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Growth promoting bacteria associated with *Opuntia cholla* rhizosphere

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Abstract. Opuntia is one of the most diverse and complex genera of the cactaceae family. This cactaceae has a wide distribution and presence in Mexico. The soil and plant microbiome, in natural ecosystems, play a crucial role on plant development under diverse conditions, impacting biodiversity across different regions. The isolation and characterization of these microorganisms allows the identification of those with agronomic potential; especially the microorganisms that act as plant growth promoters. Therefore, this study focused on the quantification and identification of bacteria with agronomic potential associated with the rhizosphere of *Opuntia cholla*. The samples of soil and roots were collected from different plants established at three different locations around La Paz, B.C.S., Mexico, with different soil properties (organic matter content, pH and electrical conductivity). The samples were taken at 15 cm dept, removing the soil and other plants at the surface. The quantification and isolation of culturable nitrogen fixing bacteria were assessed. Also, phosphate solubilization capability and antagonism against *Fusarium oxysporum* were evaluated for the isolated strains. The strain genus identified with all capabilities mentioned above were *Rhizobium, Bacillus, Pantoea, Paraburkholderia, Pseudomonas* and *Rhodococcus*. El Carrizal showed the highest amount and ratio of nitrogen fixing bacteria strains with phosphate solubilization capability and antagonism against *Fusarium oxysporum* (11, out 82 isolated strains). Also, it was the area with the highest organic matter content (1.81%, average). The identification of rhizobacteria with multiple useful properties for agriculture is a major goal for sustainable development.

Keywords: *Choya, rhizobacteria, nitrogen fixing bacteria, phosphate solubilizing bacteria, antagonism.*

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Introduction

Opuntia genus has a wide distribution and representativeness in Mexico; it is found from coastal dunes to coniferous forests. However, in Mexico, a greater presence is observed in arid and semi-arid areas, such as the Sonoran and Chihuahuan deserts (Mercado, 2014). In these areas, the soil microbiome is a useful indicator of the ecosystem's health in relation to the processes and resource management that impact biodiversity and population density (Ramírez *et al*., 2013). Soil microorganisms are directly linked to the plant species and to the soil characteristics such as organic matter, pH and electrical conductivity (Cruz *et al*., 2020). The bacteria and fungi can densely colonize the rhizoplane of different cactus species, such as *Pachycereus pringlei*, *Stenocereus thurberi* and *Opuntia cholla*, and some of these bacteria have an important role in their growth and

development (Fonseca et al., 2016).

The microorganisms associated with plants achieve diverse activities due to their biochemical versatility; for example, they carry out oxidation and reduction reactions. They also carry out other processes such as hydrolysis, precipitation or solubilization of different elements, which directly or indirectly affect the characteristics of the soil (Pérez and Chamorro, 2013). Well-defined associations of some genera of bacteria with specific plants are reported, *Rhizobium* with legumes, *Frankia* with plants of the genus *Alnus* and *Azospirillum* with grasses. The free-living bacteria of the genus *Azotobacter*, *Pseudomonas* and *Bacillus* have been also identified as beneficial for the development of some plants (Bhadrecha *et al*., 2023). The use of bacteria as biofertilizers and biocontrol agents against some pathogens has been shown to be a promising strategy for reducing the use of synthetic toxic agrochemicals (Espinosa-Palomeque *et al.,* 2025). Therefore, the objective of the present study was to characterize the microorganisms present in the rhizosphere of choya (*Opuntia cholla*) with nitrogen-fixing, phosphate-solubilizing and antagonistic activity against *Fusarium oxysporum* in three sites around La Paz, Baja California Sur, Mexico.

Material and Methods

Sample collection sites

Sampling was carried out at three different sites, the first site is situated in the area of the Northwest Biological Research Center (CIBNOR) located at 24°07´49.3´´N 110°20´05.9´´W, the second site was in San Pedro community located at 24°07´48.8” N 110°20´05.9´´W and the third site in El Carrizal community located at 23°47´04´´N 110°18´47´´W (Figure 1). These sites were chosen according to the soil texture; CIBNOR site has a sandy soil, San Pedro has a clayish soil and El Carrizal has a sandy loam soil (unpublished data provided by the soil and water laboratory of Autonomous University of Baja California Sur).

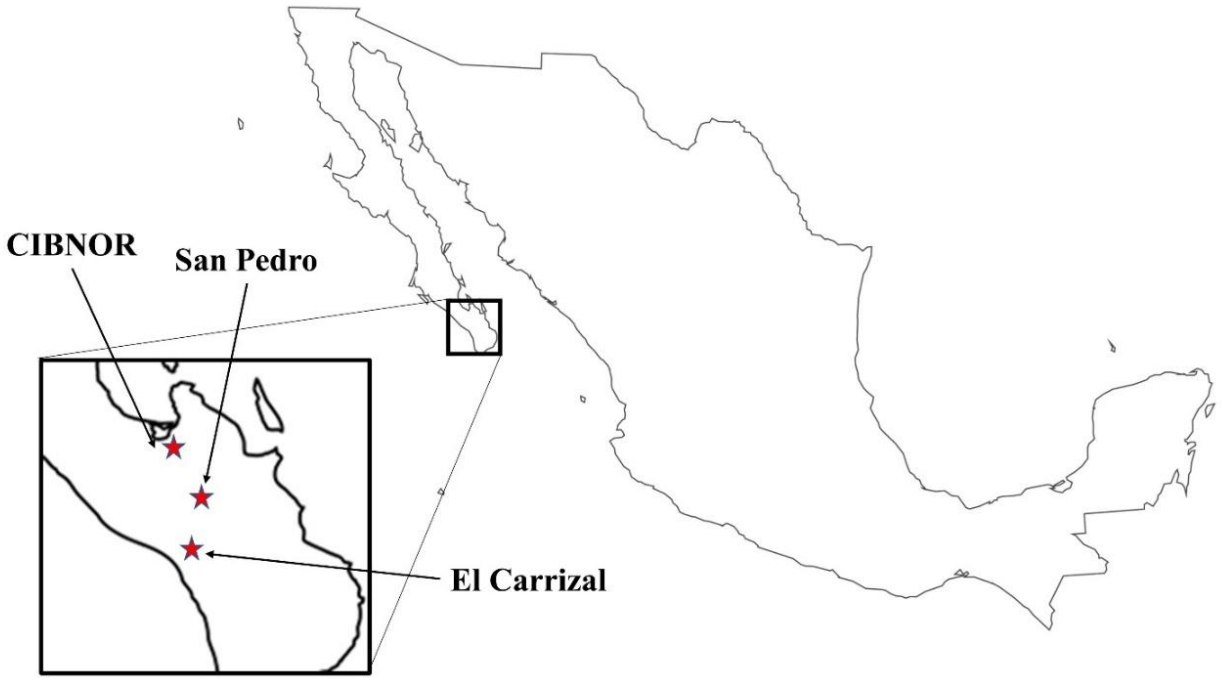

Figure 1. *Opuntia cholla* root and soil sample collection sites, CIBNOR, San Pedro and El Carrizal.

Sample processing

At each site, five choya (*Opuntia cholla*) plants were randomly selected. Once the plants were chosen, the sampling site was cleaned by removing dry plant matter above the soil. The soil was removed around the root to a depth of 15 cm. At this depth, soil and root samples were taken from each plant for edaphological and microbiological analysis (Aguirre-Garrido *et al*., 2012). For root samples, 10 g of root were cut with garden shears and placed in flasks with 90 mL of sterile 0.1 M phosphate buffer solution at pH 7.2, added with NaCl at a concentration of 20 mM (Arce-Amezquita *et al*., 2021).

Soil samples were collected by suspending 10 g of soil in the buffer solution in the same way as the root samples. In addition, another sample of approximately 50 g of soil was taken for physicochemical analysis. The sample processing was carried out in the Pharmacognosy Laboratory of the Autonomous University of Baja California Sur, located at 24°06´1.0” N 110° 18´53.7” W.

Soil analysis

The amount of organic matter in the soil samples was estimated by the chromic acid titration method proposed by Walkley and Black (1934). The pH and electrical conductivity were determined from a 1:1 solution (soil-distilled water) using a potentiometer (Hanna®, HI 9813-6, Hanna Instruments).

Microbiological analysis

The collected root and soil samples were homogenized and used to prepare serial dilutions (1:10). The collected samples at each site were considered as the 10^{-1} dilution. Subsequently, 100 µL of the obtained dilutions were placed and spread on nutrient agar and Rennie agar culture medium (Rennie, 1981) on Petri dishes to determine the total amount of microorganisms and the amount of nitrogen-fixing microorganisms, respectively. The Petri dishes were incubated at 30 °C for 48 h. The number of colony-forming units (CFU) was determined according to the type of sample and type of medium. The data obtained were reported as the number of colony-forming units per gram of original sample (soil or root).

Isolation and purification of nitrogen-fixing and phosphate-solubilizing bacteria

Nitrogen-fixing bacteria strains were isolated and purified from Rennie agar culture medium according to their colonial morphology. Colonies were selected and cultured again on Rennie agar culture medium repeatedly until pure strains were obtained. These strains were subsequently cultured on NBRIP medium (Scattareggia, 2016) to determine their phosphate solubilizing capacity by observing the appearance of transparent halos in the culture medium around the cultured colonies.

Evaluation of antagonistic activity against Fusarium oxysporum

The strains that showed nitrogen-fixing and phosphate-solubilizing activity were challenged against *Fusarium oxysporum* on potato-dextrose agar (PDA) culture medium to determine their antagonistic capacity following a method developed by our research group. For this evaluation, a spore suspension was prepared from a Petri dish with *Fusarium oxysporum* with 7 days of development. Then, Petri dishes with PDA were inoculated with 100 µL of the spore suspension at a concentration of 1×10^6 spores per mL^{-1}. After allowing the surface of the medium to dry, it was inoculated with the bacterial strains to be evaluated. The antagonistic activity was considered positive when an inhibition of the fungus development around the colonies was observed after 7 days of incubation at 30 °C.

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Molecular identification

For the identification of the bacteria of interest, bacterial strains that showed the three activities, nitrogen fixation, phosphate solubilization and antagonistic activity were selected. Molecular identification was carried out by sequencing the 16S rDNA gene. Total DNA was extracted following the recommendations of the manufacturer of the Wizard® genomic DNA purification kit (Promega Corporation). PCR was carried out using the universal primers 27F (forward): 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse): 5'- GGTTACCTTGTTACGACTT-3'. The amplicons were purified by agarose gel electrophoresis and subsequently sequenced. The sequences obtained were processed by BLAST searches in NCBI to compare with microorganisms described in this database.

Statistical analysis

The data obtained (n = 5) were analyzed using the Anderson-Darling test to verify normality and the Levene test to determine homoscedasticity, both with a confidence level of 95%. Once the condition of normality and homoscedasticity was achieved, the data were analyzed using a one-way analysis of variance (ANOVA) in Minitab®. The significant differences between means were determined using the Tukey HSD test ($P \leq 0.05$). The significant differences between soil and root samples from the same site were determined using the Student t test.

Results and Discussion

Soil-microbiota relationship

The characteristics of each sampling site have a significant influence on soil properties. In most of the Baja peninsula´s territorial extension, soils with low levels of carbon and total nitrogen content are found; also, due to the proximity of coastal areas, it is common to find salt accumulation (Endo *et al.*, 2011). The evaluations carried out on the soil at the three sites match with the description above, since a low content of organic matter was found in all samples. The higher organic matter content was found at El Carrizal site, compared to the CIBNOR and San Pedro sites. The same trend was observed for pH. On the other hand, the electrical conductivity at CIBNOR showed a higher value on average, possibly due to its proximity to the coast compared to the other two sites (Figure 2).

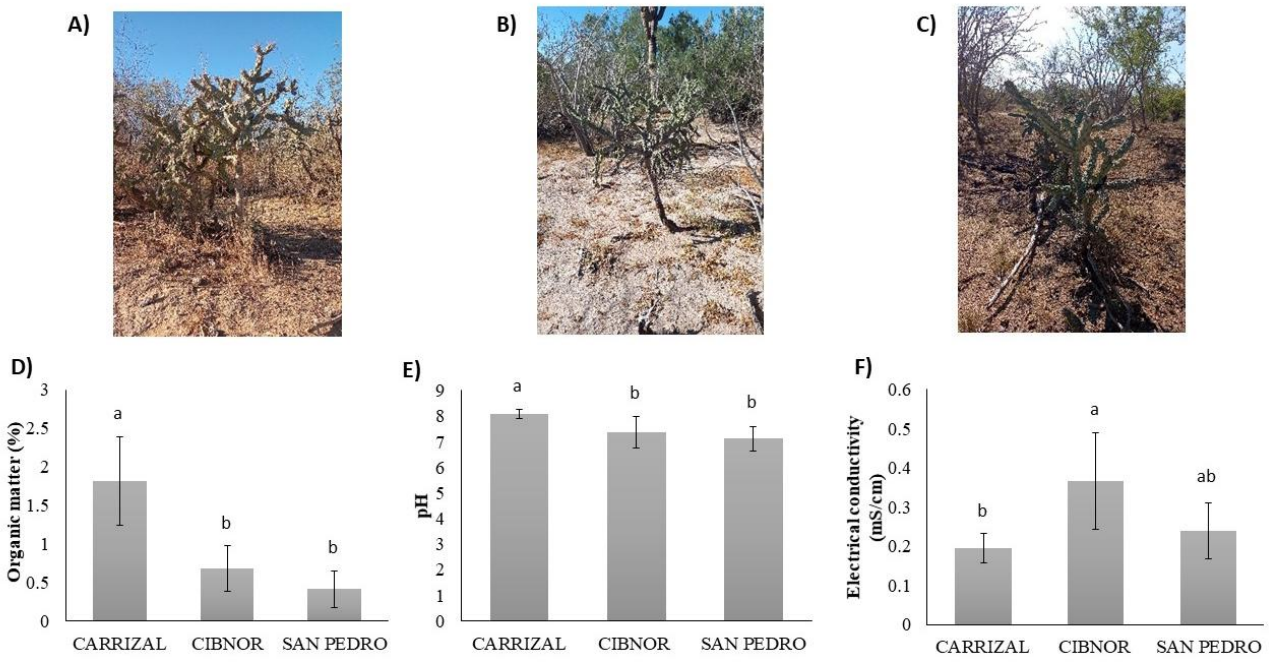


Figure 2. Photographs of an analyzed plant at A) El Carrizal, B) CIBNOR, and C) San Pedro. D) Organic matter content. E) Soil pH. F) Electrical conductivity. Different letters above the bars represent significant differences.

The number of bacteria varies in each site according to environmental factors such as humidity, electrical conductivity, organic matter content and soil pH (Bulgarelli *et al.*, 2015). In general, El Carrizal site showed a greater presence of bacteria in soil and root samples in cultures with nutrient agar. Likewise, higher presence of nitrogen-fixing bacteria in cultures with Rennie agar was found. According to Trinidad-Santos (2016), the differences in organic matter content among the sites supports the difference of CFU at each site. In the present study, an inverse relationship was observed with respect to the electrical conductivity of the soil, higher conductivity with lower presence of bacteria in the rhizosphere. In the cultures with nutrient agar, a greater amount of CFU g^{-1} of root was observed at CIBNOR and San Pedro sites compared to the soil at the same site (Figure 3). This result could be attributed to the low organic matter content in the soil of these sites. Furthermore, the variability between soil and root samples indicates that microbiological development is not only influenced by the interaction with the environment, but also by the interaction with plants (Bashan *et al.*, 2015).

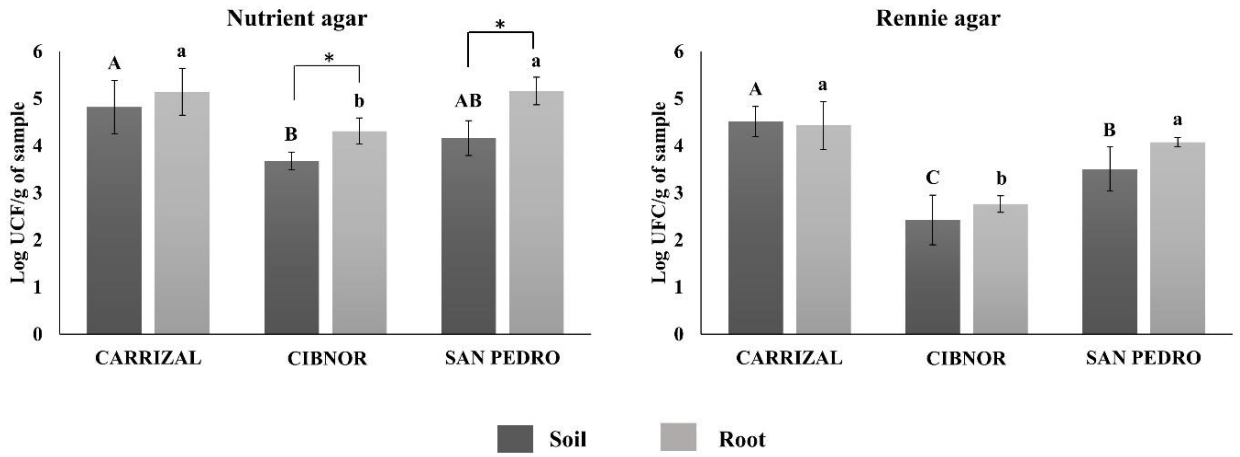


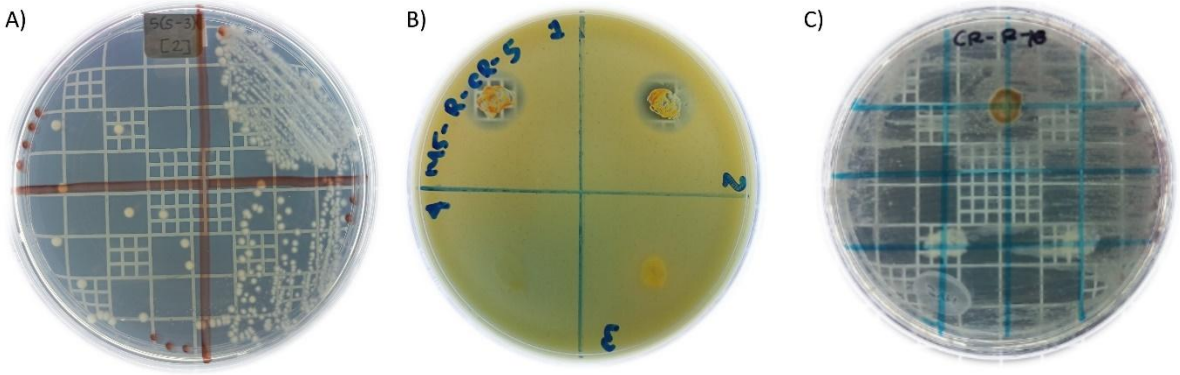
Figure 3. Overall bacterial content (nutrient agar) and nitrogen-fixing bacteria (Rennie agar) in soil and root of *Opuntia cholla* at the three sites. Different capital letters over the bars correspond to soil and represent significant differences; different lowercase letters on the bars correspond to roots and represent significant differences. The asterisks represent a significant difference between soil and root from the same site.

The microbiological development in the rhizosphere also depends on root exudates, because these exudates intervene in the process of microorganism-root signaling and recognition (Venturi and Keel, 2016). Likewise, Meraz (2019) indicates that bacteria are in a dynamic process between the soil and the roots; however, bacteria are mostly attracted to the roots due to the secondary metabolites, polysaccharides and proteins released nearby the roots. This environment promotes a beneficial association for the development of both, the plant and the microorganisms through the exchange of nutrients.

Isolation, characterization and identification of growth-promoting bacteria

In El Carrizal site, a greater number of nitrogen-fixing bacteria with phosphate-solubilizing activity and antagonistic against *Fusarium oxysporum* were found. In CIBNOR and San Pedro sites, a single strain from each site showed the three activities proposed in this study (Figure 4). It was determined that at El Carrizal site there is a greater proportion of bacteria that have multiple activities, close to 55 % of the nitrogen-fixing strains also can solubilize phosphates and 16 % of these strains have all three capabilities. In CIBNOR and San Pedro sites, the proportion of bacteria with all three capabilities is around 1.5 and 2.8 %, respectively.

In relation to these results, Rosenblueth and Martínez (2006) indicate that the highest content and diversity of bacteria is found within cacti (endophytes). In addition, Schouten (2019) points out that bacteria endophytes can increase nutrient absorption due to root growth promotion, nitrogen fixation and phosphate solubilization. Likewise, bacteria act not only as growth promoting agents but also as biocontrol agents (Toloza and Lizarazo, 2014).



	ISOLATED STRAINS		
SAMPLING SITE	NF	NF+PS	NF+PS+FA
CARRIZAL	82	45	11
CIBNOR	64	3	1
SAN PEDRO	36	8	1

Figure 4. A) Petri dishes with a nitrogen-fixing bacteria grown on Rennie agar. B) Example of an evaluation of the capacity of the isolated strains to solubilize phosphates on a Petri dish. C) Example of an evaluation of antagonism of the isolated strains against *Fusarium oxysporum*.

In the present study, all strains that showed the three activities were isolated from the root, which coincides with these authors (Table 1). The root conditions provide an environment for microbial development, and it is common to find a higher bacterial load associated with it, including nitrogen-fixing bacteria (Doncel *et al.*, 2016).

Table 1. Identification and origin of the isolated strains with nitrogen-fixing, phosphate solubilizing and antagonistic activity against *Fusarium oxysporum.*

Code*	Sample origin	Scientific name
CR-1	Root	*Rhizobium* sp.
CR-2	Root	*Streptomyces* sp.
CR-3	Root	*Pantoea dispersa*
CR-4	Root	*Enterobacter cloacae*
CR-5	Root	*Paraburkholderia fungorum*
CR-6	Root	*Paraburkholderia fungorum*
CR-7	Root	*Rhodococcus* sp.
CR-8	Root	*Bacillus subtilis*
CR-9	Root	*Enterobacter cloacae*
CR-10	Root	*Pseudomonas* sp.
CR-11	Root	*Pseudomonas aeruginosa*
CB-1	Root	*Paraburkholderia fungorum*
SP-1	Root	*Pseudomonas aeruginosa*

*The strain codes with letters CR belong to El Carrizal site, the strain code containing the letters CB belongs to CIBNOR site and the strain code containing the letters SP belongs to San Pedro site.

The identification of bacteria associated with cacti of the *Opuntia* genus is scarce. However, studies carried out on *Opuntia quitensis* demonstrated the presence of bacteria mainly of the genus *Bacillus* and *Pseudomonas* (Córdova-Rojas *et al.*, 2022). On the other hand, studies on *Opuntia ficus-indica* indicated the presence of bacteria of the genus *Pseudomonas* with plant growth-promoting and antagonistic activity against a phytopathogenic fungi in wheat (Mourad, 2019). Caballero-Mellado (1990) found the nitrogen fixing bacterium Azospirillum living associated with prickly pear cactus. This microorganism was found to natural Azospirillum populations in Opuntia roots were found to be 11,000 cells per g of fresh root. Likewise, not in *Opuntia* but in the columnar cactus *Cereus jamacaru*, strains of the genus *Rhizobium*, *Enterobacter*, *Pseudomonas* and *Pantoea* with phosphate-solubilizing activity were isolated (Lima *et al.*, 2015). In the present study, bacteria of the genus *Rhizobium, Streptomyces, Pantoea, Enterobacter, Paraburkholderia, Rhodococcus, Bacillus* and *Pseudomonas* were identified (Table 1).

Conclusions

Opuntia cholla associated with bacteria with nitrogen-fixing and phosphate-solubilizing activity, and antagonistic activity against *Fusarium oxysporum,* were isolated and characterized from three sites around La Paz, Baja California Sur, Mexico. The number of strains with all three capabilities were different at each site; therefore, the interaction soil-plant (*Opuntia cholla*, in this case) and bacteria were evidenced in the present study. Soil organic matter seems to have a more significant effect on the number of bacteria present in the rhizosphere. The higher presence of CFU in roots also supports the previous statement. This study lays the foundation for investigating the potential of useful bacteria associated with *Opuntia cholla* as a biofertilizer and/or biocontrol agents in agriculture, especially in arid and coastal areas where the environmental conditions tend to be extreme for plant development.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

All data generated or analyzed during this study are included in this scientific paper.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

Not applicable.

AUTHOR CONTRIBUTIONS

Conceptualization: GAMR., PMAA, BMA. Formal analysis: GAMR, PMAA. Investigation: GAMR. Methodology: GARM, PMAA, MRC, BMA. Resources: PMAA, MRC, BMA. Writing-original version: GAMR, PMAA, BMA. Writing-Review and editing: GAMR, PMAA, MRC, BMA. All authors have read and agreed to the published version.

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