**Analysis of microbial diversity of the edible fruit of Guamuchil** *(Pithecellobium dulce*( Roxb) Benth)

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**Introduction**
Plants are host to diverse microbiome that might have coevolved since millions of years [1]. Microorganisms – both bacteria and fungi – that associate with plants can have beneficial, neutral or harmful effects on the host. Well-known examples of beneficial plant-microbe interactions include symbioses such as nitrogen fixation by root associated Rhizobiales [2], however multiples examples of antagonic roles by potential plant pathogens are also reported [3]. Unique bacterial populations are typical for different plant organs such as leaves, roots and seeds, and can be found on the surface or within the plant tissue, how do they reach such organs is still not clear. Microorganisms which colonize inner tissues without causing any symptoms of disease are defined as endophytes, and they may play an important role in the plant development i.e. providing anti-fungal activity or as growth promoting bacteria.

Guamuchil *(Pithecellobium dulce*( Roxb) Benth) tree, a plant belonging to the Fabaceae family, is an occurring component of the Mexican deciduous and subdeciduous forests. It is considered as a multipurpose tree; is used as live fence in cultivated land, and its fruit – a bent and round shape- is used as food for cattle and humans. The seeds are protected by a white, pink or a reddish pulp, which is the edible part (Figure 1). The fruit of this species can present two contrasting flavors: sweet and bitter.

![Figure 1](image_url)

**Figure 1.** Details of fruits of *P. dulce*. a) Mature legume, shell open, exposes aril and seeds to environment. b) Immature legumes, shell completely closed, inner tissues not exposed. c) Nude fruits, aril involving the seed, during ripening color changes from white to pink intensities. d) Seeds, when mature the seed turns to dark color.

Guamuchiles trees of sweet fruit are selected for harvesting. The mature fruits featuring an opened legume with the aryl and seeds exposed to the environment are appreciated by people. The exposition of edible part of guamuchil fruits suppose interactions with pollutant in the environment and possible microbial colonization which can generate potential risk to the human health. On the other hand, as a leguminosae species, *P. dulce* can be host of microbial endophytes or as seed born bacteria which could have biotechnological traits.

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In leguminous species different microbial studies have carried out with special attention to that of economic interest i. e. *Phaseolus* species [4], but analysis of the microbial diversity of trees are poor documented. The objectives of this study are: a) to characterize morphological and at the molecular level, the microbial communities associated to the fruits of *P. dulce*, and b) to identify the taxa exclusively associated to seed and those of the pulp. Additionally, by comparison with previously reports it will be possible to identify the presence of taxa that could represent potential risks to the human health. In the present report a morphological analysis of bacterial isolated from the fruits and the main genus identified by Sanger sequencing are reported.

**Methods**

Fruits were collected from at least five different trees of *P. dulce* located in Cajititlan, in the central region of Jalisco state, Mexico. Fruits collected for microbial analysis were classified into two categories: immature (intact shell, with pulp no exposed to environmental) and mature legumes (pulp exposed to environment) were used for microbial analysis. The goal of this differentiation is to distinguish the microorganisms naturally growing in the fruit from the opportunistic colonization when the legume gets open.

Samples were sterilized according to the next procedure; immature legumes and seed of mature fruits were washed in iodum solution (8 %) and rinsed with sterile water, an additional wash was performed with ethanol (70 %) and again rinsed with sterile water. All steps were carried out in sterile area.

Microbial growth was performed by the plate emptying technic in standard count agar, with a previous microbial enrichment phase. For enrichment 90 ml of brain hart infusion was added to 10 gr of each tissue and homogenized by vigorous shaking. The homogenate was incubated during 15 days at 35 °C and after this period, dilutions ranging from $10^1$ to $10^7$ were used to plate in petri dish and incubated during 48 hours at 35 °C. Once reached the time, colonies were counted and isolated in new petri dish containing fresh media (Figure 2b). In order to assess the efficiency of the sterilization protocol, complete sterilized seed were used as control, showing no bacterial growth during the incubation period (Figure 2a).

To evaluate possible microbial colonization in mature fruits, legumes with exposed aryls (pulp) were collected. With the aim of to assess possible effects over the taxa, fruits were classified as sweet and bitter according to their flavor. Arils enrichment was processed as mentioned above, however seeds processing had slightly differences. After sterilization, seeds were dissected into inner and outer section and the last was discarded. 10 gr of the inner section were mixed with 90 ml of brain hart infusion and shaked until homogeneization. The homogenate was used for plating as previously mentioned.

Apearing colonies from each plate were count and visualized in microscopy; shape, color, consistency, elevation, morphology and gram staining was recorded. Each different colony was isolated and grown for preserve in glycerol.

![Figure 2](http://www.e-gnosis.udg.mx/index.php/trabajosinocuidad)

**Figure 2.** Sterilization of seed surface, showing not microbial growing after incubation for 48 hr at 35 °C (a), and some bacterial isolates obtained from homogenates of triturated seeds.

For molecular characterization, DNA isolation from each bacteria, and a posterior PCR amplification of the 16S marker with the 27F and 1492R primer combination was performed (figure 3a and 3b). The products of the PCR reaction were gel visualized (agarose gels) and purified. Products were sequenced by the Sanger sequencing.
methodology and electropherograms were revised by available software in the web. The obtained sequences were blasted against the NCBI database (https://www.ncbi.nlm.nih.gov/) [5] in order to identify the most related genus and/or species of each bacterial isolate.

**Figure 3.** Representative image of DNA purification (a) and PCR amplification of the 16S molecular marker (b). PCR products were gel purified and sequenced.

**Results and discussion**
Recent reports about bacterial communities associated to surfaces of fruits and vegetables have demonstrated that these tissues can harbor large a diverse populations of bacteria [6]. In the case of this work bacteria and fungi were isolated as part of the microbiota of *P. dulce* fruits. Microscopic analysis revealed the presence of at least 37 bacterial taxa (Table 1) and two different fungi (data not shown). The most abundant group – bacteria – was evaluated according to different criteria, table 1 shows the results of staining, morphology and color features of each isolated taxa. Mature fruits with almost a 73 % of the total isolated taxa resulted with more colonization respect to immature fruits, this was probably due to the exposition of the aril and seed to environmental conditions.

**Table 1.** Distribution of the bacterial isolated taxa according to gram staining, morphology and color

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Gram staining</th>
<th>Morphology</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram positive</td>
<td>Gram negative</td>
<td>Coci</td>
</tr>
<tr>
<td>Mature</td>
<td>13</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Immature</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Bacterial taxa isolated from seeds represented the 60 % of the total, it was surprising, because it was easier to suppose that by the availability or nutrients, aryls could be faster colonized by the microorganism. Seed from immature fruits also observed higher bacterial taxa than pulp. In *Phaseolus*, a relative elevated amount of bacterial groups was also reported about 50 species were endophytic to the seed [4]. Respecting to the flavor, despite approximately 60 % of the taxa appeared in sweet fruits (data not shown) the flavor factor seems not to affect the distribution of bacterial communities.

The number of taxa of bacilli was bigger than that of coccal forms, and as could be expected, they were mainly presented in mature fruits. The color of the colonies was diverse, white, yellow, orange and transparent intensities were identified, about 60 % showed a white aspect, followed by yellow colonies. Features as shape, consistency and elevation were also evaluated. Circular and fusiform shapes were the predominant shapes representing 67 and 19 % respectively. Respect to the consistency creamy and mucous...
were predominant, and according to the elevation of the colonies, taxa with a flat elevation was the common (data not shown)

The ontogeny of the fruit of *P. dulce* involves mainly two stages, an immature and a mature phase. In immature fruits the legume is completely closed, and the inner structures (aryl and seed) are not directly exposed to the environmental conditions. On the other hand, the opening of the shell that covers the edible tissue and seed is characteristic mature fruits. During ripening, aryls and seed become exposed and it is probably that new microbial communities get established in the fruit, i.e. due to pollution or by visitors to the legume.

According to the comparison of microbial growth obtained from tissues of mature and immature fruits, 10 taxa were observed exclusively in the last (5 from aryl and 7 in the seed), while 28 taxa established during fruit ripening (13 from aryl and 15 from seed). Among the identified taxa, only three resulted in both types of fruits; one from the aryl and two from seeds. As can be deduced, an elevated number of taxa were established during dehiscence, the availability of nutrients and probably contamination of external groups can be the cause of the increased communities.

The first match of the 16S sequences obtained from NCBI database revealed the presence of different bacterial genus: *Staphylococcus*, *Exiguobacterium*, *Pantoea*, *Enterococcus*, *Bacillus*, *Kosakonia*, *Micrococcus* and *Pseudomonas*, were the most represented. Phylogenetic relationships of 20 isolates sequenced is shown in Figure 4.

**Conclusions**

By collecting fruits of *P. dulce*, it was possible to isolate and characterize morphologically different taxa of bacteria, currently 37 isolates have differentiated by its external features. By molecular strategies eight different genus were identified by comparison of the 16S sequences against the NCBI database. The presented results show that putative endophytic bacteria occur in seed before the opening of the legume, and that the number of opportunistic groups increases during ripening. In seeds of immature fruits, seed-associated bacteria were observed, how beneficial for the host are these microorganisms remains not clear, it is necessary to improve further analysis. However, as has been stated for other plant-endophyte interaction it could lead to plant-growth-promoting or as defense against pathogens by the delivering of antibiotics. The colonization of pulp and seed by specific bacteria may indicate different life strategies, and they represent a new challenge to elucidate in future studies.

**References**

Figure 4. Clustering representation of the bacterial isolates obtained from Guamuchil fruits (red diamond marked) with its most related species. UPMGA analyses was performed with a bootstrap of 1000 replicates.