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Evaluation of *Bacillus* spp. antagonism against phytopathogenic fungi of Persian lime (*Citrus* x *latifolia* Tan.)

Evaluación del antagonismo de *Bacillus* spp. sobre hongos fitopatógenos de lima persa (*Citrus* x *latifolia* Tan.)

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ABSTRACT

Lima persa (*Citrus* x *latifolia* Tan.) es un cultivo de importancia económica en Veracruz, con una producción afectada por enfermedades fúngicas como muerte regresiva y antracnosis. Para reducir el uso de fungicidas químicos, se ha reportado el empleo de bacterias del género *Bacillus* como Agentes de Control Biológico (BCA). En este estudio, se evaluó el efecto antagónico *in vitro* de bacterias nativas de *Bacillus velezensis* (Bv) y dos cepas de *Bacillus subtilis* (Bs1 y Bs2) sobre los patógenos identificados como *Lasiodiplodia theobromae* y *Colletotrichum siamense*. Se midió el Porcentaje de Inhibición de Crecimiento Radial (PIRG) en placas con agar papa dextrosa y se determinó el nivel de antagonismo.

Las cepas de *Bacillus subtilis* mostraron una alta y moderada inhibición contra los fitopatógenos evaluados. Bs2 presentó los mayores porcentajes de inhibición (66.69 % y 61.78 %) frente a *Lasiodiplodia theobromae* (Las1 y Las2). Bs1 mostró un valor moderado (49.7 %) de actividad antagónica sobre *Colletotrichum siamense* (Colle). En contraste, *Bacillus velezensis* (Bv) no mostró un efecto antagónico con los fitopatógenos evaluados. Estos resultados indican que las cepas de *Bacillus subtilis* Bs1 y Bs2 presentan potencial como ACB contra los fitopatógenos que afectan la producción de lima persa.

KEY WORDS: Fitopatógeno, confrontación, inhibición, agente de control biológico.

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RESUMEN

Persian lime (*Citrus x latifolia* Tan.) is an economically important crop in Veracruz, with production affected by fungal diseases such as dieback and anthracnose. To reduce the use of chemical fungicides, bacteria of the *Bacillus* genus have been reported as Biological Control Agents (BCA). In this study, the *in vitro* antagonistic effect of native *Bacillus velezensis* (Bv) and two *Bacillus subtilis* strains (Bs1 and Bs2) was evaluated against pathogens identified as *Lasiodiplodia theobromae* and *Colletotrichum siamense*. The Percentage Inhibition of Radial Growth (PIRG) was measured on potato dextrose agar plates, and the level of antagonism was determined.

The *Bacillus subtilis* strains showed high and moderate inhibition against the evaluated phytopathogens. Bs2 exhibited the highest inhibition percentages (66.69 % and 61.78 %) against *Lasiodiplodia theobromae* (Las1 and Las2). Bs1 showed moderate antagonistic activity (49.7 %) against *Colletotrichum siamense* (Colle). In contrast, *Bacillus velezensis* (Bv) did not show an antagonistic effect against the evaluated phytopathogens. These results indicate that *Bacillus subtilis* Bs1 and Bs2 strains have potential as BCAs against the phytopathogens affecting Persian lime production.

PALABRAS CLAVE : Phytopathogen, confrontation, inhibition, biological control agent.

Introduction

Persian lime (*Citrus x latifolia* Tan.) is produced in 25 states of Mexico, with Veracruz being the leading producer, accounting for 52,990 ha of cultivated area and a production of 858,602 t, representing 53.9 % of the national output (SIAP, 2023). However, production is affected by many factors, among which fungal diseases stand out, including greasy spot (*Mycosphaerella citri*), anthracnose (*Colletotrichum* spp.), gummosis (*Phytophthora* spp.), and dieback (*Lasiodiplodia* spp.) (Sáenz-Pérez *et al.,* 2019; Leyva-Mirs *et al.,* 2021).

In Veracruz, *L. pseudotheobromae*, *L. theobromae*, *L. subglobosa*, and *L. iraniensis* have been reported as causal agents of dieback and cankers in commercial Persian lime orchards in the Martínez de la Torre, Tlapacoyan, and Papantla regions (Bautista-Cruz *et al.,* 2019). On the other hand, *Colletotrichum* spp. has been reported as responsible for brown leaf spots, leaf perforations, twig blight, as well as necrotic lesions and premature fruit drop, causing significant pre- and post-harvest losses in citrus-growing regions (Ruiz *et al.,* 2014).

Synthetic pesticides have been primarily used for disease control; however, the residues of these products spread, contaminating the surrounding environment, affecting non-target organisms, and causing severe environmental issues, including soil contamination and biodiversity loss (Gálvez-Gamboa *et al.,* 2018). An alternative to limit the application of these products and reduce the presence of pathogens in crops in an environmentally friendly method is using biological control agents (BCA) (Vinchira-Villarraga & Moreno-Sarmiento, 2019). These agents naturally occur in minimally altered or unmodified environments, where climatic and soil conditions are crucial for the survival of microbial communities (Ordóñez-Beltrán *et al.,* 2020). This natural selection process can benefit native strains characterized by their phytopathogenic activity. Among bacteria, the *Bacillus* genus species have been widely studied due to their abundance, diversity, ubiquity in various agroecosystems, and ability to sporulate, which facilitates their use as control agents. Additionally, they stand out for their metabolic traits, including the production of lipopeptides, lytic enzymes, δ-endotoxins, and siderophores, and their ability to induce systemic responses in plants (Chen *et al.,* 2020; Valenzuela Ruiz *et al.,* 2020).

Given this background, this study aimed to evaluate the *in vitro* antagonistic capacity of native *Bacillus* strains against *Lasiodiplodia* sp. and *Colletotrichum* sp., which have been reported as etiological agents of dieback and anthracnosis in Persian lime, respectively.

Material and Methods

Study strains

The experiment was conducted from May to September 2022 in the microbiology laboratory of TecNM, Tierra Blanca campus, Veracruz. Three bacterial strains of the *Bacillus* genus were used, previously isolated from a Persian lime crop under sustainable agricultural management in the "El Jícaro" region, in the Tierra Blanca municipality, Veracruz. These strains were molecularly identified at the Integral Phytosanitary Diagnostic Laboratory (LADIFIT-CP) with 100 % coverage and identity using the 8F-1492R primers, confirming *Bacillus subtilis* (Bs1), isolated from the rhizosphere, and *Bacillus subtilis* (Bs2) and *Bacillus velezensis* (Bv), isolated from the leaves. The phytopathogens used in the bioassay were isolated from diseased stem and branch samples of Persian lime from three regions in the northwest of Tierra Blanca, Veracruz. The isolated strains, Las1, Las2, and Las3, were molecularly identified as *Lasiodiplodia theobromae* with an identity percentage greater than 99 %, analyzed at the IPICYT Laboratory, S.L.P. Additionally, *Colletotrichum siamense* (Colle) was molecularly identified with 100 % coverage and identity using the ITS1-ITS4, ITS5-ITS4, GDF1-GDR1, T1BT2B, and ACT-512F/ACT-783R primers at LADIFIT-CP. The strains belong to the collection of TecNM, Tierra Blanca campus.

Antagonism assays

To evaluate the confrontation between *Bacillus* spp. and the phytopathogens, 5 mm diameter discs of the bacterial strains previously grown on PDA medium were placed at the cardinal points. Subsequently, a 5 mm diameter disc with 7-day-old mycelium of the pathogen

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was placed in the center of the plate. All plates were incubated at 29 ± 2 °C in the absence of light, and the radial growth of the pathogen was measured every 24 hours using a digital caliper (Truper® CALDI-6MP) until the control plates were fully covered (Espinoza-Ahumada *et al.,* 2019).

The measured variable was the radial growth of the phytopathogenic fungus in the presence of the bacterial antagonist. Negative control plates contained only the evaluated fungi. Using the obtained measurements, the Percentage Inhibition of Radial Growth (PIRG) was determined according to the formula proposed by Ezziyyani *et al.* (2004):

$$PIRG = \frac{R1 - R2}{R1} * 100$$

Where R1 represents the larger radius (pathogen control radius) and R2 represents the smaller radius (pathogen radius in confrontation).

The evaluation followed the method described by Krakaliya *et al.* (2017), as cited by Khuong *et al.* (2023): PIRG ≥ 60 % is highly antagonistic, 40 % ≤ PIRG ≤ 59 % is moderately antagonistic, 20 % ≤ PIRG ≤ 39 % is weakly antagonistic, PIRG ≤ 19 % is non-antagonistic.

For the bioassay, a completely randomized design was used with twelve treatments: three *Bacillus* spp. isolates were tested against three *Lasiodiplodia* sp. strains, corresponding to each sampling region (Las1, Las2, and Las3), and one *Colletotrichum* sp. strain. Each treatment included five replicates, and a control was included for each pathogenic microorganism. Normality tests were performed using the Shapiro-Wilk test, and variance homogeneity was assessed using Bartlett's test. PIRG data were subjected to analysis of variance (ANOVA) and multiple mean comparison tests (Tukey, $p \leq 0.05$) using the SAS V9.3 statistical package.

Results and Discussion

Macroscopic characteristics of the phytopathogens

From the isolates obtained from trunk and branch samples of diseased Persian lime trees in three zones of the northwestern region of the Tierra Blanca municipality, Veracruz, *Lasiodiplodia theobromae* (Las1, Las2, and Las3) was identified. These strains, corresponding to each sampling area, are associated with dieback disease. They were characterized by the development of abundant aerial mycelium with a cotton-like texture, rapid growth, and the presence of septate hyphae. The colonies initially exhibited a whitish coloration, turning gray-green after seven days (Figure 1) and becoming completely dark by day 14. The other phytopathogen was identified as *Colletotrichum siamense*, associated with anthracnose disease. It exhibited a concentric ring growth pattern, with the development of subaerial and flat white mycelium (Figure 1).

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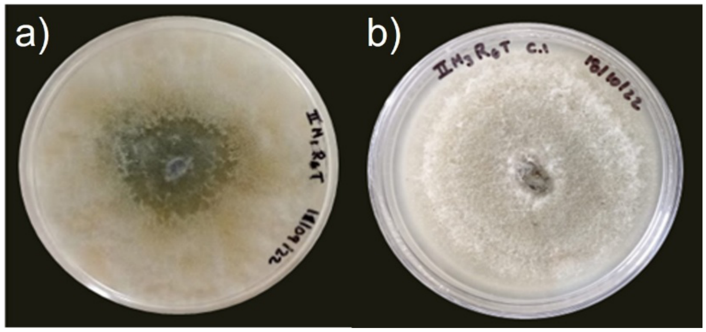

Figure 1. Phytopathogens isolated and identified in Persian lime cultures on PDA agar after 7 days of growth, a) *Lasiodiplodia theobromae* and b) *Colletotricum siamense*.

In vitro antagonistic activity of *Bacillus* spp. *vs* phytopathogens

The PIRG values obtained from the *in vitro* evaluation (Figure 2) revealed different interactions among the tested microorganisms. Based on the inhibition percentage, *Bacillus subtilis* Bs2 exhibited a highly antagonistic effect against *Lasiodiplodia theobromae* (Las1 and Las3), with inhibition values of 66.7 % and 61.8 %, respectively. Meanwhile, Bs1 demonstrated a moderate inhibition against both phytopathogens, with values ranging from 46.7 % to 49.8 % On the other hand, no antagonistic interaction was observed between Bs2 and *Colletotrichum siamense* (Colle), nor between *Bacillus velezensis* (Bv) and the two phytopathogens (Figure 2). These results contrast with those reported by Chukeatirote *et al.* (2018), where *Bacillus velezensis* exhibited a 60 % inhibition of radial growth against *Lasiodiplodia theobromae*.

The impact of *Bacillus velezensis* in biological control has been widely recognized due to the identification of gene clusters related to the synthesis of secondary metabolites with antifungal activity, such as fengycins, iturins, and surfactins, which play a key role in controlling fungal pathogens (Zhang *et al.,* 2022; Wei *et al.,* 2023). Additionally, when evaluated in plants, *B. velezensis* has been shown to reduce diseases by forming biofilms in the rhizosphere, which promote plant growth and protect against infectious microorganisms. This occurs through both the secretion of antimicrobial compounds and systematic resistance induction (Rabbee *et al.,* 2019; Moreno-Velandia *et al.,* 2021). It is important to highlight that although this study did not observe an antagonistic effect of this strain (Figure 3), different and promising results can be expected in plant development and protection under *in situ* conditions.

On the other hand, the highest inhibition values presented by Bs2 (*Bacillus subtilis*-2) against the mycelial growth of *Lasiodiplodia theobromae* strains Z1 and ZII (Las1 and Las3) (Figure 2) are similar to those reported by Sajitha *et al.* (2014), who evaluated two strains identified as *Bacillus subtilis* against *Lasiodiplodia theobromae*, obtaining inhibition values of 64.44 % and 60.74 %, respectively. The low percentage obtained from strain Bs2 against *Colletotrichum*

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siamense (16 %) contrasts with the findings of Cambero-Ayón *et al.* (2020), who achieved a mycelial inhibition percentage above 32 % in their evaluation of *Bacillus subtilis* against *C. gloeosporioides* (Colle).

Additionally, it can be highlighted that both strain Bs2 and Bs1 showed antagonistic activity against *Lasiodiplodia theobromae* (Figure 3). This aligns with the findings of Castillo-Reyes *et al.* (2015), which indicated that strains of the same species may exhibit different capabilities to inhibit the growth of numerous microorganisms. Furthermore, the origin of these strains could be a factor influencing their biocontrol characteristics, as Bs1 was isolated from leaves, while Bs2 was isolated from the rhizosphere of Persian lime plants. It has been demonstrated in several studies that native *Bacillus* strains isolated from the rhizosphere of several crops can generate an antagonistic effect on the development of phytopathogens (Castillo-Reyes *et al*., 2015; Castañeda-Alvarez & Sánchez, 2016; Yan *et al*., 2021; Balthazar *et al*., 2022). Moreover, native microorganisms as biological control agents have the advantage of maintaining ecological balance and effectively controlling pests and diseases. Being naturally adapted to the environmental and edaphic conditions of their surroundings, they have a competitive advantage over introduced species. Additionally, their high reproductive efficiency in their habitat allows them to ensure sustained and prolonged biological control (Berg & Smalla, 2009; Chavarria-Quicaño *et al.,* 2023; Martínez-Martínez *et al.,* 2023).

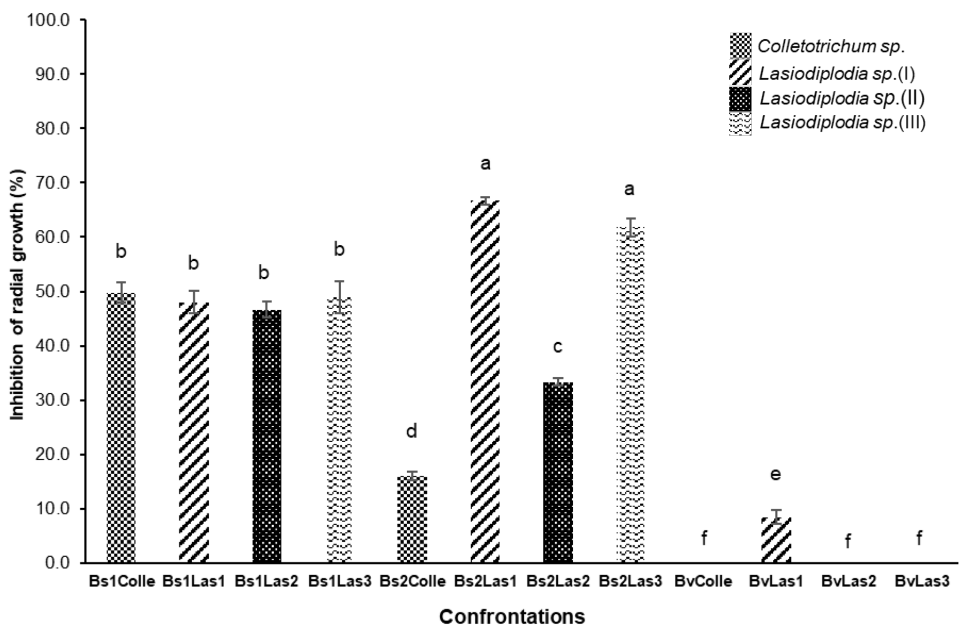

Figure 2. *In vitro* confrontation of *Bacillus* spp. against Persian lime pathogens.

(Bs1: *B. subtilis* 212s, Bs2: *B. subtilis* 314H, Bv: *B. velezensis* 614H, Colle: *Colletotrichum* sp., Las1: *Lasiodiplodia* sp. ZI, Las2: *Lasiodiplodia* sp. ZII, Las3: *Lasiodiplodia* sp. ZIII). Bars with the same letter are not statistically different, according to the Tukey test (p = 0.05).

During the confrontation assays, two distinct inhibition patterns were observed (Figure 4). The first pattern showed a clear barrier zone between the growth of the fungal pathogen and the *Bacillus* strain, delineating an inhibition zone possibly due to the production of lipopeptides such as iturins, fengycins, and surfactins, which have been identified during confrontations of *Bacillus* spp. against filamentous fungi (Sajitha & Dev, 2016; Moreno-Velandia *et al.,* 2021) (Figure 4A). This inhibition may also be attributed to competitive mechanisms for nutrients and space, as reported by Chen *et al.* (2020). The second pattern involved the formation of an interaction zone that limited the pathogen's development and induced a color change in the *Bacillus* strain. According to Chen *et al.* (2020), the production of microbial substances is the most important biocontrol mechanism for this genus (Figure 4B).

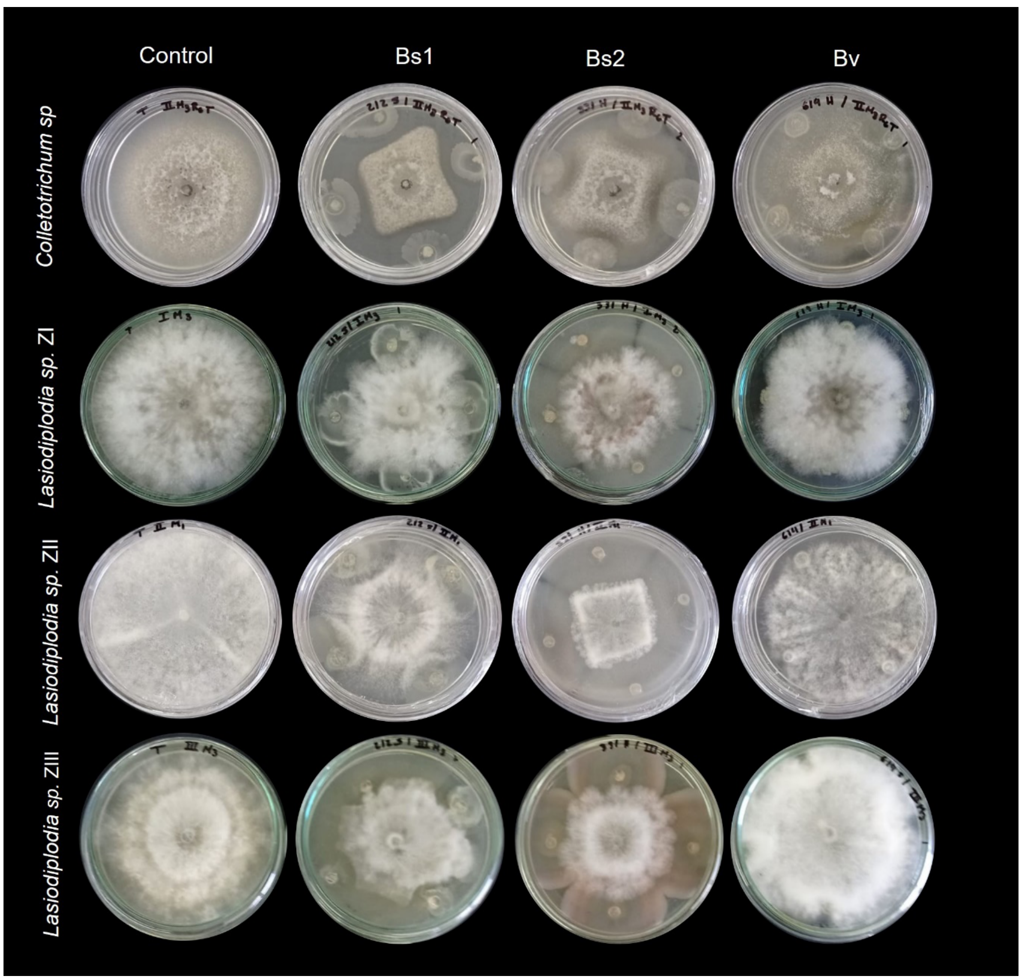

Figure 3. Inhibitory effect of three strains of *Bacillus* spp. on the growth of four Persian lime pathogen isolates at 9 days of incubation.

(Bs1: *B. subtilis* 212s, Bs2: *B. subtilis* 314H, Bv: *B. velezensis* 614H).

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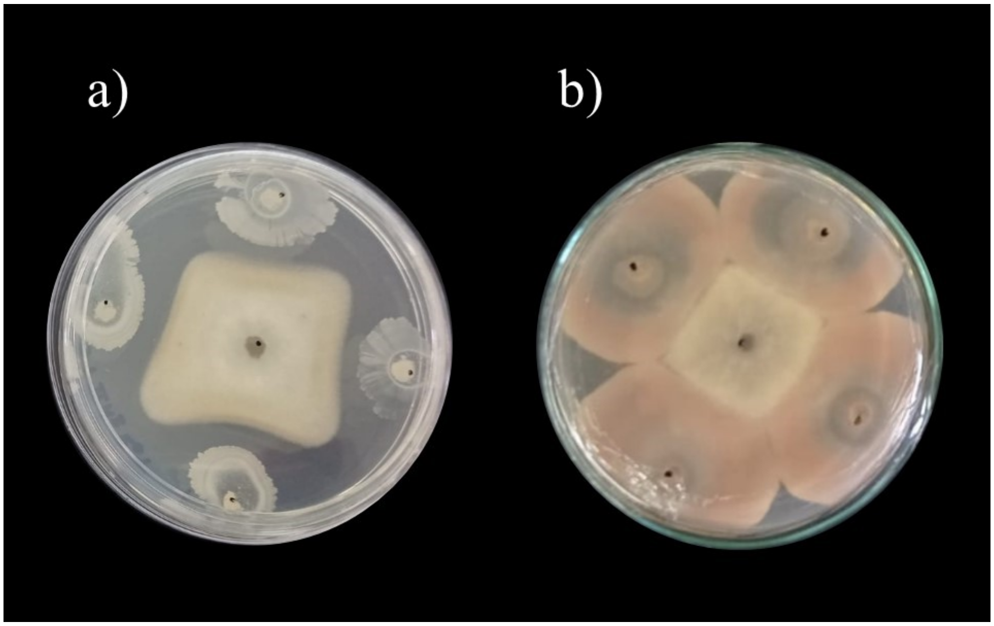

Figure 4. Inhibition patterns in confrontations.

a) Competition for space and nutrients), b) Production of microbial metabolites (formation of inhibition halo).

Conclusions

Native *Bacillus* species (Bs1, Bs2, and Bv) showed diverse antagonistic activity against two pathogens affecting Persian lime cultivation: *Lasiodiplodia theobromae* and *Colletotrichum siamense*. The level of antagonism exhibited by the evaluated *Bacillus* species was high and moderate for *Lasiodiplodia theobromae*, and mild or absent for *Colletotrichum siamense*. The response of *Bacillus subtilis* contrasts with *Bacillus velezensis*, which showed no antagonistic effect against the evaluated pathogens. In this regard, intraspecific differences among species, influenced by external factors such as the environment, may define their potential use as biocontrol agents.

Author contributions

Work conceptualization: author 1, author 2, author 4. Methodology development: author 1, author 2, author 6. Software management: author 1, author 2. Experimental validation: author

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1, author 2, author 3, author 4. Data analysis: author 1, author 2, author 3, author 5. Data management: author 1, author 2. Writing and manuscript preparation: author 1, author 2. Drafting, reviewing, and editing: author 2, author 3, author 4. Project administration: author 1, author 2. Funding acquisition: author 2.

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Conflict of Interest

The authors declare no conflict of interest.

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