department) Jujuy, Argentina. Beebread obtained from honeycombs was put in Man-Rogosa-Sharpe broth at 37ºC under microaerophilic conditions for 72 h and transferred to MRS agar for isolation [5].

Colonies were plating on pollen-agar (pollen 30 g, agar 15 g, water 1 L) under microaerophilic conditions and preserved into MRS broth + 20% (w/v) glycerol and kept at - 20° C.

2.2 Identification of Lactobacillus species

Bacterial nucleic acids extraction was performed from MRS liquid medium according to Cariaga-Martinez & Zapata (2007) [6] protocol. Bacterial regions carrying the 16S rRNA gene (~1500 pb) were amplified using the primers

Eub_1542 reverse (5′-AGAAAGGAGGTGATCCAGCC-3′ and Eub_9_27 forward (5′-GAGTTTGATCCTGGCTCAG-3′) from Invitrogen S.A., Argentina [12].

PCR amplification was performed in a 20 µL reaction mix containing 1X buffer, 2.5 mM magnesium chloride, 200 µM dNTPs, 10 ρM of each primer, and 0.5 U Taq polymerase. Amplification consisted of an initial denaturation step at 94ºC for 4 min, followed by 35 cycles at 94ºC for 40 s, 53ºC for 40 s and 72ºC for 40 s. Fragments were purified and sequencing by Macrogen Services.

The chromatograms were analyzed using BioEdit program. Sequences were compared and aligned with sequences from GenBank database using the BLAST program of NCBI network server. The sequences were deposited in GenBank database.

2.3 Phylogenetic analyses

The *Lactobacillus* 16S-rRNA sequence analysis was made using MEGA6, BLASTn, BioEdit and CLUSTAL W editors before phylogenetic tree construction. Phylogenetic analysis was made using the TNT program. Indels were used as a 5th state character. Five *Lactobacillus* strains isolated from Salta, Argentina [4, 5, 13] were included in the analysis and the *Bacillus amyloliquefaciens* RHNK22 (NZ_LMAG01000018.1) as outgroup strain.

2.4 Ascosphaera strains

Six strains of *A. apis* from of Spain provinces were isolated, identified and placed in Genbank database (https://www.ncbi.nlm.nih.gov/): P1 (KX622164) Madrid; P3 (KX622165) Zaragoza; P4 (KX622166) Ciudad Real; P5 (KX622167) Guadalajara; P6 (KX622168) Cuenca; P8 (KX622169) Barcelona. Also, one strain of *A. atra* P9 (KY810495) Castellón. All strains were kept on MY20 at 20° C in darkness [16].

2.5 In vitro antifungal activity

A) 100 µL of each *Lactobacillus* strain cultures in MRS broth (ca. 1x10^5 CFU/mL) at 37ºC for 48 h under microaerophilic conditions, were spread on pollen-agar medium. Fungal explants (7 mm in cross section) from MY20 medium, were put in the middle of each plate. They were incubated for 10 days at 30ºC under microaerophilic conditions [17].

B) 25 µL of bacterial suspensions (approx. 1x10^5 CFU/mL) were placed into wells made in pollen-agar plates and incubated under microaerophilic conditions for 4 days. Fungal explants were put at 2 cm of its and incubated for 10 days at 30ºC.

2.6 Lactic acid effects on Ascosphaera strains

To simulate inhibitory effects of lactic acid produced by bacterial strains, it was added to pollen liquid medium (pollen 30 g, water 1 L) to obtain the pH values of 2, 3 and 4. *Ascosphaera* strains were inoculated and cultures were incubated 10 days at 30˚C under microaerophilic conditions. Also the bacterial strains were inoculated into *Ascosphaera* cultures without lactic acid. The initial and final pH were measured after 10 days.

2.7 Statistical analyses

All analyses were carried out according to ANOVA and Tukey test. Results were considered significant at the $p<0,05$ level, using InfoStat [9].

3. RESULTS

3.1 Isolation and phylogenetic characterization of Lactobacillus strains

Three *Lactobacillus* strains isolated from beebread were identified, and their sequences were deposited in GenBank database. They were *L. melliventris* LSAM (MF435936), *L. kunkeei* LSAJ (MF435935) and *L. helsingborgensis* LSAI (MF435934). These are a 100% identity with other strains of the Genbank database (NCBI).

The phylogenetic tree showed that all these strains formed a monophyletic clade closely related (see figure 1). *L. johnsonii* CRL1647 and AJ5 were more associated to strains from Jujuy but *L. salivarius* C22, *L. johnsonii* IG9, *L. johnsonii* A15 formed other monophyletic clade. These strains were isolated from other materials.

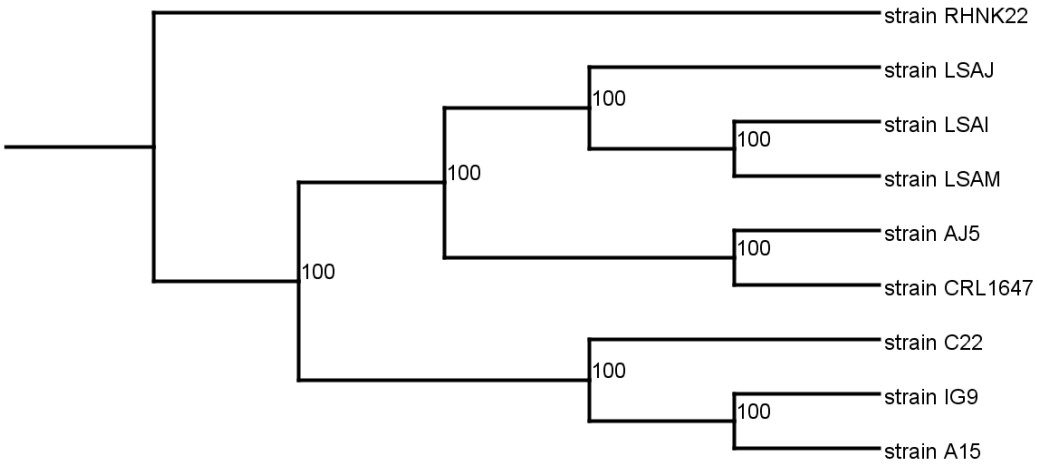

Figure 1: Phylogenetic tree based in the 16-rRNA gene sequences analyses, showing the relationship of *Lactobacillus* strains isolated from samples of Jujuy (*L. melliventris* LSAM (MF435936), *L. kunkeei* LSAJ (MF435935) and *L. helsingborgensis* LSAI (MF435934). The strains *L. johnsonii* AJ5 (EU428008), *L. johnsonii* CRL1647 (EU428007), *L. salivarius* C22 (EU428010), *L. johnsonii* IG9 (EU780913), *L. johnsonii* A15 (EU428009) were isolated in Salta from other materials. The sequences of *Bacillus amyloliquefaciens* RHNK22 (NZ_LMAG01000018.1) served as an outgroup. Numbers indicate bootstrap percentages for branch points.

3.2 Inhibition of Ascosphaera species

A) Fungal strains showed a delay in growth when they were incubated on lactic acid bacteria cultures in comparison with controls, and this effect on growth was statistically significant ($p<0.01$). Microscopic observation of fungi controls showed ascospores production at 10 days because both mating types of *A. apis* were present in explants (see table 1).

B) *Ascosphaera* strains grew without sporulation (see figure 2)

Table 1: Effect of the bacterial strains *L. melliventris* LSAM, *L. kunkeii* LSAJ, and *L. helsingborgensis* LSAI, on the growth of *A. apis* strains.

Ascosphaera apis	Growth (mm)		
	P1	P4	P6
Control	44.6±1.5	45±5	66.3±3.2
L. melliventris LSAM	38.6±3.2*	19.3±1.5*	28.6±1.5*
L. kunkeii LSAJ	11±1*	0	27.3±1.5*
L. helsingborgensis LSAI	0	0	12.6±2.5*
*Strains without ascospores after 10 days			

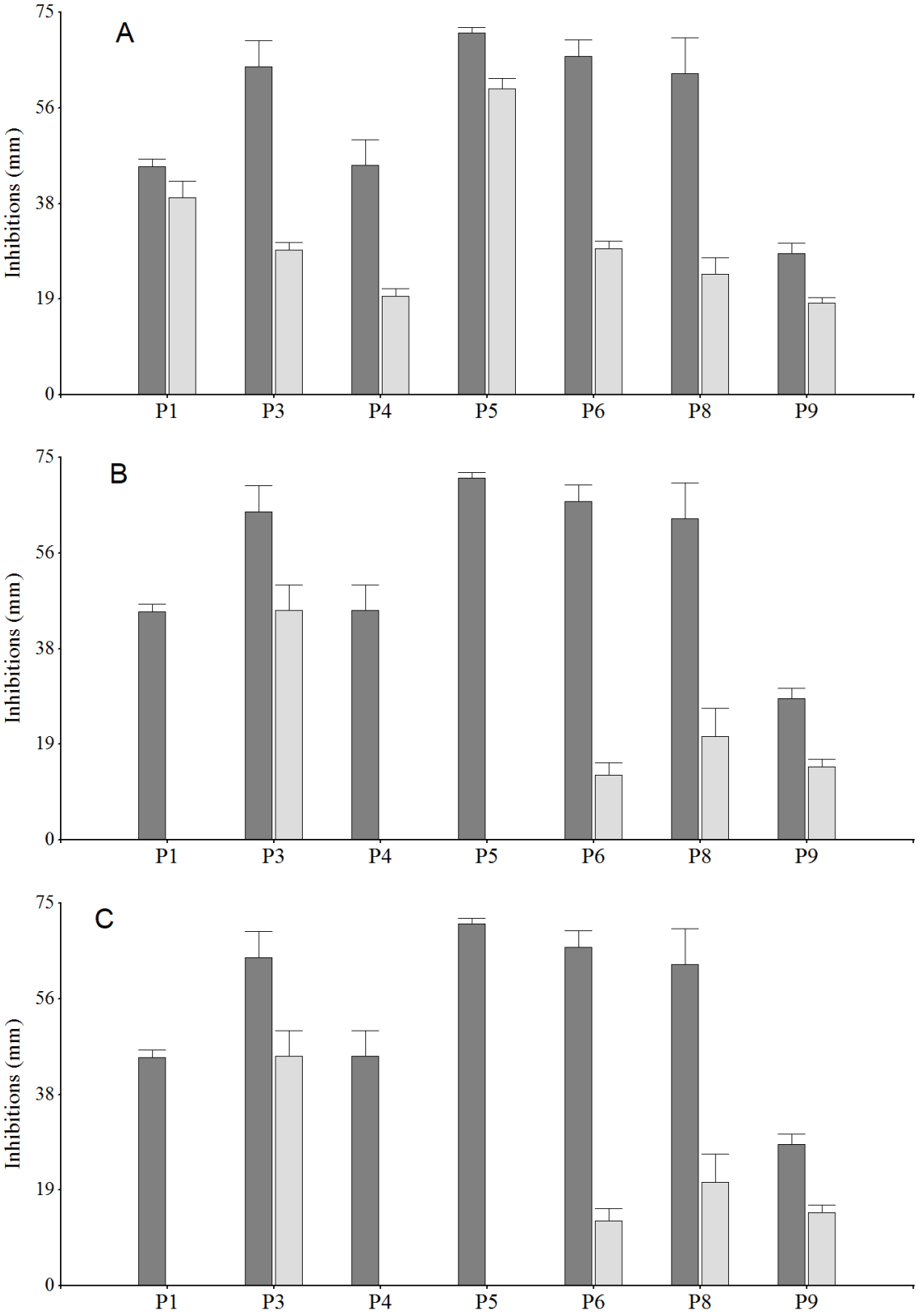

Figure 2: Effect of the bacterial strains (a) *L. melliventris* LSAM, (b) *L. kunkeii* LSAJ, and (c) *L. helsingborgensis* LSAI, on *A. apis* and *A. atra*. Dark grey bars are the fungal diameter in mm of controls, and bar grey light indicate the fungal growth in front lactic bacteria.

3.3 Lactic acid effects on fungal growth

Ascosphaera strains only grew at pH 3 and 4 but they no shown any spore production at 10 days.

4. DISCUSSION

The fungi *A. apis* and *A. atra* affect bee larvae when they ingest the spores that germinate in the hindgut. The mycelia reach the abdomen and penetrate the cuticle appearing on pupa body surface [19]. Decrease in ascospore number may contribute to the reduction of hive damage.

The gut microbiome of invertebrates is a complex association of microbial cells that collectively performs essential functions for the host. In different arthropods, included insects, gut bacteria can prevent the growth of pathogens microbes by a process known as colonization resistance and the addition of probiotic bacteria in diet can significantly improve disease resistance [10].

The lactic bacteria contribute to fermentation of corbiculae pollen and beebread production [18]. The three species of *Lactobacillus* isolated from beebread for the first in Jujuy, formed a monophyletic clade separate from bee intestinal strains isolated by other authors in Salta. These last had exhibit beneficial effect to honeybees [4, 5, 8, 11, 13].

L. melliventris and *L. helsingborgensis* were isolated from bee gut and stomach [15] but they were not mentioned as beneficial. *L. kunkei* reduced the mortality of bee larvae infected with *Paenibacillus larvae* [1].The antimycotic effects produced by lactic bacteria include organic acids, cyclic dipeptides, proteinaceous compounds, and others [7].

5. CONCLUSION

Lactic bacteria isolated in this work affected *Ascosphaera* spp. growth and sporulation. They can provide to beehive a protection against these entomopathogen fungi.

6. ACKNOWLEDGMENT

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