Exploratory 16S rRNA Metagenomic Analysis of Soil Microbial Communities in Agroecosystems of North-Central Argentina

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Abstract:

Agriculture has modified the soil structure due to the influence of external factors and processes that affect microbial biodiversity. Metagenomics is a fundamental tool for the study of soil microbial diversity because it provides information about the ecosystem diversity, including both the microorganisms that cannot be isolated in culture media and those that are no longer viable in the analyzed sample. In this work, six soil samples obtained from agroecosystems of central and northern Argentina were subjected to a preliminary 16S metagenomic analysis. Copiotrophic bacteria (Proteobacteria and Actinobacteria) were dominant and one of the samples had a dominance of an oligotrophic Phylum (Acidobacteria). Our findings

support previous evidence from traditionally managed agroecosystems and provide new insights into the diversity of soil microbiomes in Argentine regions outside the Pampas. Finally, we analyzed the most common genera with relevant species to agronomy, both beneficial and pathogenic, and their abundance and diversity in the sequenced samples.

Keywords: 16S rRNA metagenomic sequencing, bacteriome, ecosystem degradation, biodiversity, Chaco region

Introduction

The effects of anthropogenic activities are evident in landscapes and biological diversity, as well as at the intensification of erosion and climate change. These factors remark the need to modify production methods and their links with nature in order to promote a conscious and sustainable exploitation that prevents forcing the limits of soil capacity for providing its environmental services (Aizen et al., 2009; Guimarães, 1998). At the global scale, Argentina is the eighth largest country and has a low density of inhabitants, with population being concentrated in a few and quite focused spaces. Since the 20th century, the country's economy has been based mainly on exports of rural goods (Aizen et al., 2009), which are developed in more than 80% of its area and are related mainly to agricultural, livestock, and forestry practices (Bran et al., 2017).

The development of intensive agricultural practices can be analyzed at different scales, with the ecosystem-complexes scale being the minimum representation of the predominant landscape of an ecological region in our homeland country Argentina (Aizen et al., 2009; Bran et al., 2017). This is an iterating approach, since modifications in them have been detrimental to environmental quality at all the other different scales. During the 1990-2006 period, a shift in the productive structure occurred towards soybean as the main Argentine crop, with a yield increase of approximately 45% (Aizen et al., 2009). The modifications that occurred in these territories are not only at the landscape scale but also at the level of the environmental services they provide, like the loss of soil quality, water absorption, and the alteration of biodiversity (Aizen et al., 2009).

Land use patterns have several effects on soil biodiversity, food webs, and ecosystem services (Bran et al., 2017). Agricultural activity has replaced the biological processes that regulate soil structure through external factors, such as the application of fertilizers or synthetic pesticides. In this sense, changes driven by different management practices alter soil properties, causing a direct effect on microbial communities, also known as soil microbiome. For example, modifications produced by fertilizers change the soil chemical properties that may have an influence over the presence and prevalence of some bacteria groups. preventing the growth of others (Dai et al., 2018). Furthermore, chemical fertilizers have different

62 effects than those of organic fertilizers. Likewise, the overuse of nitrogen (N) causes the loss of organic
63 carbon, with an impact on microbial biodiversity, that also increases nitrification and leaching of nitrate to
64 nitrogen and reaching groundwater sources (Nowinski et al., 2008; Seneviratne, 2009; Ramirez et al., 2010).
65 Among the traditional practices, differences were also observed between fertilization with N alone and with
66 N complemented with phosphorus (P) and potassium (K) (Dai et al., 2018). The modification of microbial
67 communities due to the application of different agricultural practices has an impact on the agroecosystems,
68 since microorganisms participate in soil formation through nutrient inputs (e.g. phosphorus and nitrogen),
69 degradation of recalcitrant organic matter, and mineral weathering (Dubey et al., 2019). In this regard, a
70 better understanding of the factors determining the structure of soil microbiomes may contribute to a better
71 understanding of soil dynamics and change the approach of the most widely used traditional agricultural
72 practices.

73 Microbial diversity should be considered an indicator of soil health and an important factor to
74 ensure agriculture sustainability (Kraut-Cohen et al., 2020). Metagenomic analyses allow us to identify the
75 microorganisms that cannot be isolated and grown in culture media; consequently, the information obtained
76 using method has become a fundamental tool to correlate factors that have an influence on soil microbiota
77 in an evolving environment (Dubey et al., 2019). These analyses have been conducted in agricultural
78 systems to assess variations in soil biodiversity relative to management type (Rascovan et al., 2013). In
79 Argentina, some metagenomic studies were done in the Pampas agroecosystems (Fierer et al., 2013;
80 Rascovan et al., 2013); other productive regions of the country, however, have not been subjected to these
81 analyses.

82 In this work, we conducted an exploratory metagenomic analysis of the bacterial communities
83 present in agricultural soil samples belonging to north-central region of Argentina, including the Chaco and
84 Pampas ecosystem complexes.

85 **Material and methods**

86 ***Area of analysis***

Soil samples were collected from different agroecosystems cultivated with soybean (*Glycine max* L.) and corn (*Zea mays* L.) in Argentina (Morello et al., 2012). The agroecosystems were located in the three most populated ecoregions of the country under an extensive agricultural production that causes significant modifications of the landscape structures.

The Dry Chaco ecoregion is part of the Great South American Chaco and is characterized by a large flat terrain with a slight slope towards the east of Cordoba? province. The climate conditions are widely varied, with the highest summer temperatures exceeding 47 Celsius degrees (°C), and the lowest winter temperatures dropping to below –6 °C. Cumulative volumetric rainfall ranges from 400 to 800 mm per year and predominant soil types include Mollisols, Entisols, and Alfisols. The landscapes of this ecoregion have also suffered different transformations after cotton cells were consolidated, technological changes of agriculturalization processes have been incorporated for soybean production and cattle raising in the areas where soybean cannot be cultivated. The soils are poorly enriched, with a slight tendency to be saline. In the Central Subhumid Chaco Complex, the physiognomy is dominated by halophytic savannahs and Chaco forest, which were fragmented by land use conversion to extensive agriculture (Morello et al., 2012) (Figure 1).

The Espinal ecoregion is represented by a flat to gently undulating terrain, including a lesser extent low mountain ranges and loessial to sandy soils, Mollisols, Vertisols, and Alfisols that stand out in the Northern Complexes, with a slightly more humid and warmer climate. Cumulative volumetric rainfall ranges between 500 and 1000 mm per year, decreasing from north to south. This ecoregion is characterized by the presence of low xerophytic forests, including alternating grasslands and savannas. The loss of the natural physiognomy in this ecoregion is one of the most important of the country, and has occurred due to processes of anthropization associated with crop production and urbanization. This region includes the Pampa Pedemontana Complex, which is present almost entirely in Córdoba province (Morello et al., 2012).

The Pampas ecoregion is located the east-central region of Argentina and is an extensive plain with soft undulations in some areas and a slope from West to East towards the Atlantic Ocean. The predominant

112 climate is subtropical, with a humidity gradient that decreases from East to West and Southwest; cumulative
113 volumetric rainfall ranges from 1200 mm to 700 mm annually. Average annual temperature ranges from
114 20 to 14 °C, decreasing latitudinally. The predominant soils are Mollisols, mostly Argiudols, Haplustols,
115 Hapludols, and Natracuols (Morello et al., 2012). The dominant vegetation type is the grass steppe and the
116 species composition varies according to the local climatic and soil characteristics. This ecoregion has the
117 longest history of agricultural and livestock use in the country and is divided into two sub-regions; our
118 study was conducted in the Undulating Pampas complex, which is located in the north and is predominantly
119 a grassland. However, most of the area has been converted into farmlands, and the pastures were replaced
120 with crops, except in some relictual spaces, where agricultural activity cannot be developed (Morello et al.,
121 2012).

122 ***Sample collection and analysis***

123 Soil samples were taken with a tubular soil sampler, from the first 20 cm and as a composite of 10
124 random sub-samples of ~100 g each. We collected six samples from agroecosystems with traditional
125 agricultural management, mainly of mollisols soils. Four samples were obtained from the Central Subhumid
126 Chaco Complex (Los Juríes, Las Breñas Napenay and Concepción del Bermejo) and the other two were
127 taken from the Pampa Pedemontana (National University of Villa María, UNVM), and Pampa Ondulada
128 Complexes (Arrecifes). The samples were taken in February 2018 from soybean cultivated soils, whereas
129 UNVM was under corn management.

130 ***DNA purification, 16S rRNA sequencing and analysis***

131 Soil samples were homogenized in a high-speed mixer (High-speed Universal Disintegrator, Pro-
132 Lab Diagnosis) and DNA extraction was performed using the microbiome DNA purification kit PureLink™
133 (Invitrogen). To verify the correct DNA extraction, total DNA electrophoresis was performed on a 1%
134 agarose gel, using SYBR Safe DNA staining (Thermo Fisher Scientific) and quantified by a PICODROP
135 spectrophotometer (PICO 100 l Spectrophotometer). An Illumina library was constructed from the purified
136 DNA and sequenced using the high-performance Illumina sequencing technology of the MySeq platform
137 (Macrogen). The conserved regions V3 and V4 of the ribosomal DNA encoding the 16S rRNA gene were

138 amplified by PCR using forward and reverse primers. The obtained raw reads were then trimmed (low
139 quality trimmed regions were removed), the paired reads were then merged and a last filtering step was
140 performed to remove reads shorter than 50 bp. After reads conditioning, the taxonomic assignment of the
141 amplicons was performed using suitable tools included in Geneious R11 (www.geneious.com). Obtained
142 raw reads were merged at fastq format and stored at NCBI's Sequence Read Archive (SRA) (Leinonen et
143 al., 2011) under Bioproject Accession No. PRJNA722523.

144 Diversity indexes (number of genera, Shannon index, and equitability) were calculated to compare
145 the samples using R's package "vegan" (Oksanen et al., 2019). Moreover, the frequency of the dominant
146 phylum was analyzed to compare the results of this exploratory analysis with those obtained in other
147 productive systems of Argentina.

148 Genus abundance data, expressed as % read count, were obtained with Geneious R11 software and
149 the 16 S biodiversity tool. These results were used to compare genus diversity and abundance in each sample
150 of those genera grouped according to their agronomic relevance, namely as plant-beneficial bacteria,
151 following a categorization reported by Basharat Ali (2019). The plant-beneficial group of bacteria includes
152 members of different genera such as *Burkholderia*, *Enterobacter*, *Stenotrophomonas*, *Pseudomonas*,
153 *Herbaspirillum*, *Ralstonia*, *Bradyrhizobium*, *Ochrobactrum*, *Bacillus*, *Rhizobium* and *Staphylococcus* (Ali,
154 2019). For the plant-pathogenic group of bacteria, we used the top 10 list of bacterial plant pathogens
155 described by Mansfield et al., (2012), which includes species belonging to the genera *Pseudomonas*,
156 *Ralstonia*, *Agrobacterium*, *Xanthomonas*, *Erwinia*, *Xylella*, *Dickeya* and *Pectobacterium* (Mansfield et al.,
157 2012).

158 **Results**

159 The samples with the highest bacterial diversity were those taken from C. del Bermejo and UNVM,
160 without differences among diversity indexes. The Shannon index for C. del Bermejo and UNVM was 5.41
161 and 5.50, respectively. The values observed for this index in the other sites were Las Breñas 5.18; Napenay
162 5.05; Los Juríes 5.24 and Arrecifes 5.37. The equitability index values were: C. del Bermejo 0.73; Las
163 Breñas 0.70; Napenay 0.69; Los Juríes 0.71; Arrecifes 0.73; UNVM 0.74.

164 The most predominant phyla in all samples were Actinobacteria, Acidobacteria, and Proteobacteria,
165 showing an incidence greater than 15% (Fig. 2). Proteobacteria was the phylum with the highest incidence
166 in samples of C. del Bermejo, Los Juríes, Arrecifes, and UNVM, with more than 40% assigned by
167 comparison with each amplicon. Actinobacteria was the dominant Phylum in the sample obtained in
168 Napenay and Acidobacteria in the sample belonging to Las Breñas. Verrucomicrobia was highly
169 represented in the sample from Arrecife with respect to all the other samples, but it was not dominant in
170 any of them.

171 In addition, the most abundant genera in the sequenced samples were, as per their order of
172 abundance (% of sequence count), *Bradyrhizobium*, *Bacillus*, *Rhizobium* and *Pseudomonas* (Figure 3). In
173 samples from C. del Bermejo, Napenay, Los Juríes and Arrecife, the genus *Bradyrhizobium* was the most
174 abundant, followed by *Bacillus, Rhizobium* and *Pseudomonas*. Samples from Las Breñas and UNVM
175 showed a major abundance of the genus *Bacillus* followed by *Bradyrhizobium* and *Rhizobium.* The UNVM
176 sample showed also the most abundant proportion of *Pseudomonas* bacteria (10%). Minor genera in terms
177 of abundance were detected in the six samples and included *Burkholderia Stenotrophomonas*,
178 *Herbaspirillum*, *Xanthomonas* and *Ralstonia*. None of the following genera were detected in any of the six
179 samples: *Enterobacter*, *Agrobacterium*, *Erwinia*, *Xylella*, *Dickeya* and *Pectobacterium* (Figure 3).

180 **Discussion**

181 In this work, six samples obtained from the most productive agricultural areas of Argentina were
182 analyzed using 16S metagenomics. The samples corresponding to C. del Bermejo and UNVM showed the
183 highest biodiversity indices (Shannon index and Pielou index). Variations in these samples would be due
184 to the influence of management on bacterial biodiversity, a topic that has been widely discussed in the
185 literature (Dai et al., 2018; Huang et al., 2019). The relationships between N:P and soil available phosphorus
186 have been indicated as an influential factor in these variables. The soil nutrient content should be evaluated
187 in future studies to better understand the observed variations. N and NPK fertilization systems have
188 different effects on the dominance of some phyla over others (Dai et al., 2018; Huang et al., 2019). Some
189 research works propose the importance of evaluating changes in the proportions of copiotrophic bacteria,

190 such as Proteobacteria, which increase their frequency due to fertilization, and the proportion of
191 oligotrophic bacteria like Acidobacteria, which decreases under these conditions (Fierer et al., 2013). Since
192 NPK fertilization is frequent in agricultural management, the high frequency of Proteobacteria in the
193 analyzed samples might be expected. In experiments with different fertilization systems, a predominance
194 of this phylum has been observed (Huang et al. 2019; Dai et al. 2018; Zou et al. 2015), with a frequency
195 similar to the observed in this work.

196 The species of the phylum Actinobacteria are also characterized by being copiotrophic organisms,
197 and a high frequency is usually identified in the same environments as those where Proteobacteria is present
198 (Fierer et al., 2013; Huang et al., 2019). The increase of Proteobacteria and Actinobacteria related to high
199 nitrogen fertilization was observed, regardless of soil types or vegetation present (Fierer et al., 2013).
200 Moreover, Actinobacteria include spore-forming and gram-positive bacteria that can survive in
201 environments where soils are under extreme acidification processes; therefore, the relatively higher
202 presence of this phylum in the samples of C. del Bermejo and Napenay might be an indicator of the
203 occurrence of such processes (Dai et al., 2018).

204 In productive systems, there is a low frequency of oligotrophic phyla, like Acidobacteria and
205 Verrucomicrobia (Fierer et al., 2013; Fierer et al., 2012). In this work, Acidobacteria exhibited a relatively
206 high frequency in the samples, whereas Verrucomicrobia showed a low frequency. The frequency of this
207 phylum was relatively high in Las Breñas sample. Acidobacteria subdivisions 4 and 6 were the most
208 abundant. These... were proposed to be correlated positively with soil pH (Kielak et al., 2016); therefore,
209 the relatively higher frequency of this phylum and in particular subdivisions suggests a higher pH than in
210 the other samples.

211 The phylum Verrucomicrobia had a low frequency in most of the samples, being more abundant in
212 Arrecife and UNVM. Verrucomicrobia used to be widely present in US grasslands (up to 50%) before they
213 were managed with chemical fertilizers (Fierer et al., 2013). In this regard, the low frequency of
214 Verrucomicrobia in the analyzed samples is consistent with the hypothesis that under agricultural
215 management, the frequency of this phylum is low. Studies carried out in Argentine Pampas agroecosystems

216 proposed that one of the major regulators of microbial biodiversity is water availability as well as some soil
217 properties such as silt, organic matter, nitrogen content, pH, and salinity (Dubey et al., 2019). It was also
218 proposed that managed systems are more heterogenic than non-cultivated environments due to changes
219 caused by crop rotation and fertilization. It was also proposed that the dominant bacteria in the Pampas soils
220 might have the capacity to adapt to these changes (Carbonetto et al., 2014). Studies in Uruguay found a
221 higher frequency of phylum Verrucomicrobia in unmanaged soil samples when compared to maize-grown
222 soils (Federici Rodriguez et al., 2017). The authors found frequencies greater than 25% for Acidobacteria
223 and Proteobacteria and less than 20% for Verrucomicrobia in cultivated soils, whereas in uncultivated soils
224 the frequencies of these phyla were more than 30% Acidobacteria, between 20 and 25% Proteobacteria and
225 more than 25% Verrucomicrobia (Federici Rodriguez et al., 2017). Our results are similar to those obtained
226 by these authors.

227 The abundance and distribution of bacteria with agronomic importance were also analyzed. The
228 most common and abundant genera found were *Bradyrhizobium*, *Rhizobium*, *Bacillus* and *Pseudomonas.*
229 *Bradyrhizobium* and *Rhizobium* (rhizobia) are gram negative nitrogen-fixing soil bacteria that infect
230 legume plants (e.g. soybean, peanut, etc.) and induce nodulation, favoring the conversion of molecular
231 nitrogen (N_2) from the air into ammonia, nitrates, and other nitrogenous compounds necessary for the plant
232 metabolic functions (Verma et al., 2020). In this symbiotic association, rhizobia receives carbon-rich
233 organic compounds that are used for the production of energy. This particular group of bacteria are specially
234 needed for legume plants in soils with poor sources of N_2.

235 The third most abundant genus found was the *Bacillus*, which includes two plant-beneficial species,
236 *B. subtilis* and *B. thuringiensis*, as the most relevant species for agriculture. *B. subtilis* has been defined as
237 a GRAS (Generally Recognized as Safe) microorganism and used as a model bacterium for different studies
238 for several years. This has also been the bacterium of choice in the industry because of its friendly genetic
239 manipulation and other biotechnological properties that make it suitable for large scale fermentations and
240 the biotechnological production of proteins and enzymes (Zhang et al., 2020). *B. subtilis* has also been used
241 as plant growth promoting rhizobacteria (PGPR) and as a biocontrol agent of phytopathogenic organisms

242 such us bacteria (Bais et al., 2004), fungi (Chen et al., 2013) and nematodes (Backer et al., 2018). *B.*
243 *thuringiensis* is the gram-positive bacterium most widely studied and used for the biological control of
244 invertebrate pests in agriculture. This bacterium harbors insecticidal genes encoding δ-endotoxins (Cry
245 and Cyt proteins) and other vegetative insecticidal proteins (VIP) exhibiting toxic activity against a wide
246 range of agricultural pests as well as human disease vectors (mosquitoes) and nematodes (Palma et al.,
247 2014). The genes encoding Cry and Vip3 proteins have been successfully used for the construction of
248 transgenic plants with resistance to insect pests in agriculture; thus, these plants are used in integrated pest
249 management programs and for reducing the use of chemical pesticides (Heckel, 2020).

250 Finally, *Pseudomonas* includes both plant-beneficial and plant-pathogenic species, among the most
251 frequent and abundant genera found in the sequenced samples. Species belonging to this genus showed
252 PGPR potential (Qessaoui et al., 2019), whereas other such as *Pseudomonas entomophila* exhibited
253 interesting insecticidal potential for the control of pests in agriculture (Dieppois et al., 2015). However,
254 *Pseudomonas* also harbors plant-pathogenic species, such as different pathovars of *Pseudomonas syringae*
255 (Mansfield et al., 2012). This species was voted as number one into the top 10 bacterial plant pathogens in
256 terms of its importance (Mansfield et al., 2012).

257 Minor genera in terms of abundance but with agricultural importance were *Burkholderia*,
258 *Stenotrophomonas*, *Herbaspirillum*, *Ralstonia* and *Xanthomonas*. The genus *Burkholderia* includesboth
259 plant-beneficial and plant-pathogenic species. *Burkholderia phenazinium*, *Burkholderia megapolitana*, and
260 *Burkholderia bryophila* were described for their interesting antifungal activities, whereas *Burkholderia*
261 *caryophylli* and *Burkholderia gladioli* are well known plant pathogens (Eberl and Vandamme, 2016).The
262 *Stenotrophomonas* genus includes some species exhibit interesting biotechnological properties, such as
263 *Stenotrophomonas maltophilia*, due to their potential plant growth-promoting effects for the biological
264 control of some phytopathogenic fungi (Wolf et al., 2002). Some species of the *Herbaspirillum* genus are
265 N_2-fixing endophytic bacteria (Schmid et al., 2006). *Ralstonia* and *Xhantomonas* include several plant-
266 pathogenic species included into the top 10 list of bacterial plant pathogens (Mansfield et al., 2012), with
267 *Ralstonia solanacearum*, *Xanthomonas oryzae* pathovar *oryzae*, *Xanthomonas campestris* pathovars and

268 *Xanthomonas axonopodis* pv. *manihotis* ranking second, fourth, fifth and sixth, respectively (Mansfield et
269 al., 2012).

270 The presence of *Enterobacter* was not detected in this study, although *Enterobacter* species may
271 be found in different environmental samples (Davin-Regli et al., 2019). *Agrobacterium tumefaciens*, the
272 most representative plant-pathogenic bacterium species of the genus, and the third in the top 10 list of
273 bacterial plant pathogens (Mansfield et al., 2012), was also not detected into the analyzed samples. Species
274 belonging to Erwinia, *Xylella*, *Pectobacterium* and *Dickeya* were also included in the top ten list of plant-
275 pathogenic bacteria, ranking 7th, 8th , 9th and 10th, respectively, with *Erwinia amylovora*, *Xylella fastidiosa*,
276 *Dickeya dadantii*, *Dickeya* solani and *Pectobacterium carotovorum* and *P. atrosepticum* being the most
277 representative plant-pathogenic species of these genera (Mansfield et al., 2012); these species were also
278 absent or not detected in the analyzed samples

279 **Conclusions**

280 The results obtained in this work are the first ones focusing on the bacteriome from Argentine soils
281 that are subjected to agricultural management in areas outside the Pampas region. Although data provided
282 here are preliminary and should be considered exploratory, they provide the basis for a high-throughput
283 metagenomic study involving more than 200 samples obtained from different agricultural regions from
284 Argentina. In addition, the present results suggest that the analyzed sites had similar behavior to other
285 systems under agricultural management, providing basic information for future analyses about the evolution
286 of the microbiota in these agricultural systems.

287

Acknowledgements

This work was supported by grants PIC-UNVM 2017-2019 from Universidad Nacional de Villa María, PIP-2017-2019 GI from CONICET and PICT-2017-0087 from Ministerio de Ciencia, Tecnología e Innovación (MinCyT) Argentina.

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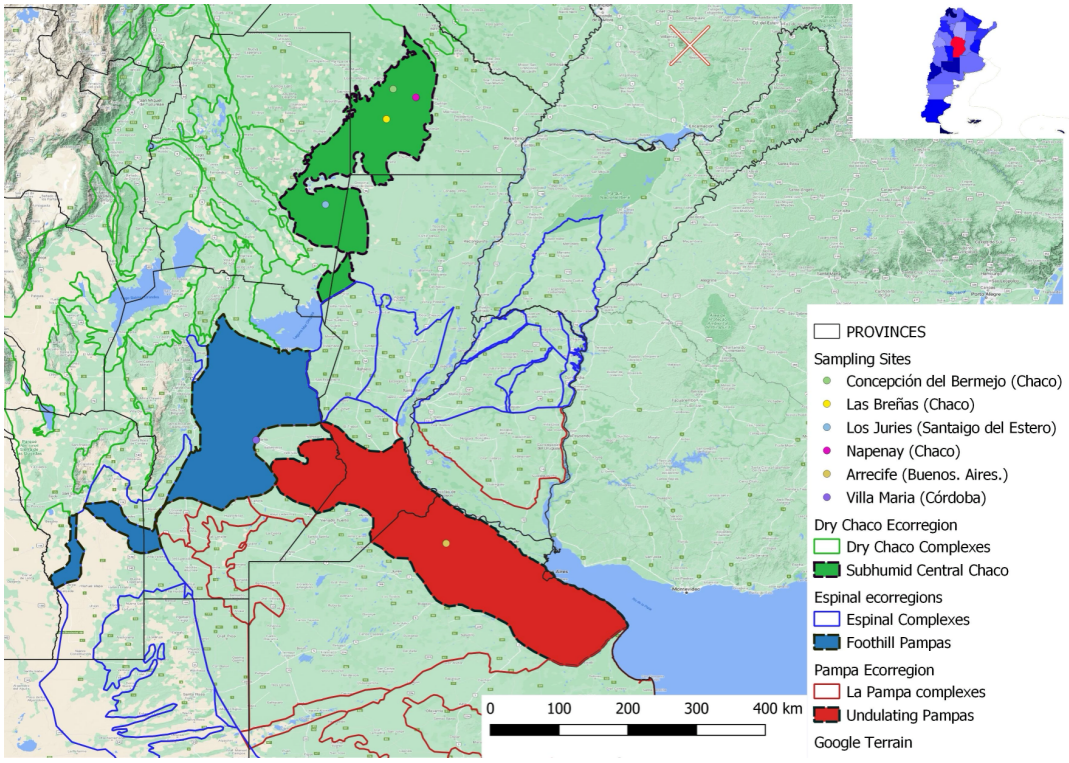
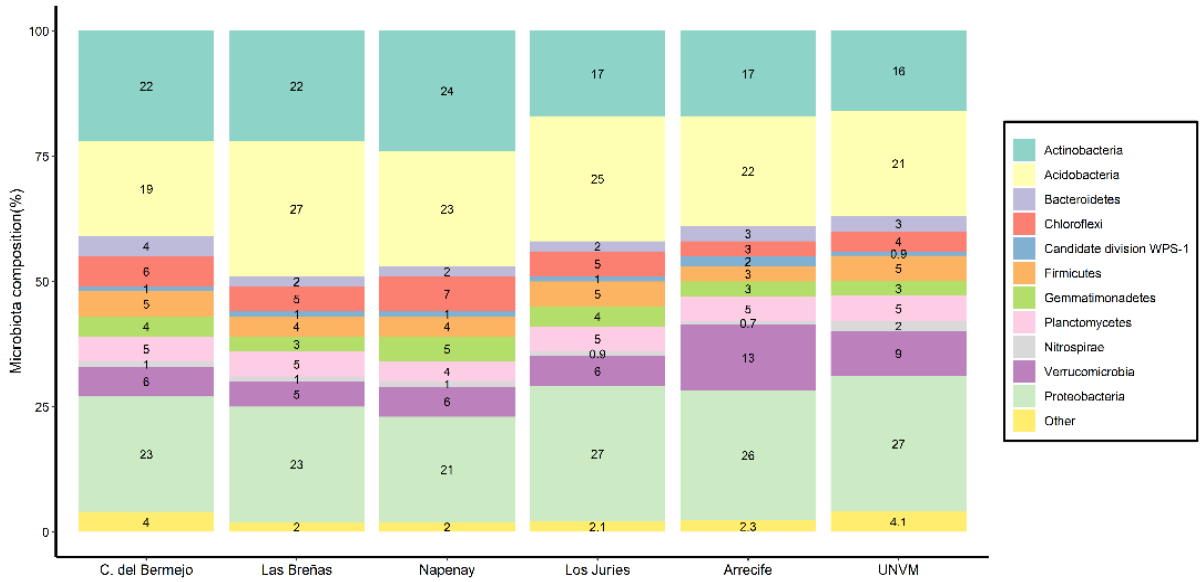
Figure captions

Figure 1



368 **Figure 2**



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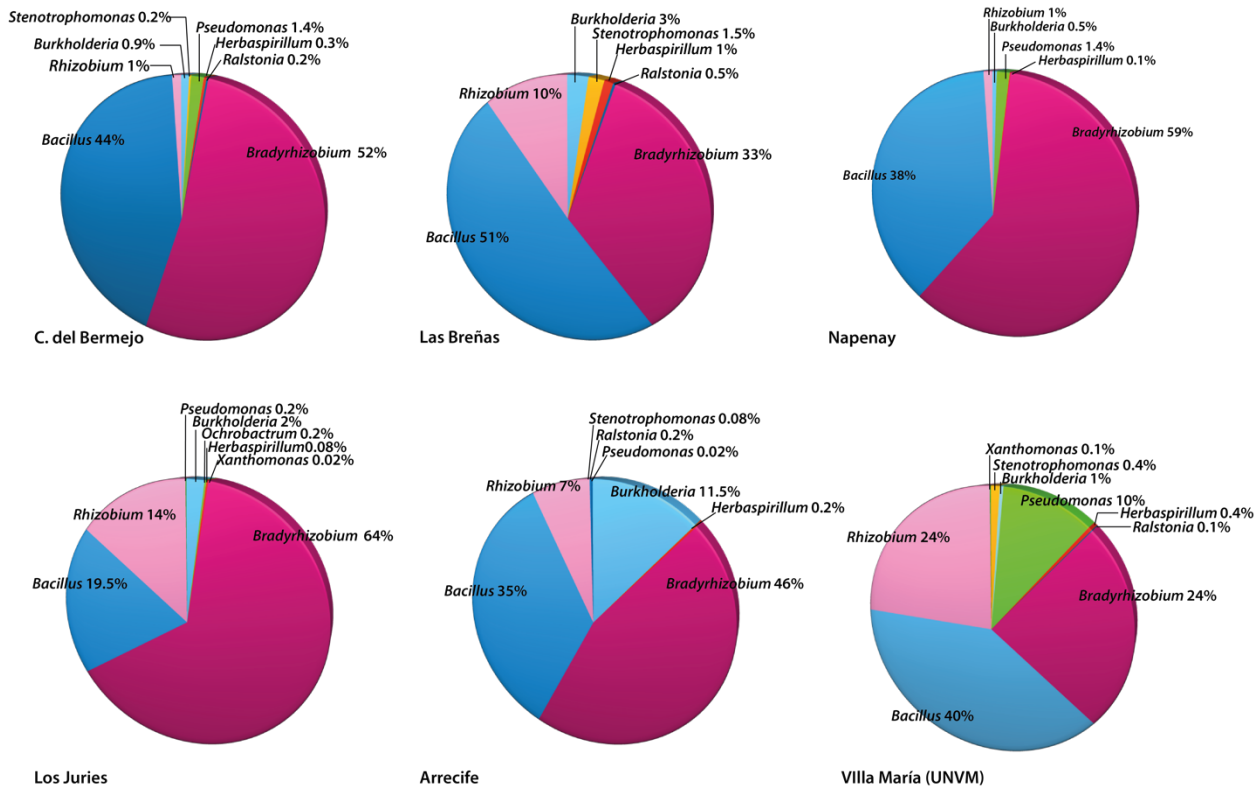
372 **Figure 3**



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378 **Figure legends**

379 **Figure 1.** Schematic map showing the sampled geographical regions.

380 **Figure 2.** Taxonomic profiles of north-center regions of Argentina. The most abundant taxa are shown in
381 the bars chart and shows the dynamics of variation in the microbial communities (Phyla) in the analyzed
382 samples.

383 **Figure 3.** Comparison among the most relevant plant-beneficial and plant-pathogenic groups of bacteria.
384 A pie graph was constructed for each sample by calculating the % read count for a given genus within the
385 group of bacteria defined in this study as agronomically relevant. Read counts and mean confidence values
386 were calculated using 16S Biodiversity tool included in Geneious R11 (www.geneious.com). Only those
387 genera exhibiting mean confidence values >50 are shown.

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