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Exploring Phosphate Solubilizing Bacterial Communities in Rhizospheres of Native and Exotic Forage Grasses in Alkaline-Sodic Soils of the Flooding Pampa

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Exploring phosphate solubilizing bacterial communities in rhizospheres of native and exotic forage grasses in alkaline-sodic soils of the flooding Pampa

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Abstract

The flooding pampa is one of the most important cattle-raising regions in Argentina. In this region, natural pastures are dominated by low-productivity native grass species, which are the main feed for livestock. In this context, previous studies in the region with the subtropical exotic grass *Panicum coloratum* highlight it as a promising species to improve pasture productivity. Cultivable phosphate solubilizing bacteria (PSB) communities associated to native (*Sporobolus indicus*) and exotic (*Panicum coloratum*) forage grasses adapted to alkaline-sodic soils of the flooding pampa were analyzed. PSB represented 2-14% of cultivable rhizobacteria and Box-PCR fingerprinting revealed a high genetic diversity in both rhizospheres. Taxonomic identification by MALDI-TOF showed that PSB populations of *P. coloratum* and *S. indicus* rhizospheres are dominated by the phylum Proteobacteria (92,51% and 96,60% respectively)

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and to a lesser extent (< 10%), by the phyla Actinobacteria and Firmicutes. At the genus level, both PSB populations were dominated by *Enterobacter* and *Pseudomonas*. Siderophore production, nitrogen fixation, and indoleacetic acid production were detected in a variety of PSB genera of both plant species. A higher proportion of siderophore and IAA producers were associated to *P. coloratum* than *S. indicus*, probably reflecting a greater dependence of the exotic species on rhizospheric microorganisms to satisfy its nutritional requirements in the soils of the flooding pampa.

This work provides a novel knowledge about functional groups of bacteria associated to plants given that there are no previous reports dedicated to the characterization of PSB rhizosphere communities of *S indicus* and *P coloratum.* Finally, it should be noted that the collection obtained in this study can be useful for the development of bioinputs that allow reducing the use of chemical fertilizers, providing sustainability to pasture production systems for livestock.

Keywords

Grasses, phosphate solubilization bacteria, alkalinity, rhizosphere communities, sustainable pasture production, biofertilizers,

1. Introduction

In natural ecosystems, plants have the ability to create a rich environment to attract and select a particular and diverse community of soil microorganisms to the rhizosphere through the release of root exudates, mucilage, and bacterial signaling hormones [1, 2]. In general, the rhizosphere is mainly enriched with bacteria that have the ability to promote plant growth and therefore are classified as plant growth-promoting rhizobacteria (PGPR) [3, 4]. This group of rhizobacteria can benefit plants through various mechanisms, such as nutrient solubilization, hormone production, and enhancement of biotic and abiotic stress tolerance, among others [5, 6]. Among PGPR, those bacteria capable of solubilizing insoluble sources of inorganic phosphates [7] are of particular importance in agriculture, since the low availability of soluble phosphorus (P) sources in soil render this nutrient limiting for plant growth. Phosphorus availability for plants is affected, among other factors, by soil pH [8].

In Argentina, areas such as the flooding pampa are of particular economic relevance for cattle breeding, one of the most important activities in the country [9, 10]. In this region, natural grasslands are the fundamental basis for livestock

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feed. However, forage productivity is limited by nutritional restrictions, derived from the high pH and sodium salt levels of the soil [11-14]. These environments are dominated by native grass species belonging to the *Poaceae* family, such as *Sporobolus indicus*, which despite being adapted to saline and sodic soils have low productivity [15]. The introduction of subtropical grass species in diverse areas dedicated to livestock farming in Argentina is shown to exert a positive impact on cattle production [16-19]. In alkaline-sodic soils of the flooding pampa, the introduction of *Panicum coloratum* doubled grass cover and soil organic matter content, compared to native species, so it is considered a promising species to improve pasture production in this region [16, 20]. Due to the previously mentioned nutritional limitations of soils, pasture production in the flooding pampa is highly dependent on the supply of nutrients, such as phosphorus and nitrogen. In this regard, the use of PGPR-based biofertilizers with the ability to improve nutrient supply to plants is a low-cost and environmentally friendly biotechnological strategy.

Even though numerous studies have highlighted the favorable impacts of PGPR on a diverse variety of plants [21-23], implementing their commercial application across a wide range of existing crops is still far from becoming a commonplace agronomic practice. One of the limitations is the still insufficient knowledge of the culturable communities of microorganisms that associate with plants, which in turn are known to be subject to change due to the influence of plant genotype on soil properties, soil microbial communities, and climatic conditions [2]. Furthermore, an important factor that greatly contributes to the success of biofertilizers, particularly in abiotic stress environments, is the ability of their microbial components to tolerate and survive adverse environmental conditions after their introduction into the soil and until the onset of their interaction with the target plants [24, 25]. In this regard, haloalkaliphilic bacteria native from saline soils and showing plant growth-promoting (PGP) characteristics enabled wheat plants to cope with different environmental stresses, such as salinity and alkalinity [26]. Similarly, studies on maize plants with alkali-tolerant rhizobacteria native to sodic-alkaline soils and with multiple PGP attributes showed beneficial effects on plant growth under alkaline stress conditions [27]. Moreover, a recent bioprospection of the phosphate solubilizing bacterial (PSB) community of the rhizosphere of *Lotus tenuis*, a naturalized legume in alkaline-sodic environments of the flooding pampa, revealed the ability of strains to solubilize phosphate over a wide pH range and to improve nutrient acquisition in *Lotus tenuis* in co-inoculation trials with nitrogen-fixing rhizobia [28].

Despite the existence of the abovementioned background, current knowledge of the structure and functionality of cultivable rhizospheric microbial communities in alkaline-sodic soils is still limited to a few plant species [29, 30]. In this regard, it is necessary to extend that knowledge to a wider range of plant species that thrive in alkaline-sodic soils,

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in order to better understand the role of microorganisms in plant adaptation to restrictive environments and thus provide the basis for the formulation of highly effective biological inputs for plant production. In the present study, we aimed to analyze and compare the genetic and taxonomic diversity of cultivable rhizospheric PSB communities associated to a native (*S. indicus*) and an exotic (*P. coloratum*) grass species (*Poaceae*), both adapted to sodic-alkaline soils of the flooding pampa. The functional diversity of rhizospheric PSB was also evaluated, through the analysis of PGP traits other than P solubilization, such as biological N fixation and indole and siderophore production, thus providing valuable information for future selection of strains able to promote growth of forage grasses in restrictive soils of the flooding pampa.

2. Materials and methods

2.1. Collection of rhizospheric samples and soil analysis

Rhizospheric soil samples were collected during August to October 2016 from *Panicum coloratum* and *Sporobolus indicus* plants growing in sodic-alkaline lowlands of the flooding pampa region. Three replicate samples were collected for each plant species: replicate 1, Manantiales (35°44′36.319″S-58°3′25.307″W); replicate 2, Punta Indio (35°16′ 15.449″ S -57°14′ 52.101″ W); replicate 3, Ayacucho (36°31'4.81"S, -58°23'32.09" W). Each replicate consisted of five plants of each grass species. After plant removal, soil tightly adhered to the roots was collected and stored at 4°C, during 24h until bacterial isolation. Physicochemical soil characteristics were determined on samples obtained from the top 0-20 cm soil layer, according to the protocols standardized by the Network of Argentinean Agricultural Laboratories (REDLAA) as described by Cumpa-Velásquez, et al [28].

The electric conductivity (EC) and pH were determined in a soil/water suspension (1:2.5; w/v). Cationic Exchange Capacity (CEC) was quantified by the ammonium acetate method (Sparks et al., 1996). The Oxidizable Organic Carbon (C) and Organic Matter (OM) were measured by the Walkley–Black method [31]. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) contents were determined by complexometric titration and sodium (Na^{+}) and potassium (K^{+}) concentrations were measured by flame photometry. Soil nitrogen (N) and phosphorus (P) were determined by the Kjeldahl [32] and Bray–Kurtz [33] methods, respectively.

2.2. Isolation of culturable rhizospheric bacteria (CRB) and phosphate solubilizing bacteria (PSB)

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Culturable rhizospheric bacteria were obtained by washing and shaking 5 g of rhizospheric soil with sterile 10 mM $MgCl_2$. Serial dilutions of the suspension obtained were plated on agarized Plate Count Agar (PCA) medium [34] supplemented with Nystatin, to prevent fungal growth. Plates were incubated at 28°C for 24 h. In order to identify the rhizospheric bacteria harboring phosphate solubilizing activity, colonies were subsequently streaked on pH 7 agarized National Botanical Research Institute's Phosphate (NBRIP) medium [35] with tricalcium phosphate as the sole P source and in Tryptone Yeast (TY) medium [36] for further conservation and subculturing. Plates were incubated at 28°C for 72h. Colonies surrounded by a clear halo in NBRIP medium were considered positive for phosphate solubilization and this phenotype was confirmed by repeatedly sub-culturing on NBRIP. Based on differential morphological characteristics of the colonies, 46 to 50 PSB isolates were selected from the rhizosphere of each plant species at each replicate site, resulting in a total of 146 PSB from *P. coloratum* and 147 from *S. indicus*. PSB selected were grown on liquid TY medium for 24 h and preserved with 30% glycerol at -80°C.

2.3. Phosphate Solubilizing Assays under alkaline-sodic conditions

An assay was performed to evaluate if PSB isolates, obtained as described before, were able to solubilize P not only under neutral pH conditions, but also under alkaline-sodic conditions. For this purpose, PSB bacteria were cultivated on Petri dishes containing 25 ml of agarized NBRIP media supplemented with different amounts of 1 M Na_2CO_3 to adjust the pH to 8 and 9, and NaCl was added to a final concentration of 200 Mm Na^+ prior to agar addition and autoclaving. Bacteria were grown in 3 ml liquid TY medium on a rotary shaker at 28°C and 180 rpm until exponential growth was reached. Following centrifugation of 1 ml aliquots of bacterial cultures at 6000 g for 5 min, pellets were washed twice and suspended in 500 µL of sterile 10 Mm $MgCl_2$. Subsequently, 10 µL aliquots of each bacterial suspension were inoculated on pH 8 and pH 9 NBRIP medium obtained as described above and incubated at 28°C for 7 days. The phosphate solubilization was recorded as the ability to form clearing haloes around the colonies. Additionally, isolates were classified by a semi-quantitative analysis based on a halo score (h), obtained by comparing the diameter of solubilization haloes with the corresponding colony. Isolates were grouped into 3 classes according to their halo scores as follows: class 1: halo diameter 0.0-0.5 times higher than the colony diameter; class 2: halo diameter 0.5-2 times higher than colony diameter; class 3: halo diameter >2 times higher than the colony diameter.

2.4. DNA Fingerprint

Each PSB isolate was grown on TY agar medium at 28°C for 24h and colonies were suspended in 20 µL of lysis

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solution (NaOH 0.01M + SDS 0.25%) and boiled at 100°C for 30 min. After addition of 0.1 ml of sterile milliQ water, cell suspensions were centrifuged at 13500 g for 5 min and the supernatant was used as source of genomic DNA for BOX-PCR amplification using the universal BOXAR1 primer (5-CTACGGCAAGGCGACGCTGACG-3) synthesized by *Invitrogen*, Argentina. PCR amplification was performed with a T18 thermo cycler (*Ivema Desarrollos* SRL., Argentina) using the following temperature profile: (i) 95°C for 6 min, (ii) 94°C for 1 min, 53°C for 1 min, 65°C for 8 min (35 cycles), and (iii) 65°C for 16 min [37]. PCR products were separated in agarose (1.5% w/v) with ethidium bromide by gel electrophoresis at 80 volts during 3h. Gels were exposed to UV light and photographed. Each agarose gel included a lane with molecular size markers (1Kb Plus Ladder DNA, Fermentas, Buenos Aires, Argentina) in order to compare the BOX-PCR patterns obtained for each isolate. The gels were then analyzed with the Bionumerics software (*Applied Maths*, Belgium, temporary Bionumerics evaluation license). Band patterns were optimized with a 3.0% tolerance. Cladograms were obtained with the Unweighted Pair Group Method with Arithmetic Averaged (UPGMA) algorithm [38] and similarity matrices were obtained using the Pearson's product moment correlation coefficient. Cluster analysis of BOX fingerprint patterns was done at 80% similarity.

2.5. MALDI-TOF MS Analysis

Identification and classification of the isolates were performed by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) and the MALDI Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany) [39]. Samples for MALDI-TOF MS analysis were prepared according to the methodology described by Lopez, et al [40]. Isolate identification was performed according to the classification described by Ferreira et al., [41]: score values <1.7, no identification; 1.70 ≤ score values <2.0, genus identification; score values ≥2.0, species identification. Prior to the present study, the original commercial database was expanded with the corresponding MS spectra of different bacterial species isolated from seeds, plants, nodules, and rhizospheric soil [40, 42, 43]. Analysis by MALDI-TOF MS was performed in the Center of Chemical and Biological Studies by Mass Spectrometry (CEQUIBIEM) of the Faculty of Exact and Natural Sciences (FCEyN) of Buenos Aires University (UBA).

2.6. Biological nitrogen fixation (BNF) assay

Bacterial isolates were grown in 1 mL TY medium on a rotary shaker at 28°C for 24h. Cells were collected by centrifugation at 6000 rpm for 5 min, washed twice, and suspended in 500 µL of sterile 10 mM $MgCl_2$ solution. Ten

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µL aliquots of the suspension obtained for each isolate were inoculated on the surface of NFb, a nitrogen-free semisolid medium [44], and were incubated at 28°C for 96h. Each isolate was tested twice; isolates that developed a white pellicle over the surface and blue color in the culture medium were considered positive for nitrogen fixation [45].

2.7. Siderophore production

Bacterial isolates were grown in TY medium and washed as described for the BNF assay. Ten µL aliquots of isolates suspended in sterile 10 mM $MgCl_2$ solution were spotted on solid pH 7 TY. After incubation at 28°C for 24h, 15 mL of Chrome Azurol S (CAS) agarized medium [46] was overlaid on top of the TY plates where the isolates were previously grown, incubated at room temperature, and analyzed at 2, 4, and 24 h post addition of CAS agar. Siderophore production was identified by the formation of yellow-orange haloes around the colonies.

2.8. Indol-3-acetic acid (IAA) production

IAA production was estimated according to the protocol of Gordon and Weber [47]. Bacteria were cultivated in 1 mL of TY medium supplemented with 10 µM L-tryptophan (Sigma-Aldrich, USA) on a rotary shaker at 28°C for 72h. After centrifugation of 100 µL aliquots of bacterial cultures at 6000 g for 5 min, the supernatants were dispensed in triplicate in a 96-well microplate. Then, 100 µL of Salkowski reagent were added to each well and the reaction mixture was incubated for 30 min in the dark. A microplate reader (Synergy H1, BioTek, USA) was used to determine the absorbance at 530 nm. The concentration of IAA was calculated using a calibration curve built with 0, 10, 20, 50, and 100 µg/mL IAA (Sigma-Aldrich, USA).

2.9. Data Analysis

The PAST software (Paleontological Statistics) 4.02 [48] was used to calculate Shannon's (H') [49] and Simpson's (1-D) [50] diversity indices from BOX-PCR fingerprint patterns and taxonomic data of the different PSB isolates. GraphPad PRISM 7.00 was used for statistical analysis of results by Students's T-test or one-way or factorial analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Wilcoxon's non-parametric test was used when the assumptions of parametric tests were not accomplished.

3. Results

3.1. Soil analysis

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All the replicates presented exchangeable sodium percentage (ESP) values higher than 15 and pH higher than 8.5 (**Supplementary Table 1**), which confirmed the alkaline-sodic condition of sampled soils.

3.2. Isolation of culturable and phosphate solubilizing bacteria

Rhizosphere soil collected from the two studied grass species was used to estimate the population size of CRB and PSB. CRB obtained from *P. coloratum* samples ranged from 6.72 x 10^5 to 5.85 x 10^6 CFU/g rhizosphere soil, and those from *S. indicus* samples ranged from 3.10 x 10^5 to 1.03 x 10^7 CFU/g rhizosphere soil. In turn, the population size of PSB from *P. coloratum* ranged from 4.16 x 10^4 and 5.06 x 10^5 CFU/g rhizosphere soil, while for *S. indicus* it ranged from 1.31 x 10^4 to 5.93 x 10^5 CFU/g rhizosphere soil. In the rhizosphere of *P. coloratum*, the percentages of PSB relative to CRB varied between 3.60% and 10.56%, while for *S. indicus* they varied from 2.88% to 13.26%. The analysis of mean CRB and PSB values revealed no significant differences between either rhizosphere environments (**Supplementary Table 2**).

3.3. Phosphate solubilization under alkaline-sodic conditions

Taking into account that PSB were isolated from alkaline-sodic soils, we analyzed not only the ability of PSB to solubilize P under neutral pH, but also under alkaline-sodic conditions. Therefore, a group of PSB isolates (selected according to the criteria described in Materials and Methods section) was tested for the ability to solubilize P under different pH and sodic conditions. The evaluation was performed at pH 8 and 9, two values within the usual pH range of alkaline-sodic soils in the flooding pampa. All the PSB isolates obtained from *P. coloratum* and a very high percentage of those obtained from *S. indicus* (98%) were able to solubilize P under alkaline-sodic conditions, at both pH 8 and 9. These high percentages suggest that in the rhizospheric communities, PSB have a high capacity to adapt to alkaline-sodic conditions of the flooding pampa. In addition, PSB were classified into three categories (1-3) by a semi-quantitative analysis based on a solubilization halo score (h), as described in materials and methods (**Supplementary Figure 1**). PSB isolated from the rhizosphere of both plant species were mainly distributed in classes 2 and 3 under both alkaline-sodic conditions tested.

3.4. Genetic and taxonomic diversity of PSB

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PSB isolated from the rhizosphere of *P. coloratum* were distributed in 96 clusters by BOX-PCR fingerprint analysis, most of which were represented by a single isolate. BOX profiles with the highest frequency of occurrence were detected in clusters I, XIV, XVI, XLIV, and LXXII (3.42; 4.79; 2.73; 6.16; and 4.11 %, respectively (**Supplementary Figure 2A**). In the rhizosphere of *S. indicus*, PSB isolates were distributed in 118 clusters according to their BOX fingerprints (**Supplementary Figure 2B**). As observed for *P. coloratum*, most of the clusters of PSB associated to *S. indicus* consisted of a single isolate. For this plant species, BOX profiles with the highest frequency of occurrence (2.72%) corresponded to PSB isolates grouped in cluster LXXIV. We were able to calculate diversity indexes of PSB associated to *P. coloratum* (H=4.345; 1-*D* =0.989) and *S. indicus* (*H'* =4.693; 1-*D* =0.997), which showed that both plant species harbor highly diverse rhizospheric PSB communities.

To taxonomically identify PSB isolated from both plant species, a MALDI-TOF MS approach was used. Most of the isolates (95 to 99%) were identified at the genus level, with score values above 1.7 (**Supplementary Table 3**). Rhizospheric PSB communities of both grasses were dominated by gram-negative bacteria of the phylum *Proteobacteria* and, to a lesser extent, by gram-positive bacteria of the phyla *Firmicutes* and *Actinobacteria*. Fifteen genera were identified in the PSB collection obtained from the rhizosphere of *P. coloratum* and 12 from the native species *S. indicus* (**Figure 1**). Eleven genera were found to be common to both rhizospheres (*Pseudomonas, Enterobacter, Pantoea, Rahnella, Citrobacter, Serratia, Escherichia, Raoultella, Klebsiella, Leclercia*, and *Bacillus*) (**Figure 1**). In addition, PSB of the genera *Salmonella, Acinetobacter, Arthrobacter*, and *Ewingella* were identified only in the rhizosphere of *P. coloratum*, while PSB of the genus *Stenotrophomonas* were found only in the rhizosphere of *S. indicus* (**Figure 1**). In the rhizospheric communities of both plants, the mean proportions of isolates of the genera *Pseudomonas* and *Enterobacter* were significantly higher than those of other genera ($P \leq 0.05$) (**Figure 1**). Richness and diversity of PSB genera (Shannon's and Simpson's indices) showed no significative differences between the communities associated with both plant species (**Supplementary Table 4**).

3.5. *In vitro* analysis of PGP abilities exhibited by PSB

In addition to P solubilization, other PGP activities are often found in PSB [51, 52]. Therefore, PSB of both plant rhizosphere communities were tested for the presence of PGP capacities such as BNF, siderophore, and IAA production.

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A high proportion of the isolates analyzed for each community (92% for *P. coloratum* and 88% for *S. indicus*) showed at least one of the abovementioned activities, while only a minor fraction exhibited none of them (**Supplementary Table 3A and B**). The analysis of PGP activities exhibited by PSB isolates of both plant communities as a whole showed that siderophore production is represented in a higher proportion ($P \leq 0.05$) of the isolates (68%) than IAA production (34%). (**Figure 2A**). The proportion of isolates showing BNF activity did not differ from the proportion of siderophore- and IAA-producing isolates (**Figure 2A**).

The comparison of the abundance of each PGP activity between both rhizospheric communities revealed that siderophore production was represented in a higher proportion in *P. coloratum* than in *S. indicus* ($P=0.035$), while no differences in the proportion of nitrogen-fixing isolates were detected between both plants. PSB from the *P. coloratum* community showed a higher proportion of IAA-producing strains than those from the *S. indicus* community, although with a low level of significance ($P=0.065$) (Two-way ANOVA) (**Figure 2B**). The analysis on the prevalence of PGP activities in the rhizospheric community of *P. coloratum* revealed that siderophore production was represented in a higher proportion of the isolates (75%) than IAA production (46%) and BNF (50%) ($P \leq 0.05$) (**Figure 2B**). For the *S. indicus* community, siderophore production and BNF were represented in a higher proportion of isolates (61% and 54%, respectively) than IAA production (22%) ($P \leq 0.05$) (**Figure 2B**).

In the rhizospheric community of *P. coloratum*, nitrogen-fixing, siderophore-producing, and IAA-producing PSB isolates comprised 8, 10, and 11 genera, respectively, as well as a very minor fraction of non-identified bacterial isolates. *Pseudomonas* and *Enterobacter* were the most abundant genera of PSB that exhibited BNF and siderophore-producing activity. In relation to IAA-producing PSB, *Enterobacter* was the most represented genus **(Figure 3).**

In the rhizospheric community of *S. indicus*, nitrogen-fixing, siderophore-producing, and IAA-producing PSB isolates comprised 9, 9, and 6 genera, respectively, as well as a very minor fraction of non-identified bacterial isolates. *Enterobacter* and *Pseudomonas* were the most predominant genera of PSB that exhibited BFN activity, siderophore, and IAA production. **(Figure 3)**.

4. Discussion

Bacteria are one of the dominant members of the rhizosphere community that allow plants to expand their functional capacities in basic and common needs, such as nutrient acquisition or pathogen suppression, enabling their adaptation

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and development in different environments. In the present study, we addressed the prospection of the community of culturable PSB from unexplored rhizospheric environments, such as those of *S. indicus* and *P. coloratum* species growing in alkaline-sodic soils.

Several studies have estimated PSB abundance in the rhizosphere of a wide range of plant species growing in environments with different physicochemical characteristics. Such studies revealed that the proportion of PSB can show significant variations, in some cases representing an important fraction (approximately 40%) of the total culturable bacteria [53]. Rhizospheric PSB abundance depends on soil nutrient levels, pH, moisture, organic matter content, as well as isolation methods and culture media used for their enumeration [54]. Some papers also reported variations in PSB abundance in soils with different vegetation cover compositions [55]. Thus, these studies showed that PSB represents a ubiquitous functional group in agroecosystems and in turn demonstrated that the abundance of PSB in rhizospheric communities are affected by a multiplicity of factors. However, the abundance and other aspects of PSB in rhizospheric communities of plants grown in alkaline-sodic soils have been scantly studied so far.

In the present study, a rich culture medium was used to isolate rhizospheric bacteria and subsequently establish the proportion of culturable bacteria that have the ability to solubilize phosphate. It is possible that the use of a rich medium may have biased the isolation toward copiotrophic bacteria over oligotrophic bacteria. Even so, prospecting for rhizobacteria growing on rich media may be of great interest to the biofertilizer industry where the use of such media is common.

The average PSB population found in the rhizosphere of *P. coloratum* and *S. indicus* growing in alkaline-sodic soils of the flooding pampa ranged from 2 to 14% of total culturable bacteria. A study by Abderrazak, Laila and Jamal [56] related to rhizospheric bacteria associated to wheat plants grown in alkaline soils of Meknes (Morocco) found PSB to represent 7% of total culturable bacteria, a proportion within the range detected in the present study.

The high number of genotypes identified by Box-PCR analysis revealed that the communities of native PSB associated to both *P. coloratum* and *S. indicus* in alkaline-sodic soils of the flooding pampa are highly diverse. Similarly, Box-PCR studies performed by Collavino, Sansberro, Mroginski and Aguilar [57] demonstrated that yerba mate (*Ilex paraguayensis*) plants cultivated in acidic soils with low P availability harbor highly diverse rhizospheric PSB communities. Thus, although the information about the diversity of rhizospheric PSB in soils with low P content is limited, it seems that a high genetic diversity is a common trait of this functional group of PGPR in soils with low

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levels of these nutrients. In addition, most of the PSB analyzed in the present study exhibited high P solubilization scores (h= 2 or 3), based on semi-quantitative analysis of their solubilization capacity. The abovementioned study also showed the presence of rhizospheric PSB with high solubilization efficiency. Likewise, seven independent studies of plant growth-promoting bacteria based on similar bioprospecting and phenotype selection methodologies revealed that a high capacity to solubilize tricalcium phosphate by bacterial isolates is associated with nutrient-poor soils [58]. Thus, this information together with results of the present study provide evidence that high phosphate solubilization efficiency is a usual feature of rhizospheric PSB communities in P-limited agroecosystems.

Research interest on PSB is based not only on the ecological role of this functional group of PGPB in natural ecosystems, but also on their potential to improve soil fertility. Soils in the flooding pampa are highly heterogeneous in several aspects, including pH. In parallel, low P content is a common trait in soils of this region. In this way, agronomic practices capable of increasing P availability would contribute to sustainable land use for agriculture and cattle breeding. In this regard, biofertilizers based on PSB would need to be effective in a wide range of soil pHs. A previous study by our group showed that PSB isolated from the rhizosphere of *Lotus tenuis* plants grown in soils similar to those of the current study were able to solubilize P both under neutral and alkaline-sodic conditions [28]. PSB isolated in the current study from the rhizosphere of *S. indicus* and *P. coloratum* also showed the ability to solubilize P under the same stressful conditions. Thus, the similarities found in both studies, regarding the plasticity of PSB to solubilize P under neutral and alkaline conditions, suggest that this capacity is a prevalent trait in rhizospheric communities of culturable PSB in alkaline-sodic environments of this region. In this way, rhizospheric communities of PSB seem to be a promising source of isolates for the development of bioproducts aimed to increase P availability in soils of the flooding pampa.

Numerous studies highlighted the importance of the chemical composition of the rhizospheric environment as a determinant of the structure of microbial communities. Plant exudates affect soil physicochemical properties in the root environment, acting either as microbial chemoattractants or repellents [59-61]. In the present study, the culturable PSB communities of the rhizosphere of *P. coloratum* and *S. indicus* showed similarities in terms of their taxonomic structure, which was dominated by *Gammaproteobacteria*, mainly of the genera *Enterobacter* and *Pseudomonas*. High abundance of *Gammaproteobacteria* in rhizospheric environments has been reported for different plant species such as maize grown on carbonate-rich alkaline soils [62], *Brachiaria* spp. grasses used as forage in marginal soils [63], species of *Poaceae* native from the Andean Puna region [64], and several halophytic species from semi-arid and

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arid regions of Pakistan [65]. Moreover, the high proportion of *Pseudomonas* and *Enterobacter* detected in the present study might be related to the previously reported ability of these taxa to chemotactically respond to a variety of exudates secreted by plants [66-68], colonize roots in diverse environments [69, 70], and metabolize a wide range of carbon compounds exudated by roots [62]. Interestingly, a recent study provided evidence that the abundance of PSB of the genera *Pseudomonas* and *Enterobacter* in rhizospheric soil of *Mikania micrantha* contributes to the increase in rhizospheric P level, thus suggesting a leading role of these bacterial genera in the adaptation and development of this plant species [71]. In this sense, the abundance of PSB of the genera *Pseudomonas* and *Enterobacter* in the rhizosphere of *P. coloratum* and *S. indicus* plants detected in the current work could be beneficial to the P nutrition of these grasses under low nutrient conditions typical of marginal soils of the flooding pampa, thus favoring their adaptation to this restrictive environment.

The communities of culturable PSB of the rhizosphere of *P. coloratum* and *S. indicus* harbored a high proportion of isolates that exhibited additional PGP abilities, such as nitrogen fixation, siderophore, and IAA production, which are of interest because of their beneficial effects on plants [2, 58, 72, 73]. A study by da Costa, Granada, Ambrosini, Moreira, de Souza, dos Passos, Arruda, and Passaglia [58] on a large number of bacteria with different PGP activities suggested that in nutrient-limited soils, plants favor interaction with tricalcium phosphate solubilizing bacteria and that siderophore production is related with the ability to solubilize this P source. It is known that siderophores not only bind Fe, but also various metal ions, and their production in the root environment may thus contribute to increase the availability of soluble phosphate sources to plants by releasing them from metals to which they are bound [58]. Interestingly, after analyzing the distribution of different PGP traits in PSB isolates, we found siderophore production to be the prevalent co-occurring activity in the PSB population of both rhizospheric environments. This observation leads us to hypothesize that, in environments with low P availability, such as the alkaline-sodic lowlands of the flooding pampa, plants recruit and interact with bacteria that exhibit both P solubilization and siderophore production activity, as a strategy to efficiently obtain soluble P.

N-fixing activity was represented in about 50 % of the rhizospheric PSB communities of both plant species analyzed in the present work. Recruitment of N-fixing *Proteobacteria* has also been reported for other forage grasses grown on low fertility soils, where this phylum is dominant [74]. Some studies have shown that *Panicum* species allocate a large portion of photosynthetically fixed C below ground, which can be assimilated into the microbial component in a short period of time [75]. Reis, dos Reis, Quesada, de Oliveira, Alves, Urquiaga, and Boddey [76] reported that some grass

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species obtain up to 41% of their N through biological N fixation and it was suggested that this is achieved by allocating large amounts of C to root exudates. Based on this background, it cannot be ruled out that the high proportion of N-fixing activity detected in both PSB communities in the present work is part of an adaptive strategy deployed by *P. coloratum* and *S. indicus* to efficiently assimilate nitrogen, a scant nutrient in sodic-alkaline environments.

Indole acetic acid production is widely distributed among rhizospheric bacteria and is estimated to be present in about 80% of such microorganisms [77]. The role of IAA-producing microorganisms in shaping plant root architecture, improving the availability of P and other nutrients [78, 79], modulating endogenous production of plant hormones to alleviate or diminish the deleterious effects of abiotic stresses and maintaining plant health is well known [80, 81].

The method routinely used to detect IAA production in bacterial populations is the Salkowski method. This method in addition to IAA also detects indole compounds not necessarily involved in plant growth promotion. Therefore, quantification of positive PSBs by Salkowski may not strictly reflect the abundance of strains with potential for plant growth promotion. On this basis, the abundance of IAA-producing bacteria associated with *P. coloratum* and *S indicus* was estimated by considering only those isolates that produced above a threshold level of 70 µg IAA equivalents /ml, a value that is higher than the average concentration found in both PSB populations. Thus, IAA production activity detected in PSB was less represented than BNF and siderophore activity in the rhizospheric environment of both *P. coloratum* and *S. indicus*. In this regard, it is worth to point out that the proportion of IAA-producing PSB was higher in the rhizosphere of *P. coloratum* than *S. indicus*. This suggests a greater need for *P. coloratum* to recruit and associate with IAA-producing microorganisms, as compared to *S. indicus*. Several reports related the adaptation of *P. coloratum* to stress conditions with the ability to develop a deep root system that allows to efficiently explore the soil profile [19, 82]. Thus, based on the high number of IAA-producing hereby found in the rhizosphere of *P. coloratum*, it can be speculated that IAA-producing PSB facilitate nutrient uptake and contribute to the ability of this plant species to thrive under growth-restrictive low nutrient-availability conditions. Finally, it is worth to highlight that PGP activities analyzed in the present work were detected in a variety of PSB belonging to different genera and phyla, thus revealing the redundancy of these functions within both *P. coloratum* and *S. indicus* rhizospheric communities. In relation to these findings, the redundancy of PGP traits is considered an important feature for microbial communities to maintain ecosystem function and stability under fluctuating environmental conditions [83].

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Enterobacter, besides being one of the most abundant genera in both cultivable PSB rhizospheric communities, was highly represented within the bacterial fraction that harbored all of the three tested activities. *Pseudomonas*, another abundant genus in the cultivable PSB community, was frequently found to be associated with the PGP activities in the rhizosphere of *S. indicus*. On the contrary, in the rhizosphere of *P. coloratum*, the genus *Pseudomonas* was mainly found to be associated with biological nitrogen fixation and siderophore production activities, but in a low proportion with indole production. Taking into account the numerous reports highlighting the strong influence of chemical properties of rhizospheric soil on the structure of bacterial communities [84, 85], differences in the composition of root exudates of both grass species could underlie the taxonomic and functional differences detected between cultivable PSB communities of both plant environments. In addition, it should be kept in mind that *S. indicus* is a native species, while *P. coloratum* was introduced in the flooding pampa approximately seventeen years ago. Thus, differences in the abundance of strains harboring specific PGP traits between the rhizosphere of both plant species could also be related to long-term dynamics of the build-up of microbial communities associated to them.

In conclusion, the present study demonstrated that microbial rhizospheric communities of *S. indicus* and *P. coloratum*, two grasses well adapted to grow in the flooding pampa, harbor cultivable PSB with multiple PGP traits. The presence of multiple PGP in a variety of bacterial taxa probably contributes to the adaptation of the abovementioned species to the restrictive environmental and soil conditions typical of this region. In this regard, the collection of cultivable rhizospheric PSB of both plant species and their taxonomic and functional characterization constitute a valuable source of information and genetic resources available for the development of efficient biofertilizers, which contribute to optimizing the use of chemical fertilizers and thus favor sustainable forage production in the flooding pampas

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Declaration of competing interest

The authors have no conflict of interest to declare.

Availability of data

All data generated or analyzed during this study are included in this published article.

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Figures Legends

Figure 1. Genus composition of the culturable community of PSB isolated from the rhizosphere of *P. coloratum* and *S. indicus*. PSB communities were identified at the genus level by MALDI-TOF MS**.** For each rhizospheric PSB community, mean percentages of each taxon were calculated and compared by

one-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate statistically significant differences ($P \leq 0.05$) and comparisons are only valid within each community. NI: non-identified isolates.

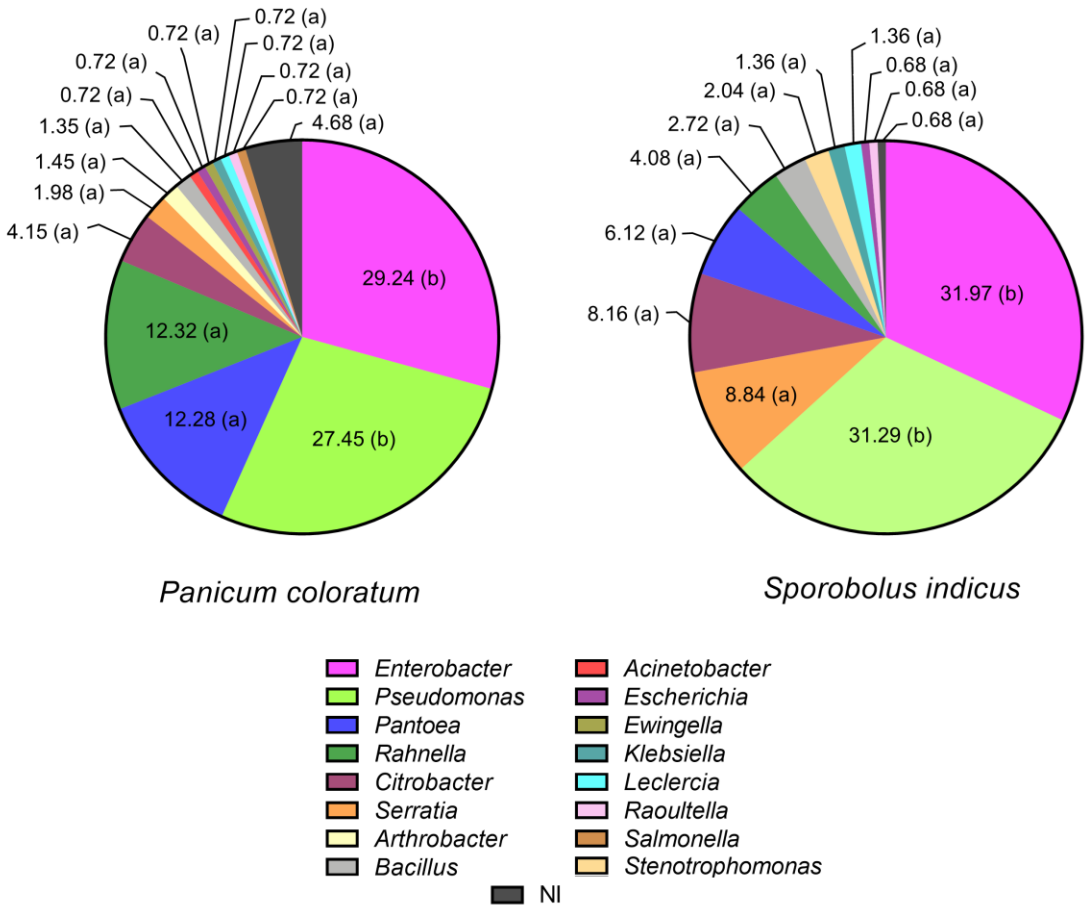

Figure 2. Quantitative representation of PGP traits analyzed *in vitro* for PSB of rhizospheric communities of *P. coloratum* and *S. indicus*. A). Total distribution of PGP traits in PSB communities. The distribution was analyzed in conjunction for both plants, without considering the plant species as a source of variation. Bars represent means ± SEM. Data were compared by one-way ANOVA and Tukey's multiple comparison test. Different letters indicate statistically significant differences ($P \leq 0.05$). **B) Distribution of PGP traits in PSB of the rhizospheric community of *P. coloratum* and *S. indicus*.** Data were analyzed by two-way ANOVA and Tukey's multiple comparison test. Capital letters indicate statistically significant differences between PGP traits of the *P. coloratum* community, while lower case letters indicate statistically significant differences between PGP traits of the *S. indicus* community. *P* values shown in the figure correspond to comparisons performed for each PGP trait between both rhizosphere communities by using a T-test.

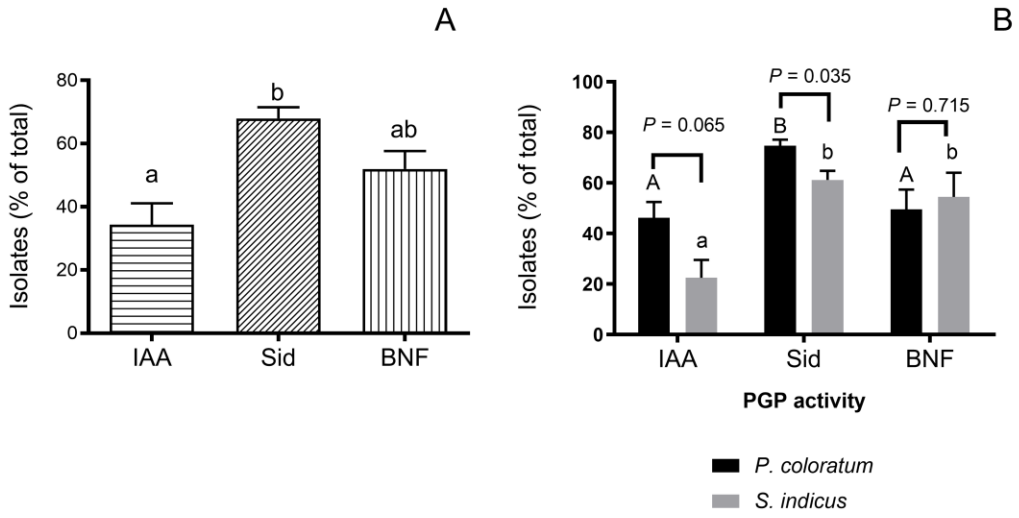

Figure 3. **Contribution of different bacterial genera to the PGP activities detected in PSB from rhizospheric communities of *P. coloratum* and *S. indicus*.** Numbers represent mean percentages of PSB isolates that exhibited a particular PGP trait. Letters indicate statistically significant differences according to one-way ANOVA and Tukey's multiple comparisons test NI: PSB isolates not identified by MALDI-TOF. Data were analyzed by one-way ANOVA and Tukey's multiple comparisons test.

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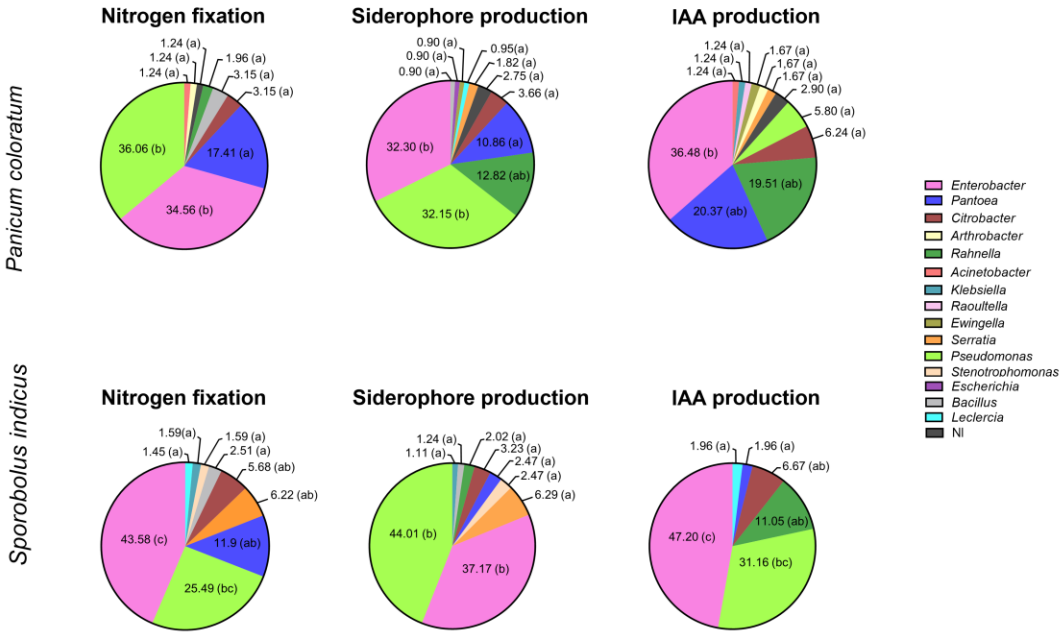

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