

# TAXONOMIC DETERMINATION OF FOUR ACTINOBACTERIA STRAINS FROM "HONEY WATERS" AND COMPOST FROM AN ORGANIC COFFEE PRODUCTION SYSTEM.

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ELECTIVE SUBJECT: MOLECULAR BIOLOGY

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## SUMMARY

The coffee industry in Colombia occupies one of the first economic fields with an annual production that reached 14 million bags of 60 kg in 2015.

There are two processing methods, dry and wet. The latter is the most used in the country and requires large amounts of water to remove a mucilaginous layer of the grain composed of sugars, proteins and lipids. This method generates solid and liquid organic wastes whose degradation involves organisms such as Actinobacteria. This group of bacteria have a high potential in the production of antibiotics, enzymes, combustible gases, among other compounds. In the present work, we intend to characterize taxonomically, through morphological data and molecular data of the 16S gene, four strains isolated from solid and liquid residues from an organic coffee crop. Finally, the results will be documented in the Laboratory of Industrial Biotechnology and Molecular Biology -CINBIN- for future projects of evaluation of its biotechnological potential.

## DESCRIPTION OF THE PROBLEM

In Colombia, coffee production and trade occupy one of the first economic fields with an annual production of 14.2 million bags of 60 kg in 2015 (FCN). However, it is considered one of the most polluting industries in the world, altering the environmental balance and quality of life of the human being (Alfaro and Rodríguez, 1994; Matuk, 1997; Alvarez et al., 2011).

Two processing systems are known in the coffee agro-industry: Dry and wet. Dry production uses dry coffee with all its outer covers and tissues when it is still in the plant or after being harvested. In this way, processing is simplified and natural coffees are obtained as a result. In wet processing, the process is more complex, since it involves the removal of a mucilaginous layer that is 16 to 22% of the weight of the ripe fruit, in order to obtain soft, washed and better quality coffees (Alfaro and Rodríguez , 1994). The latter process is the most used in Central America and Colombia (Álvarez et al, 2011). Removal of the mucilage by wet production can be done by biochemical treatment or by natural fermentation (Puerta, 2009). This method involves the use of large quantities of water, with an average consumption of 40l/ kg of dry coffee (de-pulped), contaminating water sources with organic matter from by-products (Cenicafé, 2011).

The residual waters, called "honey waters", are composed of a mixture of sugars, proteins, lipids and phenolic compounds, which by their content must be treated in purification systems (Avallone et al., 2000; Avallone et al., 2001 ; Alvarez et al., 2011). The "honey waters", when they are discharged to water sources release large amounts

of organic matter. Bacteria are responsible for degrading this organic matter using dissolved oxygen (DO). When oxygen is consumed, nitrates ( $\text{NO}_3^-$ ) and sulphates ( $\text{SO}_4^-$ ) will be metabolized, which give rise to hydrogen disulfide ( $\text{SH}_2$ ), responsible for the bad odor of the water, causing a detrimental effect on health and the environment (ANACAFÉ, 2011).

In view of the environmental problems caused by "honey waters", many strategies have been implemented to reduce water consumption and treat organic waste with artisanal anaerobic systems (Matuk, 1997, Cadena, 2005, Rodríguez and Zambrano, 2010 and Cenicafé, 2011). On the other hand, the solid organic residues of the coffee are processed by aerobic systems to produce compost and to obtain renewable energies like biogas (mainly methane), liquid biofuels like bioethanol, biodiesel and biobutanol, and solid biofuels like coal (McKendry et al., 2002), and in the case of wood chips, sawdust, pellets and briquettes (Cerdá, 2012; Lomas et al., 2001; McKendry, 2002; Minminas, 2011; Serrano & Luque, 2011).

In the present work, we intend to classify taxonomically the MFSR 17-18 isolates purified from honey-waters and ACPG 11-18 of compost residues from an organic coffee crop. For Identification macroscopic and microscopic data as well as sequence analysis of the 16S rRNA gene will be used. In the future, these strains could be used for biotechnological applications and reduce the environmental impact of the coffee industry in the country.

## THEORETICAL FRAMEWORK

In the transformation of organic matter and recalcitrant molecules (eg, cellulose, lignin and chitin) different groups of microorganisms participate, mostly bacteria of the phylum Actinobacteria (Goodfellow & Williams, 1983). Actinobacteria is one of the largest taxonomic groups within the Bacteria domain. Most are Gram positive filamentous bacteria, with high G + C content in their DNA (about 51% to 70%), morphological, metabolic and physiological variety (Gathogo et al., 2004; Ventura et al., 2007; Goodfellow Et al., 2012). In addition, it is characterized by the production of compounds with biological activity (Fenical & Jensen, 2006). Two-thirds of the antibiotics currently used are produced by actinobacteria, such as streptomycin, tetracycline, chloramphenicol and erythromycin, obtained from different species of the genus *Streptomyces* (Challis & Hopwood, 2003).

According to Zambrano (1994), each kg of cherry coffee releases 91 ml of mucilage. According to Montilla (2006), for each kg of cherry coffee 149 g of mucilage are generated and it is thought that from a kg of mucilage, 55 ml of ethanol could be obtained. The purpose of removing the mucilage is to facilitate the drying of the grain without deteriorating the quality by the effects of fermentation.

In the natural fermentation of "honey waters" the mucilage is hydrolyzed and degraded by pectins released by fungi and bacteria. The quality of the fermentation, as well as the duration of the fermentation, is determined by several factors such as temperature,

maturity of the coffee fruit and quantity of recalcitrant molecules and organic matter (Pineda, C. R, 1995).

Compost is used as a fertilizer and source of organic matter in crops because of its low N concentrations (Hartl et al., 2003). Information about the microorganisms present here could help ensure the quality of compost and the production of biofuels.

There are studies that highlight the importance of actinobacteria for degradation of organic matter and on the microbiological composition of the compost, including biochemical, morphological and molecular methods, with genera such as *Micrococcus*, *Arthrobacter*, *Nocardia*, *Pseudonocardia*, *Saccharopolyspora* and *Streptomyces* (Cruz et al., 2009; Rebolledo et al., 2008; Steger et al., 2007). However, the scientific literature on the diversity of actinobacteria involved in the biodegradation of "honey-waters" is insufficient.

In the laboratory of Industrial Biotechnology and Molecular Biology (CINBIN) were carried out two previous works on the diversity of the microbiota present in compost and honey-waters of Hacienda el Roble in the municipality Mesa de los Santos, Bucaramanga, Colombia. Since actinobacteria, like fungi, are efficient in the degradation of lignocellulose and phenolic compounds (Ramírez & Coha, 2003), it is important to characterize this group of bacteria.

## MATERIALS AND METHODS

### MACRO AND MICROSCOPICAL CHARACTERIZATION OF CRIOPRESERVED STRAINS

Cryopreserved strains were recovered in Czapek-Dox agar selective medium -Extraction of Yeast - Casamino acids (CYC), pH 7.0 (Ramirez and Coha, 2003). Cultures were incubated at room temperature ( $> 25^{\circ}\text{C}$ ) for a period of 8 Days in which the following aspects were monitored:

- Macroscopic characterization:

Each of the isolated colonies had a continuous follow-up recording observations such as morphology, type and color of the colony (Ramírez and Coha, 2003; Otero, 2011).

- Microscopic characterization.

Gram staining was performed at different culture times (3 and 8 days) (Wang et al. Al., 1999; Otero, 2011). The presence and availability of spores with Wirtz-Conklin stain 0.1% malachite green and 0.5% safranin (Schaeffer and MacDonald, 1933).

## IDENTIFICATION THROUGH 16S RNA GEN SEQUENCING

### - Molecular identification of actinobacteria.

Total bacterial DNA extraction was performed by washing with Phenol / chloroform / isoamyl alcohol according to the protocol proposed by Ausubel et al. (1992). An agarose gel electrophoresis was performed to corroborate the quality of DNA extracts.

### - Amplificación por PCR del gen 16S rRNA y secuenciación

La amplificación del gen 16S rRNA para cada cepa se realizó mediante PCR, utilizando los oligonucleótidos 27F y 1492R (Lane et al., 1991), 1159R (Blackwood et al., 2005); 338F (Guo et al., 2008) y 907R (Muyzer et al., 1993), 1159R. The obtained amplified were sequenced by capillary electrophoresis by Macrogen Inc. (South Korea).

### - Analysis of 16S rRNA gene sequences

A search for reference sequences using BlastN in the baseNCBI (National Center for Biotechnology Information) was performed. Bergey's reference sequences (2012) for distance analysis were obtained.

## GENETIC DISTANCE ANALYSIS WITH THE SEQUENCES OBTAINED AND REFERENCE SEQUENCES

With the reference sequences, alignments were constructed with the algorithm Muscle (Edgar, 2004). Matrices and distance trees were constructed by Neighbor-Joining method (Saitou and Nei, 1987) in the MEGA 7 program.

## TAXONOMIC DETERMINATION THE STRAINS WITH THE RESULTS OBTAINED FROM MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION.

Based on the analysis of the obtained sequences and the macro and microscopic data the taxonomic determination of the four strains was made. For the attribution of taxa, sequences with identity values, preferably above 95% and coverage greater than 90%, were taken into account. Sequences with genetic identity  $\geq 95\%$  were assigned to the genus category (Ludwing et al., 1998) and  $\geq 97\%$  for species (Stackebrandt and Goebel, 1994).

## RESULTS

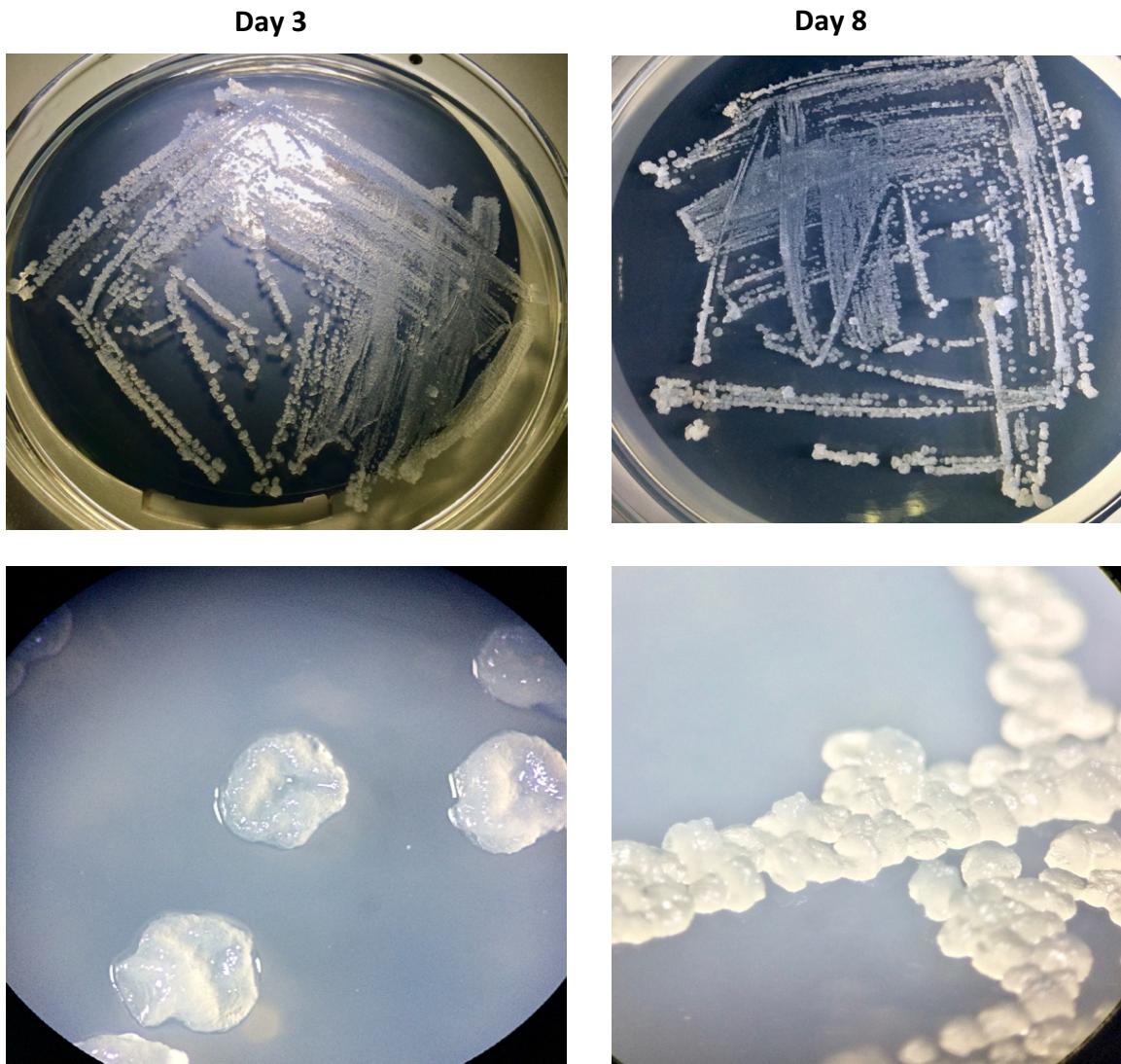
### 1. CHARACTERIZATION MACRO AND MICROSCOPIC OF THE STRAINS

The four cryopreserved strains were seeded in a selective agar-Czapek-Dox (ACD) - Extract Yeast-Casamino Acid (CYC) medium, pH 7.0 at room temperature. A replicate was made for each strain corresponding to the monitoring day three and eight.

## Strain MFSR-17

- ✓ Macroscopic characterization.

Colonies similar to molar teeth between 3-6 mm, bone color, hard consistency, granular texture, irregular margin and tendency to form protrusions, elevation of the colonies high. They do not diffuse pigment into the medium.

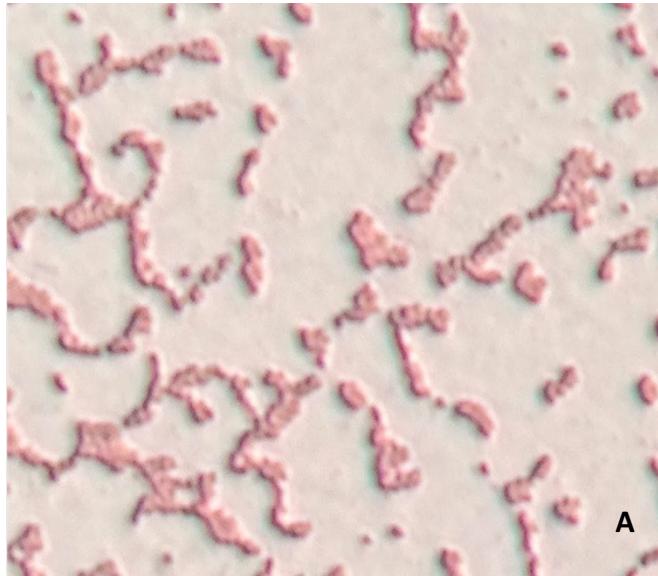


**Fig 1.** Stereoscope view day 3 and day 8.

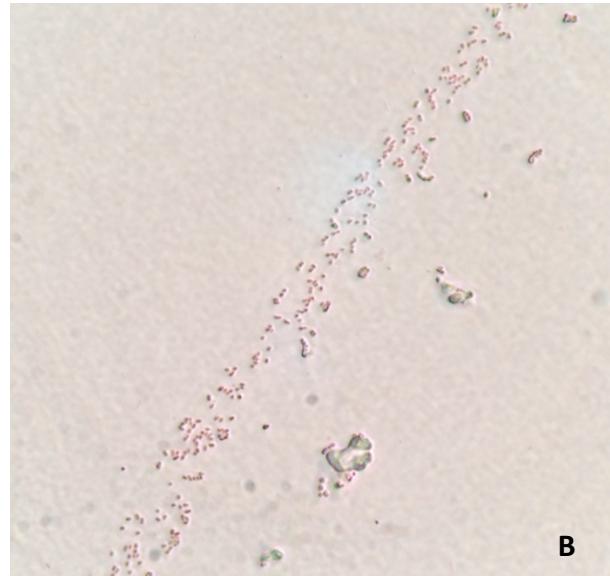
✓ Microscopic characterization.

(A.B) Gram staining in which Gram positive cocci are observed on day 3 and 8 respectively. As the strain grows, disintegration of the coconuts. C) Malachite green. No spores were found.

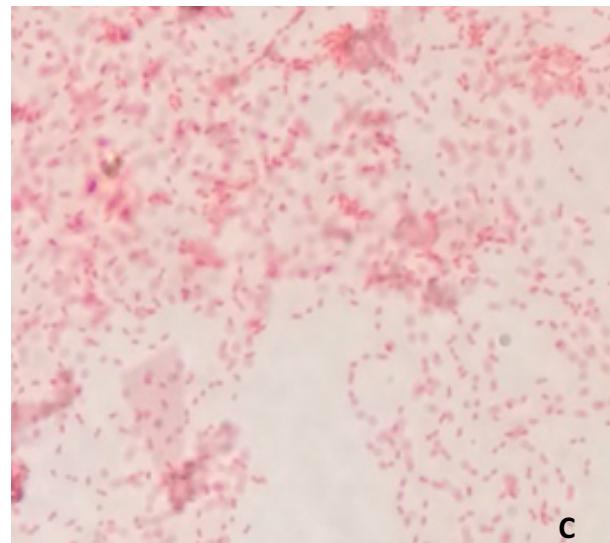
Day 3



Day 8



B

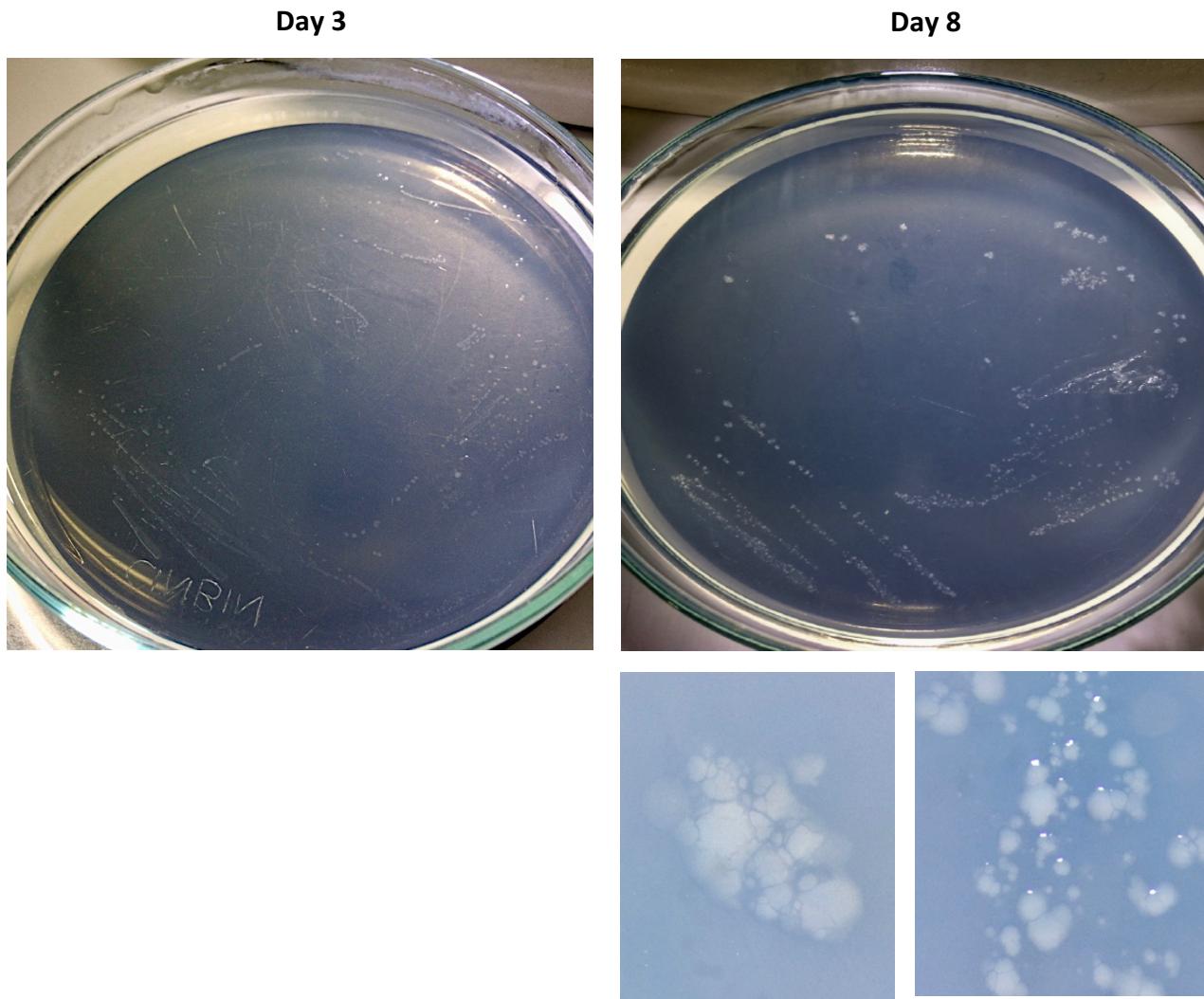


**Fig. 2. A.B.C.** Contrast microscope view 100x.

## Strain MFSR-18

- ✓ Macroscopic characterization.

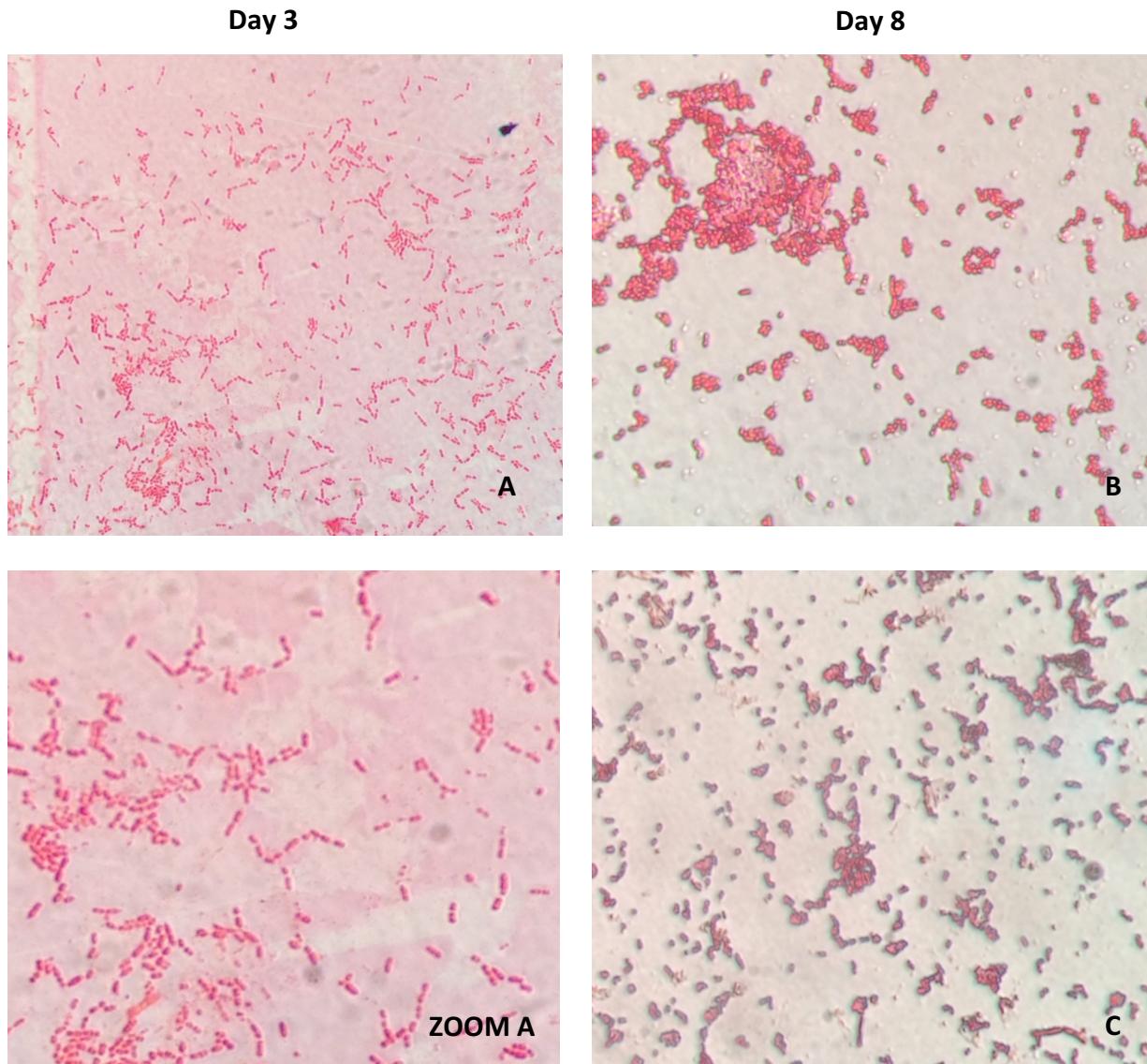
White colonies, creamy texture, convex elevation, punctate shape, whole margin and slow growth. They do not diffuse pigment into the medium.



**Fig 3.** Stereoscope view day 3 and day 8.

✓ Microscopic characterization.

**(A-B).** Gram-positive bacillary coccus-forming filaments on the third day, day eighth tend to undo filaments and form rosette agglomerates. **(C).** No spores are present with malachite green staining.



**Fig. 2. A.B.C.** Contrast microscope view 100x.

## Strain ACPG-11

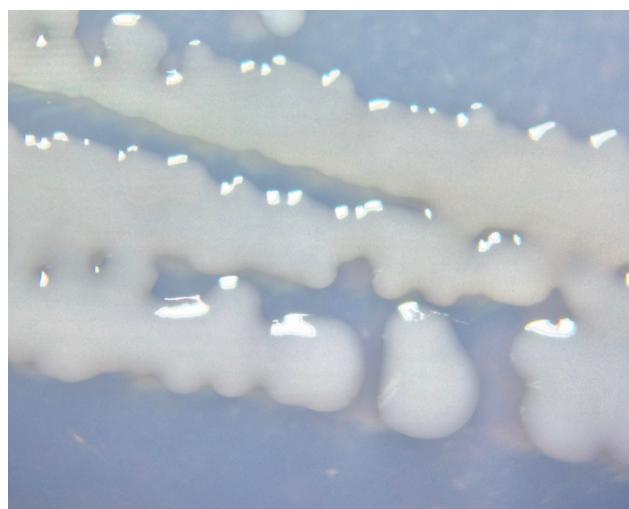
- ✓ Macroscopic characterization.

Colonies of bone color, creamy texture, irregular shape, convex elevation. At day three separate colonies of low growth are observed while at eight days they are indistinguishable. Does not diffuse pigment in the medium.

Day 3



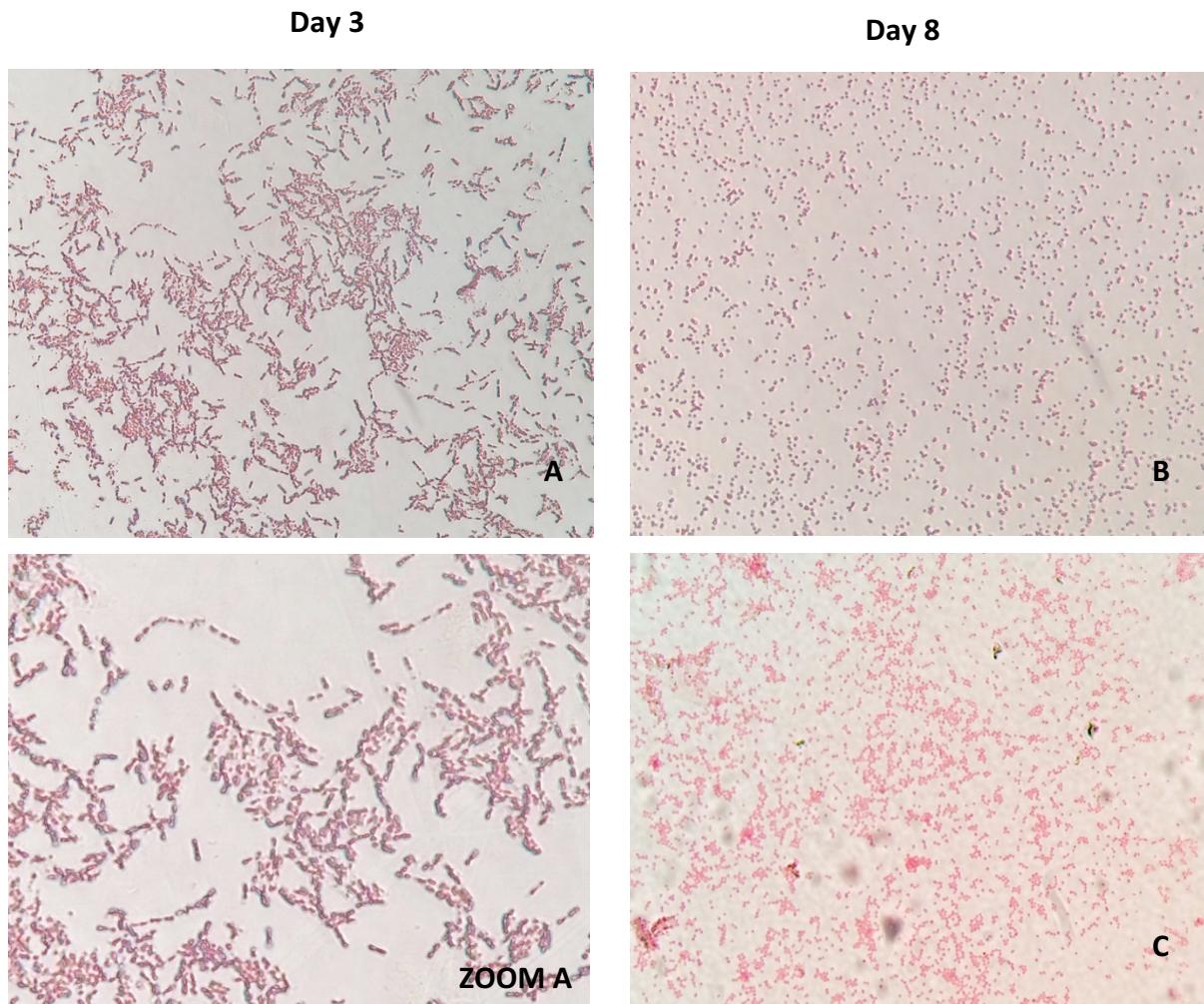
Day 8



**Fig 3.** Stereoscope view day 3 and day 8.

✓ Microscopic characterization.

(A) Gram-positive coccus tending to form filaments on the third day. (B) By the eighth day the bacteria were separated and without spatial distribution, they remained Gram positive. (C) Malachite green do not have spores.



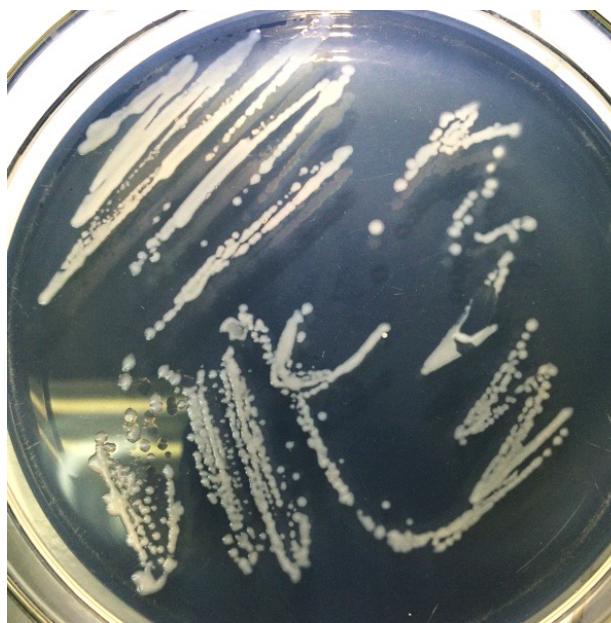
**Fig. 4. A.B.C.** Contrast microscope view 100x.

## Strain ACPG-18

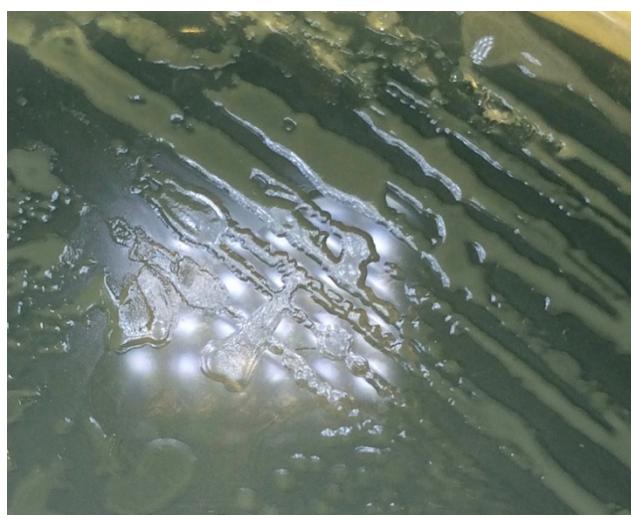
- ✓ Macroscopic characterization.

Colony of irregular shape, creamy texture, colonies without elevation. Does not diffuse pigment in the medium. Over time it tends to lose its white color and gains translucency.

Day 3



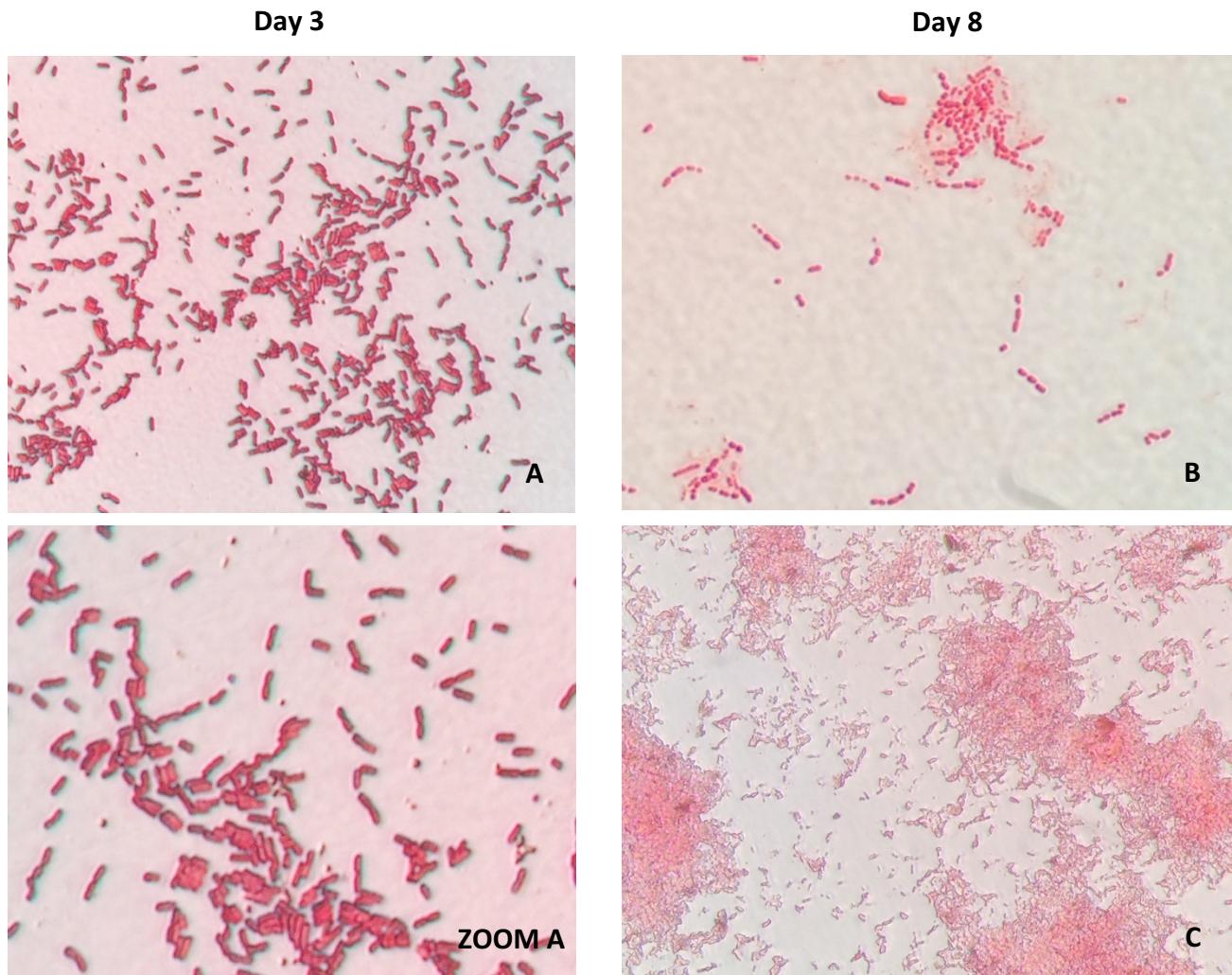
Day 8



**Fig 3.** Stereoscope view day 3 and day 8.

✓ Microscopic characterization.

**(A)** Gram positive coconuts, on the third day are united in rows of four. **(B)** On the eighth day they are separated from two bacteria forming diplococci. **(C)** Spores are evidenced with malachite green.



**Fig. 4. A.B.C.** Contrast microscope view 100x.

## 2. IDENTIFICATION OF THE ISOLATED MFSR 17-18 AND ACPG 11-18 BY SEQUENCING OF 16S RNA GEN.

### **MOLECULAR IDENTIFICATION OF ACTINOBACTERIAS.**

#### **- Total DNA extraction.**

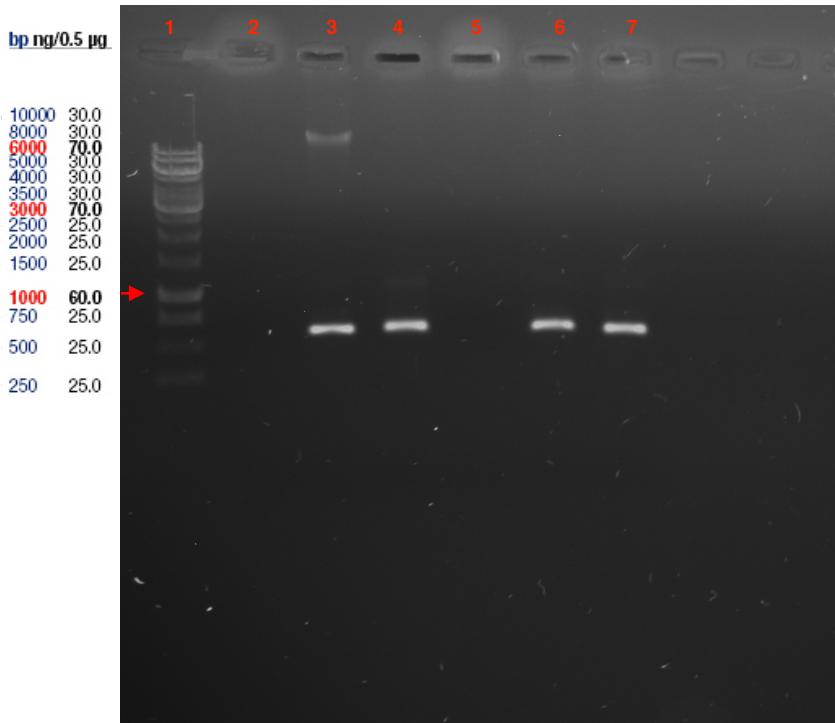
Total bacterial DNA extractions were performed by washing with Phenol/chloroform/isoamyl alcohol according to the protocol proposed by Ausubel et al. (1992), with the following modifications: after washing, a washing step with an equal volume of chloroform was added to ensure the obtaining of pure DNA. Samples were resuspended in 50uL TE RNase buffer.

#### **- Agarose gel electrophoresis.**

An agarose gel electrophoresis was performed to corroborate the quality of the extracts of DNA. Three bands were observed corresponding extracts. In the fourth lane, it was not possible to identify the presence of the sample.

### **PCR AMPLIFICATION OF THE 16S rRNA GENE AND SEQUENCING.**

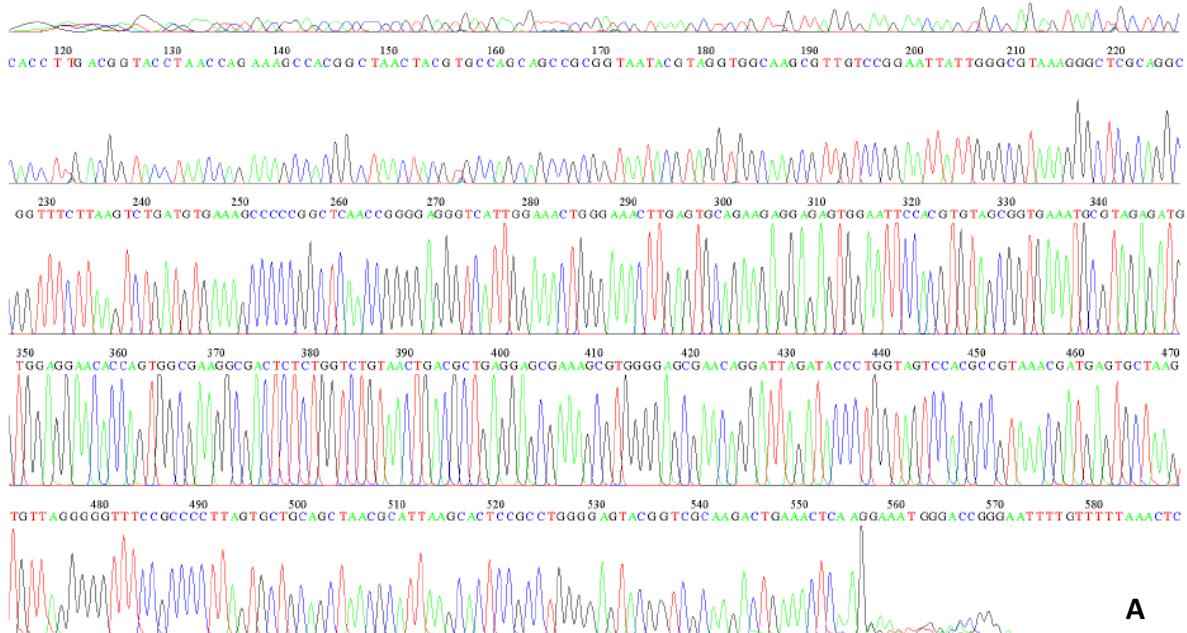
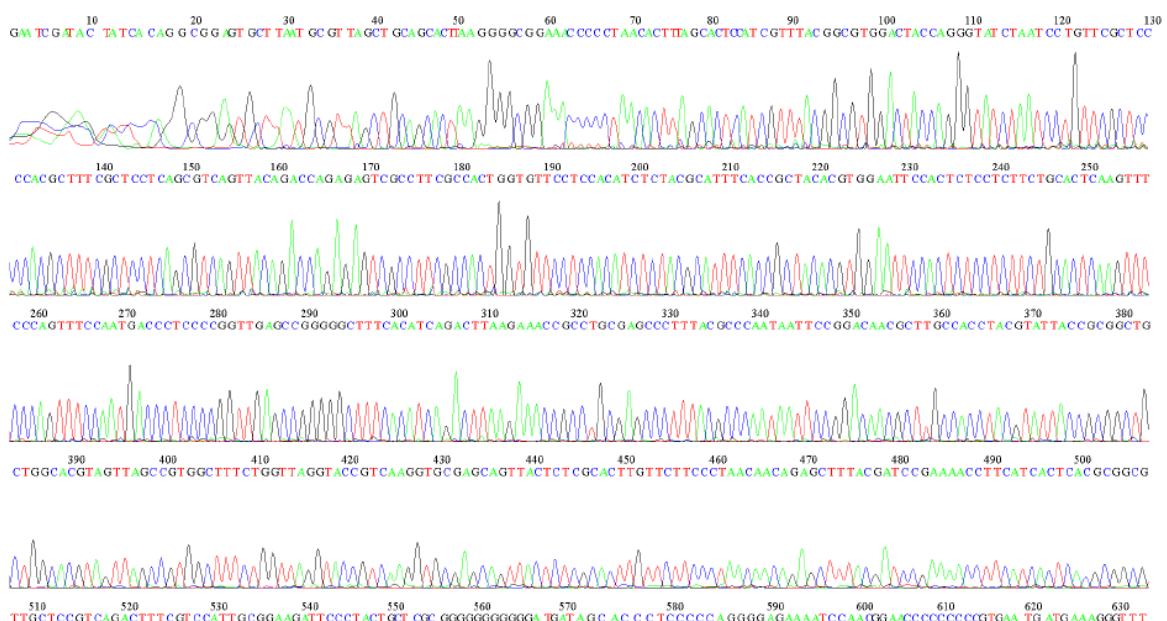
The 16S rRNA gene was amplified for each strain by PCR. Oligonucleotides 27F and 1492R (Lane et al., 1991), 1159R (Blackwood et al., 2005); 338F (Guo et al., 2008) and 907R (Muyzer et al., 1993), were used. Electrophoresis was performed to evaluate the quality of the amplified in a 0.8% agarose gel and was stained with EZ-Vision III (AMRESCO, Solon, OH, USA). Lane number 1 corresponds to the 1KB Thermo marker, in lane 2 the negative control and lane 3 the positive control. Lanes 4, 5, 6 and 7 correspond to strains ACPG18, ACPG 11, MFSR 17 and MFSR18 respectively. In lane 4 a slight indeterminate band is observed superior to the 1000pb indicated by the red arrow. In lane 6 and 7 high quality amplified are observed unlike lane 5 where there was no presence of amplified. The absence of a result in this lane could be due to the non-extraction of DNA by the washes with phenol/chloroform/isoamyl alcohol.

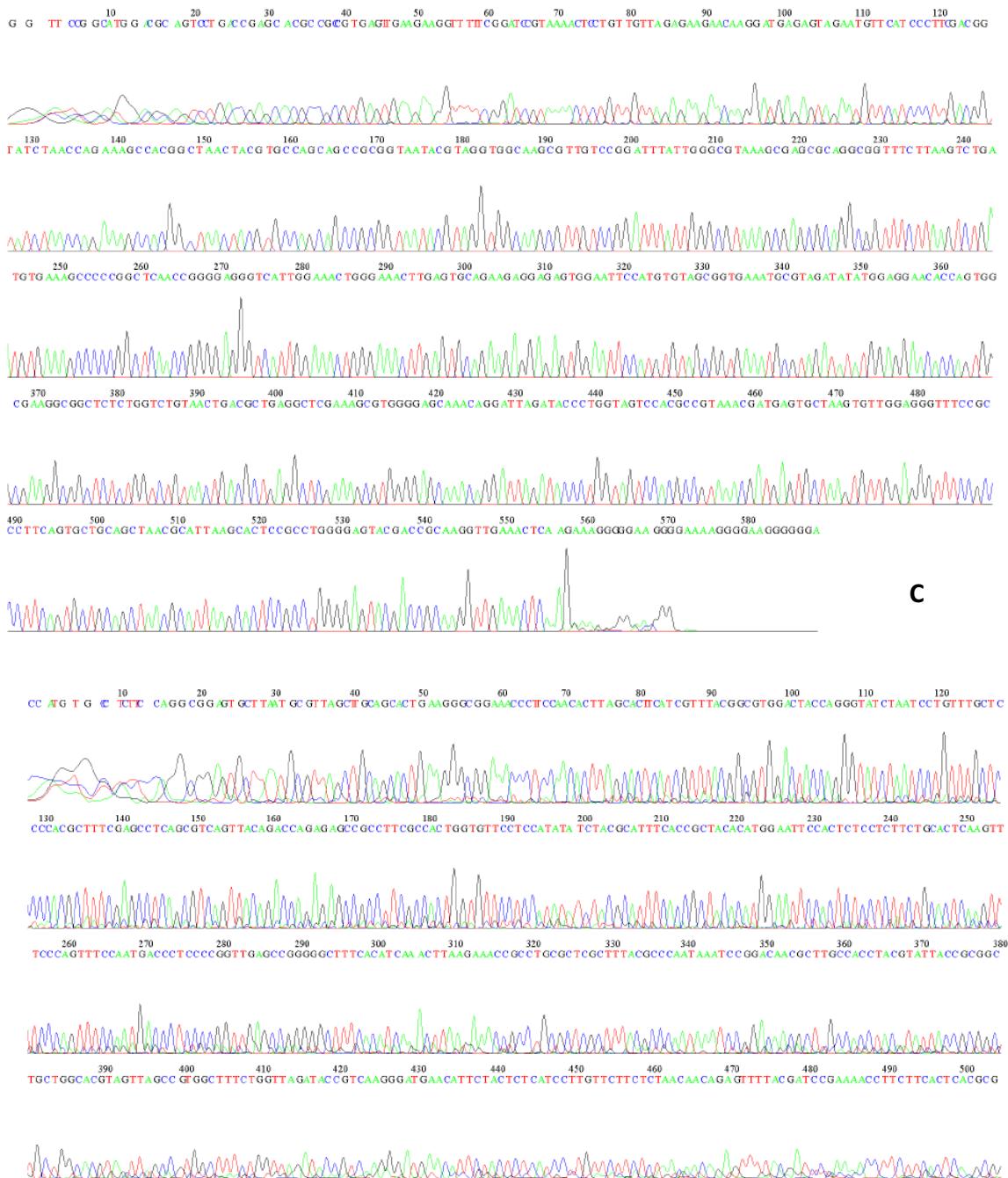


**Fig. 5.** 0.8% agarose gel with amplified DNA extraction for all four strains. Lane 1. Molecular Weight Marker 1 Kb Thermo Scientific®; 2. negative control; 3. positive control; Lane 4. ACPG18; 5. ACPG 11; 6. MFSR 17 and 7. MFSR18.

Since only two amplifiers of good quality were obtained, only strains MFSR 17 and MFSR 18 were sent sequence. Sequencing was performed by capillary electrophoresis with the two oligonucleotides with which it was performed PCR amplification by Macrogen Inc. (South Korea). The sequences received were obtained in txt format and in electropherogram Fig 6. In addition, they were verified and edited in Bioedit to avoid the presence of chimera-like anomalies and the taxa were assigned to each of the two strains.

T G TA TCGATG 10 ACGAACTCT 20 GACGGAGCA CGC 30 CGCTGAGTGT 40 GAAGGTTTCGGATCG 50 TAAAGCTCTG 60 TGTTAGGG 70 AAGAACAAAGT 80 GCGAGAGTAAC 90 TCG 100 AGTAAC 110 GC TCG


**A**

**B**



**Fig 6.** Electropherograms of the strains MFSR 17 (A. Reverse Primer, B. Forward Primer) and MFSR 18 (C. Reverse Primer, D. Forward Primer)

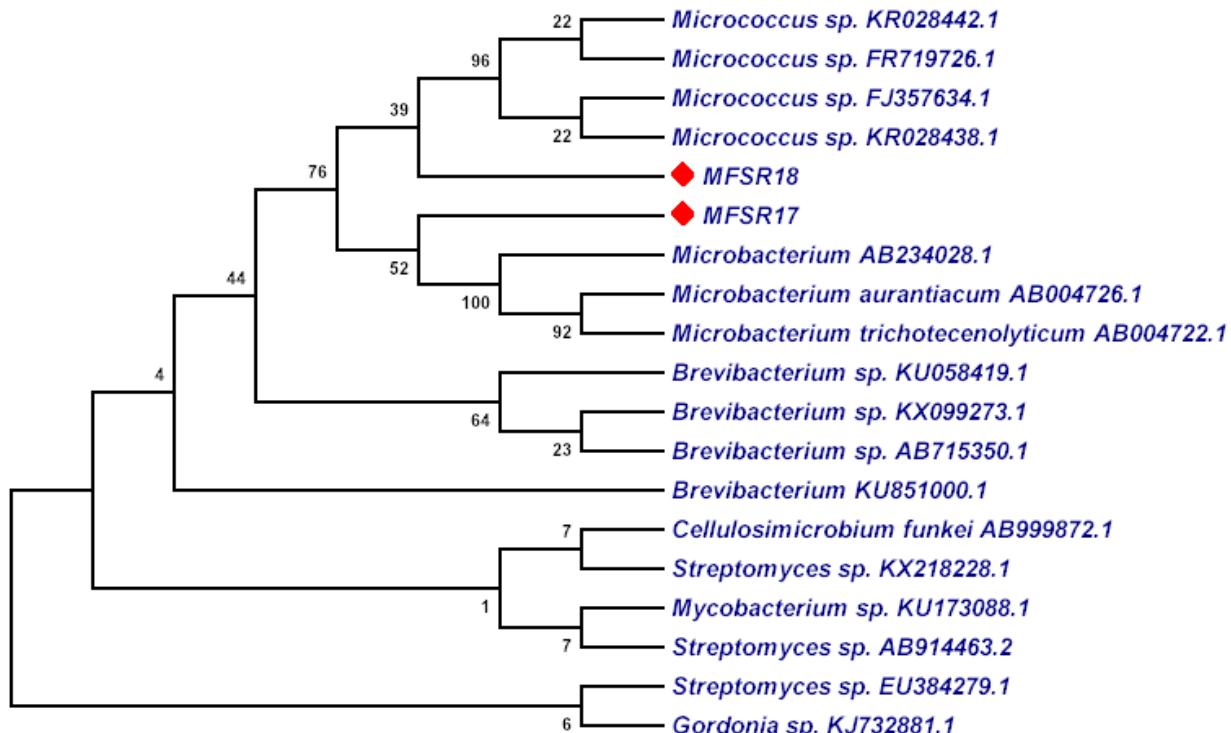
Subsequently, based on the genus determined by BlastN, the capture of reference sequences suggested in the manual of bacteriology of Bergey's (2012) and GenBank was carried out (Table 1). According to the identity values, the sequences were grouped with the taxonomic groups: *Streptomyces*, *Mycobacterium*, *Micrococcus*, *Microbacterium*, *Gordonia*, *Cellulosimicrobium* and *Brevibacterium*.

Reference Sequences			
Species	No. Access	Identity MFSR 17	Identity MFSR 18
<i>Streptomyces</i> sp.	EU384279.1	-	95%
<i>Streptomyces</i> sp.	KX218228.1	97&	95%
<i>Streptomyces</i> sp.	AB914463.2	97%	95%
<i>Mycobacterium</i> sp.	KU173088.1	97%	95%
<i>Mycobacterium</i> sp.	KU173034.1	96%	97%
<i>Cryobacterium</i>	EU852232.1	95%	-
<i>Cryobacterium</i> sp.	KR857430.1	94%	97%
<i>Micrococcus</i> sp.	KR028442.1	95%	95%
<i>Micrococcus</i> sp.	FR719726.1	95%	95%
<i>Micrococcus</i> sp.	KR028438.1	95%	96%
<i>Micrococcus</i> sp.	FJ357634.1	-	95%
<i>Microbacterium testaceum</i>	HM032885.1	96%	96%
<i>Microbacterium</i> sp.	U794390.1	-	95%
<i>Microbacterium oxydans</i>	LN890040.1	94%	95%
<i>Gordonia</i> sp.	KJ732881.1	99%	-
<i>Cellulosimicrobium funkei</i>	AB999872.1	99%	95%
<i>Brevibacterium</i> sp.	AB715350.1	97%	-
<i>Brevibacterium</i> sp.	KU058419.1	97%	-
<i>Brevibacterium</i> sp.	KX099273.1	97%	-
<i>Brevibacterium</i>	KU851000.1	97%	95%

**Table 1.** Sequences of reference taken from Bergey's (2012) and GenBank. 7 genera identified by different colors.

### 3. GENETIC DISTANCE ANALYSIS WITH THE SEQUENCES OBTAINED AND REFERENCE SEQUENCES.

An alignment in MUSCLE of the two problem sequences was performed. A distance tree was constructed with the sequences suggested in Bergey's (2012) manual of bacteriology and those housed in the Genbank database with the Neighbor-joining method in the MEGA 7 program. Fig 7.



**Fig. 7.** Distance tree inferred from sequences of the 16S rRNA gene from Actinobacteria strains by the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the relationship between the taxa analyzed. The tree is drawn to scale, with the length of the branches in the same units of the evolutionary distances used to infer the tree. The evolutionary distances were calculated using the Kimura 2-parameter method and are in units of number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions including lost gaps and data were deleted and the final data set is represented in 181 positions.

#### 4. TAXONOMIC DETERMINATION OF THE STRAINS WITH THE RESULTS OBTAINED FROM THE MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION.

Finally, the definitive taxonomic determination of the two strains sequenced complementing the morphological characterization with the molecular.

Because the alignments in BlastN aligned the problem sequences with different genus of actinobacteria, and that the genetic distance analysis was not sufficiently supported with the bootstrap, the micro and macroscopic characteristics of the strains were compared with the literature with the genera suggested by Neighbor-Joining: *Micrococcus* and *Microbacterium*.

It was found that the macro and microscopic characteristics: the type of growth of the colony, the texture of the same and the form of the cells that compose it, do not agree to the literature in Bergey's (2012) to the genera *Micrococcus* and *Microbacterium* and MFSR 17 and MFSR 18 strains.

### **DISCUSSION**

In the present work it was possible to cultivate, to characterize macro and microscopically the four strains of actinobacteria coming from honey-water and compost. However, only good quality DNA was extracted from two of these strains, MFSR 17 and MFSR 18. These extracts were then sequenced and quality sequences were obtained for taxonomic determination. The size of the sequences obtained were  $\leq 500\text{bp}$ .

The alignment in BlastN suggests for MFSR 17 seven different genera with 100% coverage and an identity  $\geq 95\%$ . Among these are *Streptomyces*, *Mycobacterium*, *Micrococcus*, *Microbacterium*, *Gordonia*, *Cellulosimicrobium* and *Brevibacterium*. MFSR-18 was aligned with the same genera as MFSR-17 but showed coverage between 95-99% lower and 95-97% identity.

In the genetic distance analysis MRSR-17 was grouped together with *Microbacterium* with a bootstrap value of 52% and close to this *Micrococcus* with 76%; MFSR-18 was grouped with *Micrococcus* with a bootstrap of 39%.

*Microbacterium* is a genus of Gram-positive cocci-bacilli with irregular rod shapes in young cultures, arranged individually or in pairs. Other clusters have a "V" shape. In older cultures, bar-shaped cells are shorter and cocci can appear, but a marked cycle between coconut-bar does not occur. Colonies appear opaque and shiny, often with yellow or orange pigmentation. It has been observed that other colonies appear whitish to orange-red. On the other hand, *Micrococcus* are Gram-positive bacteria with spherical cells of diameter between 0.5 and 3 micrometers that typically appear in tetrads. The colonies tend to be a yellowish color. Both genera belong to the order Micrococcales.

These morphological characteristics of the cells do not coincide with that observed in the laboratory for the two strains. However, it is suggested that MFSR-17 is related to *Micrococcus* rather than *Microbacterium* by the round shape of cells. Similarly, MFSR-18 corresponds to its morphology in the form of coconut-bacillus to *Microbacterium* instead of *Micrococcus*. Although both genera of bacteria are present in waters and soils, these have totally different morphologies and forms of colonies.

Therefore, it is suggested the application of methods that evaluate more physiological and morphological parameters such as the study of aerial mycelium, the motility of the cells, the use of carbon compounds and enzymes, to finally reach a more solid taxonomic determination.

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