Characterization and identification of lactic acid bacteria from Mexican stingless bees (Apidae: Meliponini)

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Abstract. Stingless bees (family Apidae; tribe Meliponini), native from Mexico, are essential in tropical ecosystems. They are responsible for the pollination of many crops, native flora, and honey production. Lactic Acid Bacteria (LAB) are a regular host of the bee microbiota. LAB provide beneficial effects such as decreasing bacterial and parasitic pathogens infections and enhancing beehive honey production. Four different stingless bee species were sampled in the southeast of Mexico (Veracruz State) and identified as Melipona beecheii, Scaptotrigona pectoralis, Plebeia llorente and Plebeia jatiformis. Twelve LAB strains were isolated from the bee gastrointestinal tract and characterised by microbiologic features, carbohydrates fermentation profile, antibiogram and phylogenetic reconstructions through distance and Bayesian inference methods, selecting two genes with hypervariable regions (16S rRNA and pheS). The species were characterised as gram-positive and catalase-negative as rods and cocci. Moreover, most of the species identified were able to use diverse polysaccharides as the only carbon source. Lactobacillaceae resulted in resistance to ciprofloxacin and Leuconostaceae to cotrimoxazole. Finally, ten strains could be identified by both phylogenetic reconstructions as Lactiplantibacillus plantarum (2), Weissella paramesenteroides (3), Leuconostoc citreum (2), and Apilactobacillus spp. (3). This is the first report of LAB isolated from Mexican stingless bees to the best of our knowledge. Keywords: Stingless bees, microbiota, Lactic Acid Bacteria, identification, pheS gene

1. Introduction
The Insects are the most diverse group of animals on the planet [1]. They are regular inhabitants of multiple ecosystems, especially in tropical regions where abundant flora and fast trophic webs have favored diversification of feeding habits [2]. The emergence of angiosperm allowed the evolution of pollination and highly specialized pollinators [3], such as Hymenoptera (bees and wasp), Lepidoptera (butterflies and moths), Diptera (flies) and Coleoptera (beetles), which are the principal pollinators [4].
Bees (family Apidae) are the major pollinator of flowering plants, providing several ecosystem services such as fertilizing 73% of native flora and crops [5].

Among bees, stingless bees (tribe Meliponini) are widely distributed in all tropical and subtropical regions of the world, being remarkably diverse in the Neotropics, with approximately 33 genera and 418 species [6]. For example, in Mexico, 46 species of stingless bees are found [7]; some of these species have cultural importance due to historical use for food production, health, and even ceremonial rituals by some native cultures of Mesoamerica [8]. The principal genera found in the region, both wild and cultivated, are Melipona, Scaptotrigona and Plebeia [9], primarily established in the perennial subtropical forest from Mexico and Central America [7]. However, stingless bee populations are threatened by several factors like habitat loss, pollution, pathogens and the introduction of exotic species, principally European honeybee (Apis mellifera), which compete for food and nesting resources with native bees [10].

Studies of insect gut microbiota have found characteristics that may benefit their hosts by degrading complex molecules like cellulose [11] and decreasing pathogen infections [12]. Currently, the majority of microbiota studies have been done in honeybees (A. mellifera), through the analysis of microbiota composition and the quantification of the microorganism abundance and diversity through culture-dependent (cultivation of microorganisms) and culture-independent (metagenomics) methods [13-15]. First studies of microbiota in Melipona fasciata (a stingless bee) found bacilli able to ferment diverse carbohydrates [16]. At present, several studies in stingless bees aim to identify its core microbiota and identify bacterial species that may enhance bee health and honey production, which is especially important for the conservation and benefit of honey-producing stingless bees [17-19].

Bee microbiota is established through feeding with plant material like nectar and pollen, with several phyla of bacteria present in its gastrointestinal tract, being Actinobacteria and Firmicutes the most abundant [16]. Several genera of lactic acid bacteria (LAB) have been related with insect hosts, especially with pollinators due to highly specialized features in lifestyle, such as Apilactobacillus, Bombilactobacillus, Fructilactobacillus, Lactiplantibacillus and Lactobacillus (family Lactobacillaceae), Fructobacillus, Leuconostoc and Weissella (family Leuconostocaceae) [20]. Particularly, Apilactobacillus kunkeei has been isolated from most studies from honeybees, stingless bees and bumblebees [21].

The ability of LAB for degrading diverse carbohydrates is a desirable trait in bee microbiota. In the gastrointestinal tract, they are exposed to a nutrient-rich environment where plant products, like polysaccharides, disaccharides and other carbohydrates are abundant, and this catabolic activity may help bees enhancing their metabolic features like phytomolecules degradation, such as lignin, cellulose and hemicellulose, as well fatty acid production, increasing host nutrient intake [22]. Another important benefit of microbiota is protecting against bacterial and parasitic pathogens through direct inhibition via production of antimicrobial metabolites like bacteriocins and peptides, and indirect inhibition due to decrease in medium pH inhibiting growth of pathogenic bacteria host immune system stimulation [23].

Identification and differentiation of LAB may be difficult due to two characteristics, the metabolic and morphological traits of different species may result in ambiguities, and it is not a reliable source for identification [24]. The genetic identity of closely related strains or genus could not be sufficient to discriminate these entities, being in some cases close to 99.8% of identity in 16S rRNA gene sequences between species [24]. To facilitate differentiation, some authors have proposed using additional genes to 16S rRNA identification, like pheS, 23S rRNA and rpoA [25-26], with the appropriate bioinformatics analysis like Bayesian inference method or maximum likelihood [27].

In the present research, LAB from the gastrointestinal tract of four different stingless bee species (Melipona beecheii, Scaptotrigona pectoralis and Plebeia jatiformis and Plebeia llorenteii) native from the subtropical region in Mexico were isolated and identified using bioinformatics analyses of 16S rRNA and pheS (phenylalanyl-tRNA synthetase) partial gene sequences. In addition, to evaluate possible desirable traits, the fermentation profile and the antibiotic resistance of the isolates were also determined.
2. Materials and methods

2.1. Stingless bee sampling and identification
Sampling was carried out in Teocelo, Veracruz, (Mexico) (19.98° N; 96.98° W). Specimens were kindly donated by "El rinconcito" bee farm. First, four of the most honey productive stingless bee species were selected and identified through morphological features: *Melipona beecheii*, *Scaptotrigona pectoralis*, *Plebeia llorente* and *Plebeia jatiformis* [28], as shown in figure 1. Then, 10 to 15 individuals of each species were sampled directly from hives and stored in ice-cold tubes for two h to euthanize bees.

![Figure 1. Spectroscopic photography of collected stingless bees.](image)

a) *Melipona beecheii*; b) *Scaptotrigona pectoralis*; c) *Plebeia llorente*; d) *Plebeia jatiformis*. Photographs by Quintos-Andrade

2.2. Isolation of LAB from stingless bee gastrointestinal tract
Five individuals of each species were aseptically dissected to extract its gastrointestinal tract with special attention on its honey stomach. Then, sections were homogenized in 1 mL of sterile saline 0.9%, aliquots of 10 μL were cultured in Man Rogosa, and Sharpe (MRS) agar (BD, France) supplemented with fructose 2% (Meyer, Mexico) at 30 and 37 °C for 72 h under microaerophilic conditions. Colonies corresponding to gram-positive and catalase-negative rods and cocci were selected and subcultured until isolation [27]. Isolates were preserved at -20 °C in 25 % glycerol (Golden Bell, Mexico) until further analysis.

2.3. DNA isolation, amplification and sequencing of genes 16S rRNA and pheS
The genomic DNA extraction was carried out using Wizard Genomic kit (Promega, USA), according to the manufacturer instructions. Genes *rRNA 16S* and *pheS* were selected for the identification of LAB. PCR amplification programs consisted of (1) 7 min at 95 °C, (2) 35 cycles of 1 min at 94 °C+1 min at 63 °C+1 min at 72 °C and (4) a final 10 min at 72 °C for 16S *rRNA* gene with primers UniBac-F/R [29] and (1) 7 min at 95 °C, (2) 35 cycles of 1 min at 94 °C+1 min at 61 °C+1 min at 72 °C and (4) a final 10 min at 72 °C for *pheS* gene with primers pheS-21F/22R [25] in a T100 thermal cycling (BIO-RAD, USA). PCR products were sequenced using Genetic Analyzer 3130xl sequencer (Applied Biosystems, USA) to obtain 816 and 495 bp sequences, respectively.

2.4. Phylogenetic analysis of genes 16S *rRNA* and *pheS* by distances and Bayesian inference
A total of 94 sequences corresponding to both genes of several species of LAB were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/) and aligned to sample sequences using MUSCLE
algorithm in UGENE v3.3.0 [30]. The alignment of pheS gene was translated to amino acids. Two methods of phylogenetic analysis were evaluated. First, the distances method was made using PAUP v4 [31], for phylogenetic reconstruction F84 model and BLOSSUM62 matrix were used for 16S rRNA and pheS alignments respectively with 1000 bootstrap replicates with NNI approach. Second, the Bayesian inference method was evaluated using BEAST v2.6.3 [32] for phylogenetic reconstruction GTR model and BLOSSUM62 matrix for 16S rRNA and pheS alignments, respectively with a strict molecular clock, Yule model and considering gamma function. All trees obtained were annotated and edited using iTOL v5 [33].

2.5. Carbohydrate fermentation profile

Sixteen sugars were tested. Eight monosaccharides: arabinose, fructose, galactose, glucose, mannitol, mannos, sorbitol and xylene; seven di or tri-saccharides: cellobiose, lactose, maltose, melibiose, raffinose, sucrose and trehalose; and one glycoside: salicin (all from Sigma-Aldrich, USA). MRS broth without dextrose was supplemented with 0.01% of bromocresol purple (BD, USA). Then, each carbohydrate was added at a final concentration of 2% (w/v). Next, each isolate was added at 1% and incubated for 75 at the optimal temperature of each strain (30 to 37 °C). The test was carried out in 96 wells microplates [34]. A color-changing in media from purple to yellow was considered as positive evidence of carbohydrate fermentation.

2.6. Antibiotic resistance

The disk diffusion method was used to determine antibiotic resistance according to the manufacturer [35]. Amoxicillin 10μg, amoxicillin/clavulanic acid 15μg, erythromycin 15μg, cotrimoxazole 25μg, cephalexin 30μg, cephalozin 30μg, cefuroxime 30μg, ciprofloxacin 5μg, ofloxacin 5μg, piperacillin 100μg, azithromycin 15μg and tetracycline 30μg were used as antibiotics. For this analysis, a Combi Disc kit (Accutrack, Mexico) was used.

3. Results and discussion

3.1. Isolated LAB strains

A total of twelve strains corresponding with the usual phenotype of LAB were isolated from collected stingless bees. Their microbiological features are described in Table 1. The most number of strains was isolated from M. beecheii (5 spp.) followed by S. pectoralis (4 spp.) and P. jatiformis (3 spp.). Unfortunately, no LAB strains were recovered from P. llorentei. Similar numbers of isolated strains are reported in other culture-dependent studies [12, 15, 19].

| Isolated strains | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 | M10 | M11 | M12 |
|------------------|----|----|----|----|----|----|----|----|----|-----|-----|-----|
| Growth (°C)      | 30-| 20-| 20-| 20-| 20-| 15-| 20-| 20-| 20-| 20-  | 20-  | 20-  |
| Shape            | R  | R  | R  | R  | R  | O  | O  | O  | O  | O    | O    | O    |
| Clustering       | S  | S  | S  | C  | P  | C  | C  | P  | C  | P    | P    | P    |
| Gram             | +  | +  | +  | +  | +  | +  | +  | +  | +  | +    | +    | +    |
| Catalase         | -  | -  | -  | -  | -  | -  | -  | -  | -  | -    | -    | -    |
| Colony colour    | W  | W  | W  | W  | W  | T  | W  | T  | B  | W    | W    | B    |
| Colony size (mm) | 1.5| 1  | 1.5| 1.5| 1.5| 1.1| 1.1| 1  | 1.2 | 1.5  | 1.5  | 1.5  |
| Aggregation      | +  | +  | +  | -  | -  | +  | +  | +  | +  | -    | -    | +    |
| Gas production   | +  | +  | +  | -  | +  | +  | +  | +  | -  | +    | +    | +    |

Notes: R, rod cells; O, ovoid cells; C, short chains; P, pairs; W, white; T, translucent; B, beige; +, positive reaction; -, negative reaction; Mb, strains isolated from M. beecheii; Sp, strains isolated from S. pectoralis and Pj, strains isolated from P. jatiformis
The microbiological characteristics of strains Mb-1, Mb-2, Mb-3, Mb-4 and Sp-2 exhibited the usual phenotype of lactobacilli [24], forming white, punctiform colonies of gram-positive, catalase-negative rods. Similar to strains Mb-5, Sp-1, Sp-3, Pj-1 and Pj-2, which produced similar colonies of gram-positive, catalase-negative ovoid cells, according to the usual phenotype of Leuconostocaceae family [24]. Strains Sp-4 and Pj-3 showed a different cell morphology of big beige colonies with creamy texture of gram-positive, catalase-negative large rods. Additionally, strains Mb-1, Mb-2, Mb-3, Sp-1, Sp-3, Sp-4 and Pj-3, presented an evident aggregation phenotype, forming a clear precipitate when cultured on broth. This characteristic is relevant for host health, facilitating colonization surfaces in the gastrointestinal tract [36].

3.2. Genetic identification of LAB strains
Through distance method by 16S rRNA alignment, a monophyletic clade with strains Mb-1, Mb-2 and Mb-3 were recovered as members of *Apilactobacillus* genus with high confidence. However, it was not possible to circumscribe them in a specific taxonomic entity because they were recovered as a monophyletic group among other *Apilactobacillus* species in the phylogenetic reconstruction. Strains Mb-4 and Sp-2 were recovered as *Lactiplantibacillus*, Mb-5, Pj-1 and Pj-2 as *Weissella paramesenteroides* and Sp-1, Sp-3, Sp-4 and Pj-3 as members of *Leuconostoc*. With *pheS* alignment, most recoveries were conserved. However, *pheS* reconstruction better differentiated highly similar species *i.e.* *Lactiplantibacillus plantarum* from *Lactiplantibacillus pentosus* [24, 25] and grouping strains Mb-4 and Sp-2 and other strains of *L. plantarum*. Nevertheless, lower accuracy recognizing basal clades was found, mixing genus from Lactobacillaceae and Leuconostocaceae families. In contrast, through Bayesian inference method, both reconstructions resulted in fewer ambiguities, showing that this approach is better for these sequences. 16S rRNA phylogeny (Figure 2a) resulted in similar species clustering, identifying Sp-1, Sp-3, Sp-4 and Pj-3 as *Leuconostoc citreum*. With *pheS* phylogeny (Figure 2b) none strain was recovered as a member of *Leuconostoc* genus.

![Figure 2. Phylogenetic tree of isolated strains constructed through Bayesian inference method. a) Partial sequence of gene 16S rRNA; b) Translated protein of partial sequence of *pheS* gene. Principal genus related to bees (*Apilactobacillus; Lactiplantibacillus; Weissella; Leuconostoc*) is highlighted in grey blocks. Isolated strains are highlighted in bold type.](doi:10.1088/1755-1315/858/1/012010)
Considering microbiological and genetic characters, eight strains were identified without discrepancies and strongly supported by genetic and morphological evidence. Mb-1, Mb-2 and Mb-3 were identified as *Apilactobacillus* spp. Mb-4 and Sp-2 as *L. plantarum* and, Mb-5, Pj-1 and Pj-2 as *W. paramesenteroides*. Strains Sp-1 and Sp-3 presented some discrepancies between 16S rRNA and *pheS* phylogenies but, according to cell morphology and 16S rRNA phylogeny, are identified as *L. citreum*. Isolated species concur with previous microbiota analysis from stingless bees [13-16, 36-39]. The last strains Sp-4 and Pj-3, showed several discrepancies in genetic identification and microbiological features, hence being unable of circumscribing these strains in a taxonomic entity. According to the information of these analyses, we propose that isolated strains correspond to species shown in table 2.

**Table 2. Genetic identification of LAB isolated strains**

| Bee species         | Identified species         | Strain |
|---------------------|---------------------------|--------|
| *M. beecheii*       | *Apilactobacillus* sp.    | Mb-1   |
|                     | *Apilactobacillus* sp.    | Mb-2   |
|                     | *Apilactobacillus* sp.    | Mb3    |
|                     | *Lactiplantibacillus* plantarum | Mb-4 |
|                     | *Weissella paraamesenteroides* | Mb-5 |
| *S. pectoralis*     | *Leuconostoc* citreum     | Sp-1   |
|                     | *Lactiplantibacillus* plantarum | Sp-2 |
|                     | *Leuconostoc* citreum     | Sp-3   |
|                     | *Undetermined* LAB        | Sp-4   |
| *P. jatiformis*     | *Weissella paraamesenteroides* | P2-1 |
|                     | *Weissella paraamesenteroides* | P2-2 |
|                     | *Undetermined* LAB        | P2-3   |

Notes: Species determined according to phylogenetic reconstructions and microbiological characteristics.

### 3.3. Carbohydrate fermentation profile

The carbohydrate fermentation profile is shown in Table 3. Strains identified as *Apilactobacillus* spp. showed a typical phenotype of homofermentative lactobacilli, with unusual gas production, probably due to CO2 production from other metabolic routes [38]. Strains identified as *L. plantarum*, *W. paramesenteroides*, *L. citreum* and unidentified strains resulted heterofermentative, showing a great capacity of hydrolyze complex carbohydrates from vegetable origin present in pollen and other plant structures (arabinose, cellobiose, fructose, maltose, mannitol, melibiose, raffinose, sucrose, sorbitol, and xylose) [22, 39]. This is related to bees’ feeding habits, providing bacteria with a nutrient-rich environment from pollen and nectar [19]. The ability to ferment diverse carbohydrates is a desirable trait for possible use as zootechnical probiotic for bees [40], since LAB microbiota may enhance nutrient intake from plant food [19, 22].

### 3.4. Antibiotic resistance

All strains were sensitive to most antibiotics (Table 4). However, most strains identified as Lactobacillaceae and Leuconostocaceae families showed resistance to ciprofloxacin and cotrimoxazole, respectively. It has been previously reported constitutive resistance to fluoroquinolones in lactobacilli [41] and cotrimoxazole in *Leuconostoc* [42].
4. **Conclusions**

A total of twelve LAB strains from the gastrointestinal tract of Mexican stingless bees were isolated in the present research. Five strains were isolated from *M. beecheii*, including *Apilactobacillus*, *L. plantarum* and *W. paramesenteroides*; four strains from *S. pectoralis*, *L. plantarum*, *L. citreum* and an unidentified LAB; and, finally three strains from *P. jatiformis* two strains of *W. paramesenteroides* and an unidentified LAB. Unfortunately, no LAB strains could be isolated from *P. llorentei*.

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**Table 3. Carbohydrate fermentation profile of isolated strains**

| Strains       | Mb-1 | Mb-2 | Mb-3 | Mb-4 | Mb-5 | Sp-1 | Sp-2 | Sp-3 | Sp-4 | Py-1 | Py-2 | Py-3 |
|---------------|------|------|------|------|------|------|------|------|------|------|------|------|
| **Monosaccharides** |      |      |      |      |      |      |      |      |      |      |      |      |
| Arabinose     | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | +    | +    |
| Fructose      | +a   | -    | +a   | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Galactose     | -    | -    | -    | +    | +a   | +    | +    | -    | +    | +    | +    | +    |
| Glucose       | +a   | +a   | +a   | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Mannitol      | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Mannose       | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Sorbitol      | -    | -    | -    | v a  | -    | +    | -    | -    | -    | -    | -    | -    |
| Xylose        | -    | -    | -    | +    | +    | +    | +    | +a   | +    | +    | +a   | +    |
| **Di-tri-saccharides** |      |      |      |      |      |      |      |      |      |      |      |      |
| Cellobiose    | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Lactose       | -    | -    | -    | +    | -    | +    | +    | +a   | -    | +    | +    | +    |
| Maltose       | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Melibiose     | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Raffinose     | -    | -    | -    | v    | +    | +    | +    | +a   | v    | -    | -    | -    |
| Sucrose       | +a   | +a   | +a   | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Trehalose     | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Glycoside     | -    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +a   | +    |

+, positive reaction; +a, weak positive reaction; -, negative reaction

| **Table 4. Antibiotic resistance profile of isolated strains** |
|---------------------|-------|-------|-------|
| Strain              | Mb-1  | Mb-2  | Mb-3  |
| Amoxicillin         | +     | +     | +     |
| Amoxicillin/        | +     | +     | +     |
| Clavulanic acid     | +     | +     | +     |
| Erythromycin        | +     | +     | +     |
| Cotrimoxazole       | -     | +     | +     |
| Cephalexin          | -     | +     | +     |
| Cefazolin           | +     | +     | +     |
| Ciprofloxacin       | -     | +     | +     |
| Ofloxacin           | +     | +     | +     |
| Piperacillin        | +     | +     | +     |
| Azithromycin        | +     | +     | +     |
| Tetracycline        | +     | +     | +     |

+, sensitive ; -, resistant
Similar LAB species were identified between the three stingless bee species, showing that these bees have resilient microbiota related to their specific environmental conditions and diet. Although this study may be limited to cultivable species of bacteria, culture-dependent methods allow to evaluate and characterize proper trains of bacteria that may be useful for future applications. Besides, isolated strains may participate in honey production or fermentation, making them useful in fermented food processing or as zootechnical probiotics for bees.

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