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*Research Article*

# Genomic study and characterization of *Bacillus safensis* and its potential use in white shrimp (*Penaeus vannamei*) farming

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**ABSTRACT.** *Bacillus safensis* has emerged as a promising probiotic candidate in aquaculture due to its metabolic versatility and antagonistic activity against pathogenic bacteria. In this study, two morphotypes of *B. safensis* (BS1-A and BS1-B) were characterized using biochemical and genomic analyses, and their effects on the productive performance of white shrimp (*Penaeus vannamei*) were evaluated. Both morphotypes exhibited Gram-positive, rod-shaped cells; punctiform colonies with entire margins and flat elevation; carbohydrate fermentation; indole production; lack of motility; and the ability to produce protease and cellulase enzymes. Whole-genome sequencing of BS1-A revealed a genome size of 3,686,099 bp. Average nucleotide identity (ANI) analysis showed 99.999% identity between BS1-A and BS1-B and 97.46% identity with the reference strain PgKB20. Shrimp bioassays were conducted over six weeks using three treatments in a juvenile white shrimp culture system: control (1% molasses), BAC (1% molasses plus *B. safensis* at a final concentration of $2.50\times10^{7}$ CFU $\text{mL}^{-1}$), and COM (1% molasses plus a commercial probiotic at the same concentration). At the end of the experimental period, significant differences ($P < 0.05$) were observed among treatments in growth performance, weight gain, feed consumption, and survival. Under the experimental conditions evaluated, the results suggest that *B. safensis* BS1-A and BS1-B contribute to improved digestion and nutrient absorption from balanced feed, thereby enhancing shrimp growth performance.

**Keywords:** *Bacillus safensis*; probiotic; shrimp; genome; aquaculture

## INTRODUCTION

In recent years, aquaculture has become the fastest-growing sector worldwide (Wenning 2020). However, bacterial infections have significantly hindered global development over the past decades, causing substantial economic losses (Kaktcham et al. 2018, Reda et al. 2018, Magray et al. 2020, Kang et al. 2022). Moreover, the excessive use of antibiotics to control pathogenic bacteria has increased the prevalence of antibiotic-resistant bacteria and led to environmental contamination on farms (Limbu et al. 2021, Kang et al. 2022). Consequently, several studies have focused on developing new biological control strategies, with probiotics emerging as a viable alternative to mitigate the impacts of these chemotherapeutic agents (Hoseinifar et al. 2018).

According to the World Health Organization (WHO), probiotics are live microorganisms that confer health benefits to the host when administered appropriately (Kang et al. 2022). Therefore, in recent years, some species, such as *Bacillus* sp., *Clostridium butyricum*, *Enterococcus* sp., and *Rhodopseudomonas* sp., have been widely used (Wang et al. 2019). Their

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application in aquaculture has demonstrated improvements in growth, survival, and immune response of organisms, as well as in disease control and water quality (Ringo et al. 2018, Zabidi et al. 2021, Kang et al. 2022, Rohani et al. 2022, Yilmaz et al. 2022). Several studies have shown that probiotics isolated from shrimp or fish farming systems are more effective in aquaculture than those isolated from unrelated sources, as they are adapted to the same environment (Zhang et al. 2022). Therefore, the selection and application of autochthonous probiotics from aquaculture systems have become increasingly important.

Characterizing probiotic strains isolated from culture systems or organisms is a primary requirement for selecting candidate strains. Among the essential traits, it is crucial to verify their ability to persist, adhere to, and colonize the intestine and evaluate their antioxidant and antibacterial properties (Xie et al. 2023, Yang et al. 2023). Another critical aspect is genome sequencing and functional annotation, which provide effective tools for exploring the molecular mechanisms of probiotics, thereby aiding in the selection of beneficial strains (de Melo-Pereira et al. 2018). In this regard, bacterial genome analysis focuses on identifying molecular markers that confer probiotic properties, such as adhesion, antimicrobial production, and biological safety (Kapse et al. 2019), including the absence of virulence genes or toxin production.

The genus *Bacillus* is one of the most commonly used effective probiotic supplements in aquaculture (Abdel-Tawwab et al. 2022, Monier et al. 2023). It produces various extracellular enzymes, tolerates UV light, and withstands both low and high temperatures, allowing it to remain dormant for extended periods (Nicholson 2004). Various studies have demonstrated that they can significantly improve the physiological, morphological, hematological, and immunological conditions of aquatic organisms when administered at optimal levels in the culture water.

Most studies have investigated the probiotic traits of *Bacillus* species using *in vitro* and *in vivo* models (Kavitha et al. 2018). In the case of *Bacillus safensis*, one of the most widespread species found in a wide range of marine and terrestrial habitats (Singh et al. 2013, Lateef et al. 2015, Wu et al. 2021), some studies have explored its antibacterial activity (Abdelli et al. 2019, Vinh et al. 2022) and its enzymatic profile (Kothari et al. 2013).

These characteristics indicate that this bacterium has great potential for biotechnological applications across various industries (Rekik et al. 2019, Thite et al. 2020). However, information on its genome and some of its biochemical properties is scarce. Therefore, the present study aimed to characterize the genome and biochemical properties of two *B. safensis* morphotypes to evaluate their potential as probiotics in white shrimp (*Penaeus vannamei*) farming.

## MATERIALS AND METHODS

### Isolation and identification

The *B. safensis* used in this study was isolated from a marine sponge (Galicia-Nicolás et al. 2018) and initially exhibited two distinct colonial morphotypes, one large (BS1-A) and one small (BS1-B). Both morphotypes were evaluated under identical experimental conditions, and all procedures were applied equally. To verify that the morphotypes represented natural phenotypic variants rather than mutants or contaminants, whole-genome sequencing was performed at Plasmidsaurus Lab (San Francisco, USA), complemented by biochemical metabolic profiling. Bacteria were cultured on tryptic soy agar supplemented with 2.5% NaCl (trypto-casein soy agar, TSA+) at 35°C for 24 h. Cells were harvested by centrifugation (3,600 g, 4°C, 10 min), adjusted to an optical density of $OD_{600} = 1$ in 2.5% NaCl saline solution, and quantified as colony-forming units (CFU $mL^{-1}$) by plating serial dilutions on TSA+.

### Characterization of *Bacillus safensis*

The cellular morphological characterization of BS1-A and BS1-B was conducted by microscopic observation and Gram staining. According to the methodology described by Mac Faddin (1980) and Díaz et al. (1995), with modifications, the following biochemical characterization tests were performed: motility, indole, and ornithine production in motility, indole, and ornithine (MIO) medium (BD Bixon). Simmons' citrate, catalase, oxidase, and methyl red using the strains *Escherichia coli*, *Klebsiella aerogenes*, and *Staphylococcus aureus* as positive and negative controls, as appropriate. The fermentation of different carbohydrates, including glucose, lactose, mannitol, sorbitol, arabinose, trehalose, xylose, galactose, and maltose, was evaluated. As part of the physiological characterization, growth at temperatures ranging from 4 to 45°C and the effects of different NaCl concentrations (0-13%) were determined. Specific culture media were used to assess protease, amylase, and cellulase production. The culture media used were as follows: 1.5% gelatin agar, revealing agent: acidified mercuric chloride (Mac Faddin 1980); 1% starch agar, revealing agent: Lugol's iodine; 1% carboxymethyl cellulose (CMC) agar, revealing agent: 0.05% Congo red (Viteri et al. 2015).

### Inoculum preparation and typical growth curve

A colony of BS1-A and BS1-B was taken from a TSA+ plate grown for 24 h and inoculated into a flask containing 20 mL of TSB+2.5% NaCl (TSB+). The culture was incubated at 30°C and 100 rpm for 18 h. This culture was adjusted to an optical density of 1.0 (600 nm) and used to inoculate 200 μL of fresh TSB+ in a microplate well (ratio of 1:10). The microplate was incubated at 30°C in a microplate reader and programmed to measure optical density every hour at a wavelength of 600 nm for 48 h (MultiskanGo, Thermo Scientific). Three independent replicates were performed for each bacterial growth curve.

The growth curves for BS1-A and BS1-B were established by plotting bacterial cell numbers over time, and the specific growth rate was determined using an empirical growth model (Eq. 1). To the growth curve, bacterial concentration was estimated as cells per milliliter from colony-forming units by plating serial dilutions on TSA+.

$$\mu = \mathrm{Ln}\,(N/No) / \Delta T \qquad \text{(Eq. 1)}$$

where: N: final cell number; No: initial cell number; ΔT: time period from No to N.

### Dot-spot technique for antagonistic activity assay

Pathogenic bacteria of the genus *Vibrio* (*V. parahaemolyticus*, *V. diabolicus*, and *V. harveyi*) were obtained from the collection of the Phage and Microalgae Biotechnology Laboratory at CIBNOR. They were identified by genome sequencing (unpubl. data). All isolates were cultured separately on TSA+ supplemented with 2.5% NaCl for 24 h at 30°C before the experiment. Suspensions of the pathogenic bacteria were adjusted to an approximate concentration of $10^7$ CFU $mL^{-1}$ based on an absorbance of 1 at 600 nm ($OD_{600}$) and evenly spread onto TSA+ plates. The concentrated suspension of each isolate containing pathogenic bacteria was inoculated onto the plates. Morphotypes BS1-A and BS1-B were evaluated in triplicate by adding 5 μL of each one onto sterile filter paper disks at a concentration of $10^8$ CFU $mL^{-1}$, based on an absorbance of 1 at 600 nm ($OD_{600}$). As a control, three filter paper disks containing 30 μg of chloramphenicol were placed on the opposite side of the plates (Chen et al. 2024). Plates were incubated at 30°C for 24 h. After incubation, disks exhibiting inhibition halos were considered to show antagonistic activity, and the diameters of the inhibition zones were recorded (Kewcharoen & Srisapoome 2019). To confirm antagonistic activity, the procedure was repeated twice for isolates that exhibited inhibition halos against at least one *Vibrio* species.

### Genomic DNA of BS1-A and BS1-B

Genomic DNA from morphotypes BS1-A and BS1-B was extracted using the QIAamp DNA Microbiome Kit according to the manufacturer's instructions. DNA quality was evaluated by electrophoresis on 1% (w/v) agarose gels, and its concentration was measured using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

### Bioinformatic analysis

Whole Plasmid Sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis. The assembled genome was initially annotated and inspected with Prokka 1.14.6 (Seemann 2014), then annotated with the NCBI Prokaryotic Genome Annotation Pipeline. The average nucleotide identity was calculated using FastANI v1.1.0 (Jain et al. 2018). Data were analyzed with Alien Hunter 1.1.0 (Vernikos & Parkhill 2006) to identify potential horizontal gene transfer events, and with Phastest 3.0 v1.0.1 (Wishart et al. 2023) and VirSorter2 (Guo et al. 2021) to identify phage sequences. ResFinder v4.7.2 (Clausen et al. 2018, Bortolaia et al. 2020), AMRFinderPlus v4.0.23 (Feldgarden et al. 2021), and the Resistance Gene Identifier (RGI) v6.0.0 were used to search for resistance genes using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2023). Vfanalyzer was used to search for virulence factors in the VFDB (Liu et al. 2018). Finally, antiSMASH 7.0 (Blin et al. 2023), Prism4 (Skinnider et al. 2020), and BAGEL4 (van Heel et al. 2018) were used to find secondary metabolic clusters. The circular genome representation was obtained using the PROKSEE server (Grant et al. 2023). The pangenomes of morphotypes BS1-A and BS1-B were analyzed and compared with those of four closely related strains from the NCBI database. We downloaded 36 complete *B. safensis* genome records from the NCBI databases, used the FASTA files as input for Prokka, and conducted a pangenome analysis using Roary v3.13.0 (Page et al. 2015). The resulting core gene alignment was used as input for trimAl v1.5.rev1 (Capella-Gutiérrez et al. 2009) and finally RaxMLv8.2.12 (Stamatakis 2014) were used to obtain the maximum likelihood phylogenetic tree using 100 bootstrap replicates. The gene presence/absence matrix obtained from Roary and the tree generated in RaxML were used as input to Phandango (Hadfield et al. 2017) to generate a pangenome heatmap.

### Probiotic and feed preparation for bioassay

Preserved working stocks in pure glycerol at -18°C were inoculated into flasks containing 20 mL of TSB+ and incubated with agitation at 110 rpm at 30°C for 24 h. Each morphotype was centrifuged in 50-mL tubes at 3,600 *g* for 15 min, and the supernatant was decanted. The obtained pellets were resuspended in 20 mL of 2.5% saline solution, vortexed, and the optical density (λ 600 nm) was measured to determine the volume required to achieve a final concentration of $2.50\times10^7$ CFU $mL^{-1}$ in the experimental culture tanks.

A shrimp-balanced feed was formulated (Table 1) and manufactured in the aquaculture nutrition laboratory at CIBNOR. All dry ingredients were mixed until homogenized in a 1.5 L mixer (Kitchen-Aid™, USA), then oil-based ingredients and warm water (350 mL $kg^{-1}$ at 60°C) were added, and the mixture was homogenized until a wet dough formed. The mixture was passed through a meat grinder (0.5 hp Torrey®, MX) through a 2-mm die-cutting. Finally, the pellets were dried for 10% moisture at 45°C for 10 h in a forced-air oven and stored at 4°C. The feed was analyzed for dry matter (Method 930.15; AOAC 2005), crude protein (Ebeling 1968), total lipids (Method 2003.05; AOAC 2005), crude fiber (Method 978.10; AOAC 2005), and ash (Method 942.05; AOAC 2005). Nitrogen-free extract (NFE) was estimated by difference.

### Shrimp bioassay with *Bacillus safensis*

Pacific white shrimp *P. vannamei* post-larvae (~80 mg) were kindly donated by the commercial laboratory Biomarina SA de CV located in Mazatlán, Sinaloa, Mexico. The shrimp were acclimated in 800-L tanks to the following conditions: dissolved oxygen ≥ 4.5 g $L^{-1}$, 28.2 ± 0.4°C, pH 7.8 ± 0.3, $NH_3 < 0.5$ ppm, 37 of salinity, and photoperiod of 12:12 h: light:dark (fluorescent light). Shrimp were fed a commercial diet (Purina 35% protein) for three weeks before the experiment. After the acclimation period under laboratory conditions, a six-week experiment was conducted to evaluate three treatments: control (no probiotic), BAC (a mix of equal parts of BS1-A and BS1-B at a final concentration in the experimental tanks of $2.5\times10^4$ CFU $mL^{-1}$), and commercial probiotic (COM, Hatchery Prime which contains *Bacillus* sp. at a final concentration in the experimental tanks of $2.5\times10^4$ CFU $mL^{-1}$). All treatments received 1 mg $L^{-1}$ molasses in the water daily, which was increased to 1.5 and 2 mg $L^{-1}$ during the fourth and fifth weeks of the experiment, respectively, to provide additional carbon to control ammonia in the culture water due to increased shrimp biomass and feed ration. The respective probiotic and molasses were added daily throughout the experimental period. Each treatment consisted of four replicates, with 11 shrimp (wet average weight of 0.5 ± 0.3 g) randomly distributed in 60 L fiberglass tanks with a controlled photoperiod and a temperature of 28 ± 0.5ºC, and continuous aeration. Throughout the experimental period, tank water samples were taken weekly for water quality analysis in terms of total ammonia nitrogen (TAN), nitrites, nitrates, phosphate, and pH (API Marine, saltwater master test kit, FL, USA), and total sedimentable solids (TSS) particles were quantified per triplicate in mL $L^{-1}$ using 1-L Imhoff cones every four days. Shrimp were fed to satiety at an initial rate of 10% shrimp biomass, divided into two rations (09:00 and 14:30 h). Feeding rates were adjusted based on the remaining feed

**Table 1.** Ingredient composition (g $kg^{-1}$ diet) and proximate analysis (% dry basis) of feed. [a]PMA S.A. de C.V., Jalisco, MX. [b]PIASA S.A. de C.V., Baja California Sur, MX. [c]Molino San Cristóbal, Sonora, MX. [d]Cedrosa SA de CV, Estado de México, MX. [e]Sigma Aldrich, Missouri, USA. [f]Vitamin and mineral premix: detailed content in Peña-Rodríguez et al. (2020). [g]Prilabsa, Sinaloa, MX.

| Ingredients | g $kg^{-1}$ diet |
|---|---|
| Fish meal[a] | 180 |
| Soybean meal[b] | 341.3 |
| Wheatmeal[c] | 400 |
| Soy lecithin[d] | 35 |
| Fish oil[a] | 25 |
| Alginic acid[e] | 10 |
| Vitamin-mineral premix[f] | 5.3 |
| Antioxidant BHT[e] | 0.4 |
| Vitamin C[g] | 1 |
| Choline[e] | 1 |
| Sodium biphosphate[e] | 1 |
| Total | 1000 |
| **Proximate analysis** | **% dry basis** |
| Crude protein | 34.3 ± 0.5 |
| Lipids | 8.7 ± 0.1 |
| Crude fiber | 0.43 ± 0.1 |
| Ash | 6.7 ± 0.1 |
| NFE | 49.8 |

observed during a daily 10% water exchange performed by siphoning. Biometrics were performed weekly until the end of the experiment, and the following parameters were evaluated: final weight (g), percentage of weight gain (WG), specific growth rate (SGR% $d^{-1}$), feed conversion ratio (FCR), and survival.

### Statistical analysis

Shrimp performance and water were analyzed using tank averages (n = 4), tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test), and then subjected to one-way ANOVA, followed by Tukey's multiple-comparison tests if applicable (95% confidence level). For water quality parameters, a two-way ANOVA was conducted to evaluate the effects of time (week) and treatment, with Tukey's *post-hoc* tests applied when significant effects were detected. All statistical analyses were carried out using R software (version 4.4.1).

## RESULTS

### Biochemical and physiological characterization of *Bacillus safensis* morphotypes BS1-A and BS1-B

Both morphotypes exhibited Gram-positive rod-shaped morphology, punctiform colony shape, entire edge, flat elevation, and smooth bright color. They were positive for ornithine, capable of fermenting a range of carbon sources, negative for motility and indole, positive for catalase, and demonstrated growth starting at 15°C. The two morphotypes of *B. safensis* differed in biochemical activity despite being the same species. It was determined that BS1-A was citrate-positive and grew in 10% NaCl, whereas BS1-B was citrate-negative and grew only up to 6% NaCl (Table 2). Both *B. safensis* morphotypes tested positive for protease and cellulase production (Table 3); however, their quantitative activity still needs to be determined.

### Bacteria growth curve

The growth curve for both *B. safensis* morphotypes was carried out for 48 h; the constructed graph of natural logarithms of the cells per milliliter as a function of the culture time indicates that the lag phase of growth is practically non-existent and that the logarithmic phase of growth ends at 18 h of culture for both morphotypes, (Fig. 1). The phase of cell death was not determined for this experiment, so the reading values remained static for the rest of the culture time, indicating no diauxic growth, as observed in other bacteria. The statistical analysis of the specific growth rate shows no difference between the two determinations ($P > 0.05$), indicating high similarity in the medium-uptake efficiency of BS1-A and BS1-B.

### Screening of bacteria with antagonistic activity bacteria

Both morphotypes of *B. safensis* were tested for their antagonistic activity against three pathogenic *Vibrio* species. The results showed that both could inhibit the growth of at least one *Vibrio* bacterium, with one BS1-A exhibiting strong activity against all three tested *Vibrio* species (Table 4).

### Identification of *Bacillus safensis* BS1-A and BS1-B

After sequencing, a total of 157,576 reads were obtained, with an rN50 value of 3,651 bp and a coverage of 98.13X. Detailed assembly statistics are presented in Table 5. The *B. safensis* BS1-A genome was uploaded to the NCBI database (BioProjectID: PRJNA1363193) and consists of 3,686,099 bp and a GC content of 41.76% (Fig. 2). The Average Nucleotide Identity (ANI) between BS1-A and BS1-B and the *B. safensis* reference genome strain PgKB20 (NZ_CP043404.1) showed a 97.46 ANI value. The genome of the second morphotype was sequenced and showed an ANI value of 99.999 between *B. safensis* BS1-A and BS1-B.

Among the genomic features, phage genes encoding terminase, structural proteins, and holins were identified using two software tools, Phastest and VirSorter, providing evidence of a prophage in *B. safensis* BS1-A. The antiSMASH analysis identified several gene clusters associated with the synthesis of secondary metabolites, including lychenisin, plantazolicin, and bacilysin. However, when analyzing antibiotic resistance genes using CARD and the NCBI Gene database, both programs (RGI and ResFinder) identified the cat86-1 gene in *B. safensis* BS1-A, showing 90-100% identity across other *Bacillus* sp. strains reported in NCBI.

Genome annotation identified 3,781 genes, including phage-related genes encoding terminase, structural proteins, and holins. These features were detected using both PHASTest and VirSorter, providing evidence of a prophage in *B. safensis* BS1-A. Additionally, antiSMASH analysis revealed multiple gene clusters associated with the biosynthesis of secondary metabolites, including lichenysin, plantazolicin, and bacilysin. Analysis of antibiotic resistance genes using the CARD and NCBI Gene databases showed that both RGI and ResFinder identified the cat86-1 gene in *B. safensis* BS1-A, which exhibits 90-

**Table 2.** Biochemical and physiological characterization of *B. safensis* BS1-A and BS1-B. NA: not applicable; (-) negative; (+) positive.

| | Morphotype | | Controls | | |
|---|---|---|---|---|---|
| | BS1-A | BS1-B | *E. coli* | *K. aerogenes* | *S. aureus* |
| Motility | - | - | + | + | - |
| Indole | - | - | + | - | - |
| Ornithine | + | + | + | + | - |
| Simmons citrate | + | - | - | + | - |
| Catalase | + | + | NA | NA | + |
| Oxidase | - | - | - | NA | NA |
| Methyl red | - | - | + | - | NA |
| Carbohydrate fermentation: | | | | | |
| Glucose | + | + | NA | NA | NA |
| Lactose | + | + | NA | NA | NA |
| Mannitol | + | + | NA | NA | NA |
| Sorbitol | + | + | NA | NA | NA |
| Arabinose | + | + | NA | NA | NA |
| Trehalose | + | + | NA | NA | NA |
| Xilose | + | + | NA | NA | NA |
| Galactose | + | + | NA | NA | NA |
| Maltose | + | + | NA | NA | NA |
| Temperature: | | | | | |
| 4°C | - | - | NA | NA | NA |
| 15°C | + | + | NA | NA | NA |
| 25°C | + | + | NA | NA | NA |
| 30°C | + | + | NA | NA | NA |
| 35°C | + | + | NA | NA | NA |
| 45°C | + | + | NA | NA | NA |

**Table 3.** Extracellular enzyme production (protease, amylase, and cellulase) by strains. (-) negative; (+) positive.

| Hydrolysis test | Morphotype | |
|---|---|---|
| | BS1-A | BS1-B |
| Protease | + | + |
| Amylase | - | - |
| Cellulase | + | + |

100% sequence identity to homologs reported in other *Bacillus* species deposited in NCBI.

We compared *B. safensis* BS1-A with 36 *B. safensis* strains retrieved from the NCBI database and used them to construct a pangenome model. The core genes were used to construct a maximum-likelihood phylogenetic tree, which showed that *B. safensis* BS1-A clustered separately from the other strains but was closely related to *B. safensis* strain FUA2117 (Fig. 3) and revealed a group of unique genes annotated as hypothetical proteins. Within the accessory genes *B. safensis* BS1-A shared with *B. safensis* FUA2117, there is an interesting cluster of genes involved in cell wall reshaping, such as lytB and lytC_3 (Baidya et al. 2020)

## Shrimp performance

Significant differences among the treatments ($P < 0.05$) were observed in growth parameters and FCR at the end of the experimental period (Table 6). Specifically, the BAC treatment exhibited higher final weight ($F_{2,9} = 6.22$, $P = 0.02$, $\eta^2 = 0.61$), WG ($F_{2,9} = 6.26$, $P = 0.02$, $\eta^2 = 0.61$), and SGR ($F_{2,9} = 6.80$, $P = 0.01$, $\eta^2 = 0.63$) than the control treatment, but no significant difference compared to the COM treatment. The FCR was significantly lower in BAC treatment with respect to control ($F_{2,9} = 5.44$, $P = 0.03$, $\eta^2 = 0.58$). All treatments showed similar survival rates (93-96%). In terms of water quality parameters (Fig. 4), TAN, nitrite, nitrate, phosphate, and TSS increased significantly over time ($P < 0.001$). Regarding the treatment effect, only TAN ($F_{2,36} = 8.85$, $P < 0.001$, $\eta^2 = 0.33$) and TSS ($F_{2,36} = 26.2$, $P < 0.001$, $\eta^2 = 0.59$) exhibited significant differences among treatments. The interaction between time and treatment also had a significant effect on TAN

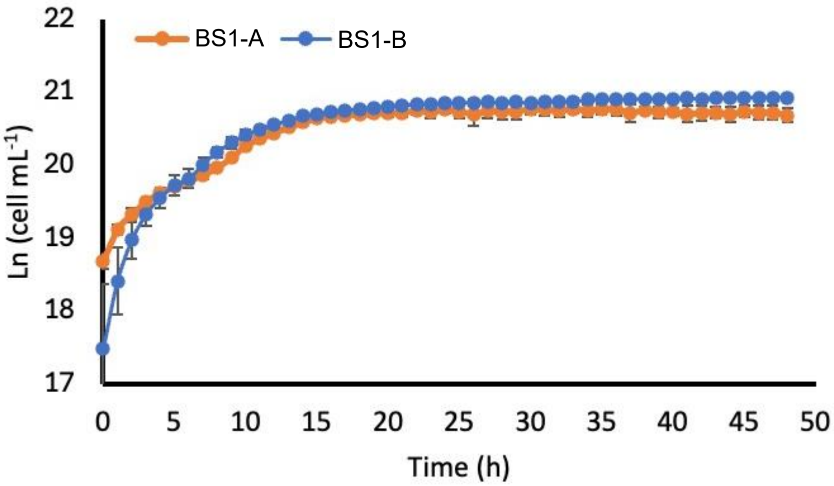

**Figure 1.** Typical growth curve for *Bacillus safensis* strains on tryptic soy broth+2.5NaCl. Bars indicate standard deviation for three independent experiments (n = 3).

**Table 4.** Antagonistic activity of *Bacillus safensis* morphotypes BS1-A and BS1-B to three *Vibrio* pathogenic bacteria. sd: standard deviation.

| *Bacillus safensis* | Inhibition zone diameter (mean ± SD) (mm) | | |
|---|---|---|---|
| | *V. parahaemolyticus* | *V. diabolicus* | *V. harveyi* |
| 1. BS1-A | 22.3 ± 0.8 | 22.7 ± 0.6 | 24.23 ± 0.6 |
| 2. BS1-B | | 16.5 ± 0.5 | 4.3 ± 0.6 |
| Control chloramphenicol 30 µg | 20.3 ± 0.7 | 19.3 ± 0.6 | 20.7 ± 1.1 |

($F_{2,36} = 2.29$, $P = 0.033$, $\eta^2 = 0.39$), with concentrations in week six being significantly higher in the control treatment (1.2 ± 0.2 mg $L^{-1}$) compared with BAC (0.8 ± 0.1 mg $L^{-1}$) and COM (0.75 ± 0.2 mg $L^{-1}$). Similarly, TSS showed a significant effect ($F_{2,36} = 3.92$, $P < 0.001$, $\eta^2 = 0.52$), with values more than 35% higher in the control treatment during week 6 compared with BAC and COM (Fig. 4).

## DISCUSSION

Biochemical tests provide a rapid, straightforward method for preliminary identification of bacteria based on the presence or absence of specific enzymes. They also allow determination of their metabolic and physiological properties, enabling them to metabolize different substrates. Both *B. safensis* morphotypes tested positive for ornithine. They fermented the carbon sources examined in this study, demonstrating their ability to degrade and metabolize various sugars for carbon and energy. These results contrast with those of Singh et al. (2013), who reported no reaction with mannitol or galactose.

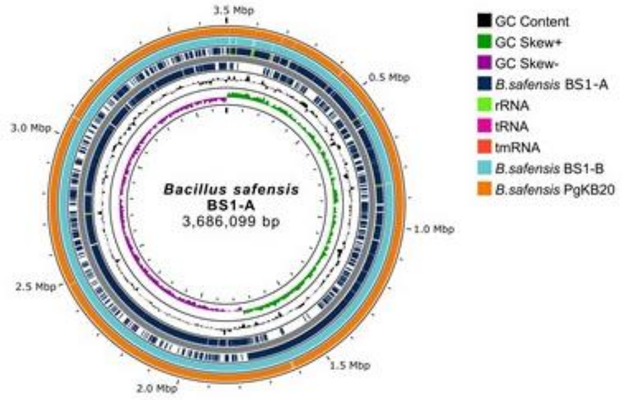

**Figure 2.** Genome of *Bacillus safensis* BS1-A. Circular representation of the BS1-A genome. From the outside to the center: BLAST comparison with *B. safensis* PgK20 and BS1-B sequences; coding genes of BS1-A; genomic G+C content; and genome GC skew value distribution.

During the phenotypic characterization of *B. safensis*, two colonial morphotypes (BS1-A and BS1-B) were observed in the same isolate, suggesting intra-strain phenotypic variation. This phenomenon has been

**Table 5.** Sequencing and assembly statistics of the bacterial genome, including read quality metrics and genome assembly parameters (genome size, coverage, number of annotated genes, rN50, and rNG50).

| Metric | Value |
|---|---|
| Total bp sequenced | 382,148,349 bp |
| Total number of reads | 157,576 reads |
| Longest read | 33,518 bp |

Assembly stats

| Contig(s) | Length | Genome size | Coverage | Number of genes annotated | rN50 | rNG50 |
|---|---|---|---|---|---|---|
| Genome | 3686099 bp | 3.69 Mb | 98.13x | 3781 genes | 3651 bp | 17,267 bp |

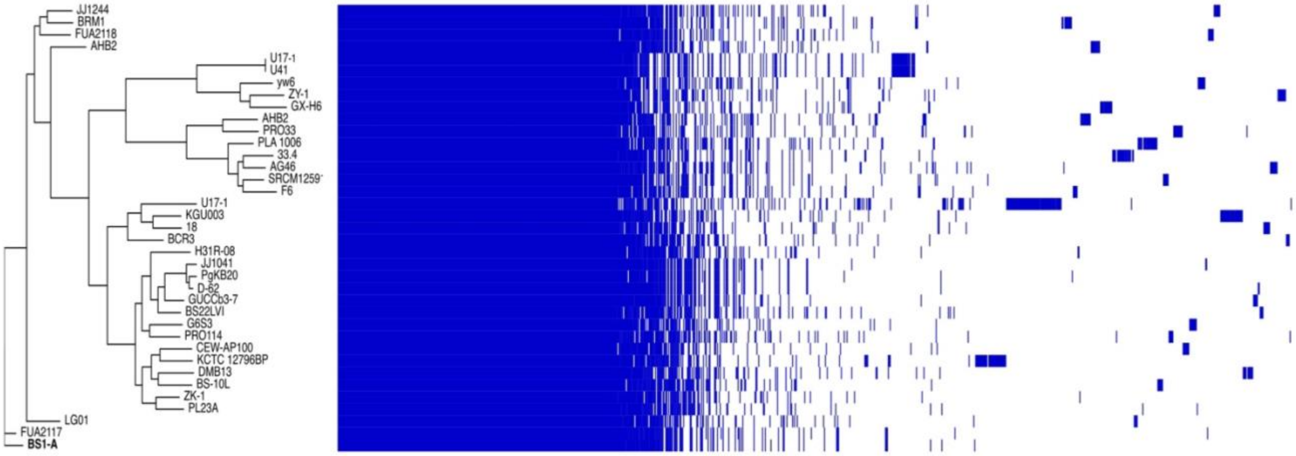

**Figure 3.** Pangenome analysis for 38 *Bacillus safensis* genomes. The phylogenetic tree on the left was constructed in RaxML using the maximum likelihood method, and the core gene alignment was obtained from Roary. The pangenome distribution is visualized in a heatmap where the rows correspond to the strains in the phylogenetic tree, and the columns represent specific orthologous groups. Genetic presence is indicated in blue, and absence is shown in white.

widely documented in the genus *Bacillus*, where a single bacterial lineage may give rise to distinct colonial morphotypes as a result of phase variation, phenotypic plasticity, or regulatory changes, without necessarily implying taxonomic differences (Di Franco et al. 2002, Arnaouteli et al. 2021).

In model species such as *B. subtilis*, colony morphology is closely associated with extracellular matrix production and biofilm formation, processes regulated by complex genetic networks in which the master regulator SinR plays a key role. Alterations in the activity of this regulator can generate colonies with contrasting phenotypes, such as smooth or wrinkled morphotypes, which represent differentiated adaptive strategies within the same bacterial population (Leiman et al. 2014, Richter et al. 2018). Recent studies have shown that these morphotypes may coexist as functional subpopulations, differing in physiological traits, extracellular enzyme production, and competitive abilities (Arnaouteli et al. 2021).

Furthermore, reversible transitions between colonial morphotypes, such as opaque and translucent colonies, have been reported in different species of the genus *Bacillus*, and are associated with variations in gene expression and ecologically relevant properties (Diabankana et al. 2022). In *B. mycoides*, drastic changes in colony morphology have been described as resulting from modifications in growth and cellular organization, even when strains share the same genetic background (Di Franco et al. 2002).

In *B. safensis*, colony morphology has generally been described as white, round colonies with undulate margins; however, the emergence of morphological variants within the same isolate is consistent with the genus's phenotypic plasticity (Satomi et al. 2006). Therefore, the presence of morphotypes BS1-A and

**Table 6.** Zootechnical parameters of growth, food consumption, and survival for *P. vannamei* shrimp after 42 days with low water exchange and application of experimental (BAC) and commercial (COM) probiotic bacteria. The values in the table represent the replicate means ± standard error. Different letters denote significant differences ($P < 0.05$) among treatments, as determined by Tukey's test. WG: weight gain; SGR: specific growth rate; FCA: feed conversion ratio; S: survival.

| Treatments | FW (g) | WG (%) | SGR (% $d^{-1}$) | FCA | S (%) |
|---|---|---|---|---|---|
| Control | $4.99 \pm 0.05^a$ | $898 \pm 10^a$ | $5.48 \pm 0.02^a$ | $1.48 \pm 0.02^b$ | 96 ± 3 |
| BAC | $5.49 \pm 0.17^b$ | $998 \pm 30^b$ | $5.70 \pm 0.07^b$ | $1.40 \pm 0.0^a$ | 96 ± 3 |
| COM | $5.42 \pm 0.1^{ab}$ | $978 \pm 23^{ab}$ | $5.66 \pm 0.05^{ab}$ | $1.42 \pm 0.0^{ab}$ | 93 ± 4 |

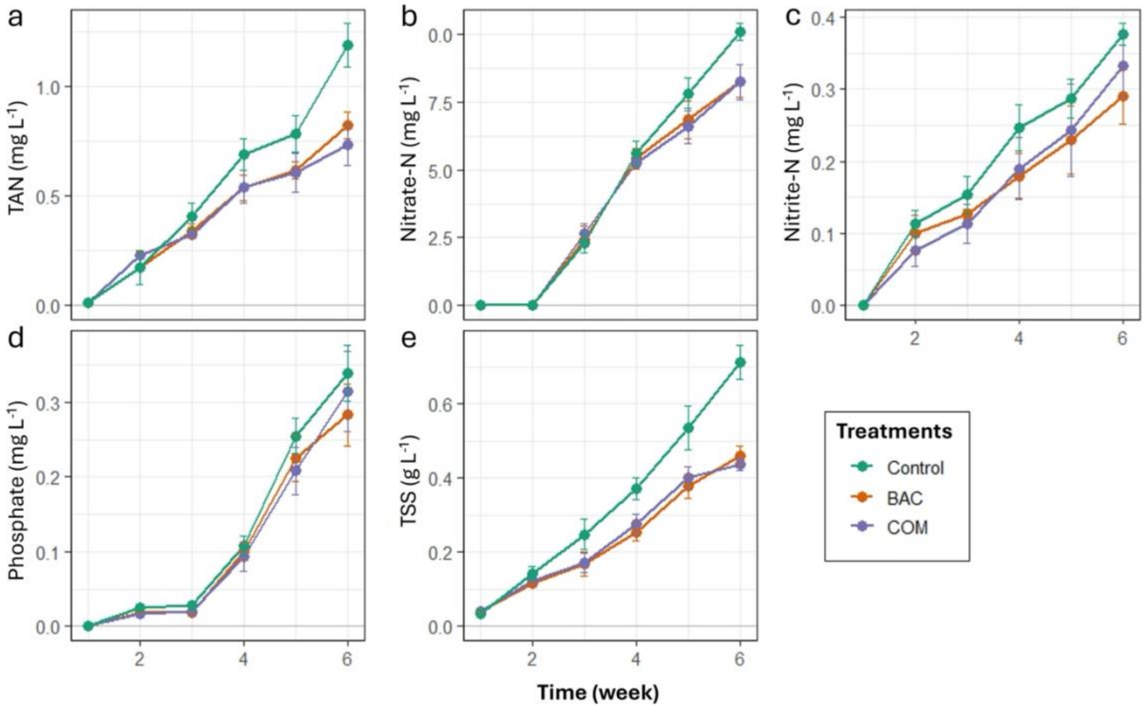

**Figure 4.** Temporal variation of water quality parameters during the experimental period under different treatments. Mean (± standard error) values of a) total ammonia nitrogen (TAN), b) nitrate ($NO_3$-N), c) nitrite ($NO_2^-$-N), d) phosphate ($PO_4^{3-}$), and e) suspended solids (SS) measured weekly in tanks subjected to different treatments: control, mix of equal parts of BS1-A and BS1-B (BAC), and commercial probiotic (COM).

BS1-B may partially explain differences observed in certain biochemical and physiological tests, reinforcing the idea that intraspecific variability is a common feature of bacteria in the genus *Bacillus* (Sitdhipol et al. 2012).

The temperature growth range of 15-45°C observed in this study suggests that *B. safensis* BS1-A and BS1-B are mesophilic bacteria abundant in nature; however, this differs from other reports, which indicate a range of 25-42°C for these bacteria (Satomi et al. 2006, Singh et al. 2013). The tolerance to salinity presented by the *B. safensis* BS1-A and BS1-B coincides with that reported by Satomi et al. (2006), who described growth at 0-10% NaCl for a strain *of B. safensis*, and Sánchez-Ortiz et al. (2015), who reported a tolerance to NaCl of 9-12% for a strain of the genus *B. subtillis*. Recent studies have shown that this bacterium exhibits high salinity tolerance, even when 16% NaCl is used in culture. In probiotic strains, tolerance to a wide range of salinities is desirable, given the environments the strain must face before reaching the host. Both morphotypes were oxidase negative, suggesting that they may be facultative anaerobic and do not require oxygen to colonize specific tissues or organs, an expected property of a probiotic strain, coinciding with what was described by Álvarez-López et al. (2014), who explained that the genus *Bacillus* presents species with positive or negative oxidase reaction. The two morphotypes presented an adverse reaction to the indole and motility test, so it can be inferred that they do not present the tryptophanase enzyme that allows for the hydrolysis of tryptophan and that do not present structures for locomotion, such as flagella or cilia, respectively, coinciding with previous reports (Álvarez-López et al. 2014, Lateef et al. 2015). Several authors have reported that biochemical characteristics may vary even within the same species (Raja & Omine 2012). Variations in biochemical activity between morphotypes of the same strain, as those observed in BS1-A and BS1-B. Similar observations were made by Jiménez et al. (2008), who compared the biochemical characterization of *B. licheniformis* strains and noted differences despite their shared species classification. In the case of qualitative determination of enzymes, *B. safensis* morphotypes BS1-A and BS1-B tested positive for protease and cellulase enzyme production but not for amylase enzymes, which has also been reported by other authors who also observed no amylase activity in *B. safensis* strains (Satomi et al. 2006, Singh et al. 2013), which coincides with our findings. On the other hand, the production of the enzymes protease and cellulose by both morphotypes allows the release of amino acids and the availability of simple sugars and energy, respectively, allowing the microorganisms to use them directly and efficiently, results that coincide with those reported by Álvarez-López et al. (2014), Lateef et al. (2015) and Andreani et al. (2017). In addition, authors such as Dat et al. (2019) reported that the presence of protease and cellulose enzymes in a strain of *Bacillus* sp. improved water quality in aquaculture.

The morphotypes BSA-1 and BSA-2 of *B. safensis* demonstrated the ability to inhibit the three tested pathogenic *Vibrio* species, which is consistent with the study conducted by Huynh-Phuoc et al. (2022), who found that *B. safensis* VQV1 and VQV8 inhibited the growth of four *Vibrios*. Similarly, a study by Rodrigues e Silva et al. (2018) found that *B. safensis* SG-32 inhibited the growth of 20 of 26 bacterial isolates (excluding *Vibrio* spp.) from the same oil reservoir. These results demonstrate the antagonistic activity of *B. safensis* against various *Vibrio* species and its potential to prevent disease in aquatic systems. In recent studies, *B. safensis* has been recognized as an important species for scientific and industrial studies (Huynh-Phuoc et al. 2022). However, to date, no reports of inhibition of pathogenic *Vibrio* in this species have been published. Therefore, the morphotypes used in this study have shown strong potential to inhibit pathogenic *Vibrio* bacteria. As mentioned in the results section, they might even inhibit antibiotic-resistant *V. diabolicus*.

The bacteriophage genes identified in the *B. safensis* genome are present in many *B. safensis* strains. This phage sequence is homologous to the *Brevibacillus* phage, Jimmer 1 (Tirumalai et al. 2018). In addition, the cat86 gene encodes a chloramphenicol acetyltransferase (CAT), an enzyme that confers resistance to chloramphenicol and other phenicol antibiotics through acetylation-mediated inactivation. This resistance mechanism has been extensively documented in members of the genus *Bacillus*, where cat-type genes may be either constitutively expressed or inducible, depending on their regulatory architecture (Mongkolsuk et al. 1984, Duvall et al. 1985). The detection of cat86-1 in *B. safensis*, showing 90-100% sequence identity with homologous genes reported in other *Bacillus* spp. deposited in the NCBI database, is consistent with previous genomic studies describing chloramphenicol resistance determinants in environmental and industrial *Bacillus* strains (CARD ARO:3002672). Although high sequence identity suggests conservation of cat86-1 within the *Bacillus* lineage, sequence similarity alone does not provide sufficient evidence regarding the evolutionary origin or mobility of the resistance determinant. For strains proposed for application in aquaculture as probiotics or biocontrol agents, it is essential to determine whether cat86-1 represents an intrinsic chromosomal feature or an acquired gene associated with mobile genetic elements, such as plasmids, transposons, or integrative conjugative elements. This distinction is particularly relevant in aquatic environments, where microbial communities are highly dynamic and horizontal gene transfer may be enhanced by selective pressures (Baidya et al. 2020). *B. safensis* and closely related *Bacillus* species have been increasingly investigated for their antagonistic activity against *Vibrio* spp., owing to their capacity to produce antimicrobial compounds and to compete effectively within aquatic microbial communities. However, the presence of antibiotic resistance genes in candidate probiotic strains raises biosafety concerns, as these bacteria may serve as reservoirs or vectors for the dissemination of resistance in aquaculture systems (FAO/WHO 2002). Accordingly, international guidelines for probiotic evaluation emphasize that strains intended for use in food or feed applications should not harbor transferable antibiotic resistance genes (FAO/WHO 2002, EFSA 2023). Recent genome-based studies have reported the presence of cat86 in *B. safensis* strains isolated from diverse environments, suggesting that this gene may constitute part of the species' or closely related taxa' accessory genome (Bezuidenhout et al. 2025, Kamilari et al. 2025). Nevertheless, in silico detection alone does not necessarily correlate with phenotypic resistance. Therefore, complementary analyses-such as minimum inhibitory concentration (MIC) assays for chloramphenicol, gene expression analysis, and detailed examination of the genomic context of cat86-1 are required to assess its functional relevance and potential risk.

Overall, the identification of cat86-1 in *B. safensis* BS1-A highlights the importance of incorporating comprehensive genomic safety screening into the evaluation of beneficial bacteria intended for aquaculture applications. While this finding does not automatically preclude the use of BS1-A as a biocontrol or probiotic candidate against *Vibrio* spp., it underscores the need for a balanced risk assessment that integrates antimicrobial efficacy with considerations of antibiotic resistance and environmental safety. The presence of bacillibactin and bacilysin gene clusters in both morphotypes is important because their gene products have the potential to serve as sources of antibacterial compounds (Chakraborty et al. 2022, Islam et al. 2022).

*B. safensis* tolerates a broad spectrum of habitats due to its genotypic and, therefore, physiological capacities (Lateef et al. 2015). One is related to its ability to tolerate high salt concentrations (Atif et al. 2023). However, the best growth results are obtained with concentrations below 5% NaCl. In this work, NaCl was added to the culture medium at a concentration of 2.5%, ensuring growth results comparable to those at the start of the bacterial growth kinetics to those reported by Huynh-Phuoc et al. (2022), whose cultures reached the exponential phase at 4 h. Still, unlike the study above, BS1-A and BS1-B reach their maximum growth at 18 h, while those reported by Huynh-Phuoc, even at 24 h, have not yet achieved the stationary phase, indicating a faster growth capacity for *B. safensis* in trypticase soy broth than that for marine broth used in the work of Huynh-Phuoc.

Regarding the potential benefits of *B. safensis* in shrimp culture, the application of this bacterium as a probiotic in the culture water improved shrimp growth and feed conversion ratio compared to the control (without bacterial application) and did not differ significantly from a commercial probiotic (COM). Several *Bacillus* species are classified as probiotics in aquaculture, with demonstrated benefits including growth promotion and immune and disease resistance in various aquatic species, such as white shrimp (Nayak 2021). The application of a 1:1 mixture of two strains of *Bacillus subtilis* at $10^5$ and $10^8$ CFU mL$^{-1}$ in the *P. vannamei* shrimp culture water resulted in improved growth rate and FCR compared to the untreated control (Zokaeifar et al. 2014). The use of *B. subtilis* in feed has also been described to improve *P. vannamei* growth and FCR compared to a control feed (Olmos et al. 2011). These observable benefits could be partly attributed to the improvement in enzymatic digestive activities and beneficial gut microbiota (Zokaeifar et al. 2014, Olmos et al. 2020). On the flip side, sometimes probiotics may not directly impact shrimp growth, but they do improve physiological parameters and water quality (Ferreira et al. 2015, Llario et al. 2019).

On the other hand, to the best of our knowledge, there is no previous study on the use of *B. safensis* as a probiotic in *P. vannamei*. Nevertheless, in Nile tilapia (*Oreochromis niloticus*), the application of *B. safensis* (NPUST1) in feed ($10^5$ to $10^7$ CFU g$^{-1}$ for eight weeks) improved growth, innate immunity, and resistance against *Streptococcus iniae* (Wu et al. 2021). Regarding water quality, both *B. safensis* morphotypes reduced total ammonia nitrogen compared to the control treatment. This property has previously been reported for *B. safensis* in domestic wastewater (Kim et al. 2021). Moreover, the capacity of *B. safensis* to ferment various carbon sources may be associated with the lower total sedimentable solids observed in treatment BAC compared to the control. Different *Bacillus* species have been reported to promote ammonia denitrification and remove organic carbon from shrimp wastewater, while also contributing to pathogen control (Boopathy et al. 2015, Sampath et al. 2025). These results suggest that *B. safensis* may play a dual role in improving water quality and maintaining a more stable microbial environment, thereby supporting better culture performance and system sustainability.

## CONCLUSIONS

This study identifies *B.safensis* as a potential probiotic for shrimp culture water, improving feed utilization as evidenced by a reduced FCR. The two morphotypes, BS1-A and BS1-B, shared core physiological and biochemical traits but exhibited intra-species variability in citrate utilization and salinity tolerance, indicating metabolic versatility under aquaculture conditions. Both morphotypes produced protease and cellulase enzymes, suggesting a role in nutrient digestion and absorption that may enhance shrimp growth. Genomic analyses confirmed the identity of *B. safensis*. They revealed gene clusters associated with secondary metabolite biosynthesis and accessory genes involved in cell wall remodeling, which may support environmental adaptation. Although a prophage region and the cat86-1 gene were detected, further evaluation is required to assess their functional and biosafety implications. Overall, these results support the potential application of *B. safensis* in shrimp aquaculture; however, additional studies are needed to elucidate its physiological effects, including its effects on digestive enzyme activity and modulation of the microbiota.

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### Credit author contribution

M.A. Liñan-Vidriales: conceptualization, validation, methodology, formal analysis and writing-original draft; E. Quiroz-Guzmán: funding acquisition, project administration, supervision, review and editing; A. Peña-Rodríguez: funding acquisition, project administration, supervision, review and editing; N.A. Ochoa-Álvarez & J.M. Ramírez-Orozco: methodology, validation, supervision, review and editing; I. Ramírez-Sánchez: methodology, data curation, formal analysis, review and editing; L. Rojo-Arreola: methodology, formal analysis, review and editing

### Conflicts of interest

The authors declare that they have no conflicts of interest.

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