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Research article

Bacillus mojavensis isolated from aguamiel and its potential as a probiotic bacterium ☆

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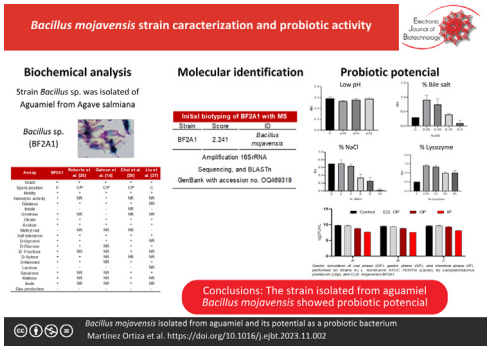
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Millenary fermented beverages are a source of industrially important microorganisms. Aguamiel and pulque are traditional Mexican beverages of pre-Hispanic origin, with a microbial diversity that contributes to the different fermentations (lactic, alcoholic and acetic). The aim of this research was to characterize the *Bacillus mojavensis* (BF2A1) strain isolated from aguamiel and determine its probiotic potential. The strain was identified through Mass Spectrometry (MS), molecular techniques, as well as morphological and biochemical profiling. The probiotic activity of the BF2A1 strain and its response during the gastric simulation was determined.

Results: The strain BF2A1 is a Gram-positive, spore-forming bacillus, positive for catalase, gamma-hemolysis, citrate, ornithine, grows at 7.5% NaCl, and acetoin, but negative for motility, indole, and methyl red. Its taxonomic identity was determined as *B. mojavensis* both by MALDI-TOF MS and sequencing of 16S rDNA. Its probiotic potential was demonstrated as BF2A1 was tolerant to pH 2 (OD_{620nm} 0.289 ± 0.012), 0.3% bile salts (OD_{620nm} 0.103 ± 0.089), 8% NaCl (OD_{620nm} 0.254 ± 0.096), and 1% lysozyme (OD_{620nm} 1.342 ± 0.078) compared to the probiotic strain *Lactobacillus leichmannii* ATCC 7830TM (Laclei). The antagonistic effect of BF2A1 against *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *and Candida albicans* (ATCC60193) showed 25.5%, 8% and, 65% inhibitory growing effect, respectively. BF2A1 in the gastric simulation showed only a reduction of 1–2 log CFU/mL and showed

☆ Audio abstract available in Supplementary material.

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after the intestinal phase a survival rate of 84.4% as compared to the control strains.
Conclusions: This study shows that BF2A1 isolated from aguamiel is a bacterium with probiotic properties that can be used in different areas of Biotechnology.
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1. Introduction

Mexico is recognized for its natural richness and great diversity in flora and fauna. The Agave genus, belonging to the *Agavaceae* family, has 310 reported species, 272 are found in Mexican territory, and 135 of them are endemic to arid and semi-arid regions. The most concentrated zone of these species lies between the states of Puebla and Oaxaca in Mexico [1]. The Agave plant is used for the production of non-distilled beverages including pulque, which is obtained from the *Agave mapisaga* [2], *Agave atrovirens* [1], and *Agave salmiana* species [1]. The most used for obtaining a sap known as "aguamiel," distinguished by its yellowish color and herbaceous odor, is obtained by tapping the mature maguey plants. Various authors have reported on the nutritional characteristics of aguamiel, highlighting its micronutrient content, which includes calcium, phosphorus, iron, thiamine, riboflavin, and important prebiotic components like inulin. Inulin promotes the development and metabolism of beneficial microbiota in the colon, thus contributing to the human health [2,3,4]. The microbiota associated with aguamiel and pulque includes probiotic bacteria that are associated with significant health benefits [4,5]. Different microorganisms have been reported to be isolated from these beverages [5], including bacterial species such as *Lactobacillus paracasei, Lactobacillus sanfranciscensis, Lactobacillus* spp., *Leuconostoc citreum, Acetobacter orientalis, Leuconostoc mesenteroides* [6], *Leuconostoc kimchii, Erwinia rhapontici* [3], *Enterobacter* spp., and *Acinetobacter radioresisten*, as well as yeasts such as *Clavispora lusitaniae* (before *Candida lusitaniae*) and *Kluyveromyces marxianus* [1].

The *Bacillus* genus comprises 293 species and subspecies [7]. Some species within the *Bacillus subtilis* group of biotechnological interest include *B. subtilis, B. vallismortis, B. amyloliquefaciens, B. atrophaeus, B. licheniformis, B. mojavensis, and B. tequilensis* [8], which have been widely used for the production of enzymes, antagonistic agents (antimicrobials), exopolysaccharides, biosurfactants, and even used as biological control agents and plant growth promoters [9]. In recent years, this genus has gained recognition as an essential group in the production of secondary metabolites of biotechnological interest, applications in the industrial, pharmaceutical, medical, environmental, and agricultural fields [10]. Due to the diversity of applications, some of these species have shown potential as probiotics for humans [11].

The presence of the probiotic (e.g., *Bacillus* spp.) presents an alternative for the food industry to create innovative products such as functional or symbiotic foods [12,13]. The Bacillus genus has been identified in various fermented foods including as soybean curd (*B. subtilis, B. amyloliquefaciens, B. licheniformis, B. safensis*), fermented milk (*B. safensis, B. cereus*), yogurt (*B. atrophaeus*), fermented algae (*B. pumilus*), onion and garlic roots (*B. mycoides*), salt marshes (*B. clausii, B. gibsonii*), and rice wine (*B. pumilus*) [14]. In Mexico, several traditional pre-hispanic beverages contain bacteria from the Bacillus genus, such as tepache: *B. subtilis, B. megaterium*; pulque: *Bacillus* sp., *B. licheniformis* [3]; pozol: *Bacillus* sp.; tejuino: *B. Bacillus megaterium, Bacillus safensis*; tibicos: *B. mexicanus, B. brevis, B. polymyxa, B. circulans, B. coagulans, B. firmus, B. macerans,* and *B. pumilus* [13,15].

The FAO/WHO recommends the use of specific microorganisms as a preventive measure to maintain the balance of intestinal microbiota [12]. This helps promote intestinal integrity and motility, inhibit the growth of pathogenic and infectious bacteria, and modulate the immune system [13,16]. Strains of the Bacillus genus have been used in food production [17]. The US Food and Drug Administration describes them as "Generally Recognized As Safe" (GRAS) [17,18,19]. The spore-forming capacity exhibited by *Bacillus* species provides important advantages over non-sporulated microorganisms such as the *Lactobacillus* genus, particularly regarding thermal tolerance and resistance to desiccation. This enables the preservation of integrity and viability for extended periods. Moreover, spore survival under low pH conditions favors viability when encountering the gastric barrier [17]. In sporulated bacteria like *Bacillus*, spores can withstand physicochemical stress during their passage through the gastrointestinal tract, ensuring their survival in the intestine, and subsequent colonization as a vegetative cell [17,19].

The versatility of the *Bacillus* genus opens opportunities for a wide range of applications making it a promising candidate for utilization in the food industry. In this study, we aimed to characterize the BF2A1 strain isolated from aguamiel using microbiological, biochemical, and molecular techniques. The primary objective was to establish the probiotic potential use in both the food and pharmaceutical industries.

2. Materials and methods

2.1. Isolation and morphological identification

Samples of aguamiel were collected from the Municipality of Nanacamilpa de Mariano Arista, in the state of Tlaxcala, Mexico. Isolations were performed through successive dilutions, and strains were purified by cross-streaking on petri dishes. The selected strain was designated as BF2A1. To assess its macroscopic characteristics, it was inoculated on Man, Rogosa and Sharpe Agar (MRS, Merck, KGaA Germany), Soybean Trypticase Agar (TSA, DB Bioxon, Mexico City, Mexico), and Salt and Manitol Agar (MSA, DB Bioxon, Mexico). The strain was incubated at 37°C for 24–48 h on each culture medium. Morphological features on each culture medium [20] were determined using an optical Olympus CX22LED microscope (Olympus Corporation, Tokyo, Japan).

2.2. Biochemical profile of the isolated strain

A series of tests were conducted to characterize the BF2A1 strain: i) Catalase test, ii) determination of hemolytic activity, iii) mobility test, iv) indole and ornithine test (MIO medium, DIBICO, Cuautitlán Izcalli Mexico), v) citrate assimilation test (Simmons Citrate Agar, DIBICO, Mexico), and vi) production of acetoine and acids (Voges Proskauer- Methyl red broth, Becton Dickinson-Bioxon, Mexico City, Mexico) [20].

In addition, to assess sugar assimilation and gas production, Phenol Red broth (DIBICO, Mexico) was used, supplemented with

D-Glycerol, D-Glucose, D-Fructose, D-Xylose, Sucrose, Lactose, Maltose, Mannitol, and Inulin. Hemolytic activity was determined using culture medium Blood (DIBICO, Mexico) [20].

2.3. Biotyping by mass spectrometry

The initial taxonomic identification of isolates was performed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) on a MICROFLEX LT platform (Bruker Daltonik), This was carried out following the Direct Transfer method described by the manufacturer. The spectra were compared to BDAL v.10 database within the Biotyper 3.1 software (Bruker Daltonik).

2.4. Molecular identification

Genomic DNA extraction was performed using the Quick Start Protocol Dneasy Ultraclean Microbial Kit (Qiagen). For amplification of the 16sRNA gene, the universal primers 27F-YM (5́AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5́TACCTTGTTACGACTT-3́) were used. The amplification conditions included an initial denaturation at 98°C for 2 min (1 cycle), followed by 28 cycles of denaturation at 98°C,10 s; annealing at 48°C, 15 s; extension at 72°C, 12 s. Subsequently, a final extension step was performed at 72°C for 5 min and maintained at 4°C for 10 min.

The PCR product was analyzed through horizontal agarose gel electrophoresis at 1%, running at 120 V for 30 min. The resulting PCR product was sequenced using the Sanger capillary sequencing method at the Langebio Genomic Services Laboratory, CINVESTAV-Irapuato, Mexico. The obtained sequence of 1412 bp was edited and corrected using BioEdit (version 7.2.5) and compared to Data Base NCBI. The sequence was deposited in GenBank with accession no. OQ469319. The BF2A1 strain is stored in the microbial culture collection of CINVESTAV-Zacatenco and is assigned the code number B-2078.

2.5. Probiotic activity tests

2.5.1. Biological material

For this study, the *L. leichmannii* strain ATCC 7830TM (LacLei) and *Lactiplantibacillus plantarum* (Lbp) previously identified by the workgroup (sequence deposited in GenBank under accession number OQ642147), were used as a control. Pathogenic strains, including *E. coli* (ATCC 25922), *S. aureus* (ATCC 13076), *K.* (ATCC 10031), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 60193) were provided by Public Health Institute of the State of Puebla.

2.5.2. Tolerance to low pH

The preinoculum of BF2A1 was prepared in MRS broth medium (Merck, KGaA Germany), incubating at 37°C for 48 h. The optical density (OD) of the inoculum was adjusted to 0.1 (OD_{620nm}), before adding it to the tubes containing MRS medium with different pH levels ranging from 2.3 to 5, adjusted with 1 M HCl. The OD reading (OD_{62nm0}) was t measured at 4 h using a Microplate reader (Thermo Fisher-Scientific) [21,22].

2.5.3. Tolerance to bile salts

MRS broth was prepared with Bile salts at concentrations of 0.05, 0.1, 0.15, 0.3, and 0.5% (w/v) using BB0225 (Bio Basic). The broth was then inoculated with 2% (v/v) of the BF2A1 strain (adjusted to OD 0.1) and incubated at 37°C for 24 h. The OD_{620nm} was determined using a UV–visible Spectrophotometer (Model VE-5100 Company Scientiphyca Vela Quin Mexico City, Mexico) [20,21,22].

2.5.4. Lysozyme tolerance

MRS broth was prepared by adding varying concentrations of lysozyme (0.5, 1, 2, and 3% w/v). The broth was then inoculated with 2% (v/v) of the BF2A1 strain (adjusted to OD 0.1) and incubated at 37°C for 2 h. The growth was assessed by measuring the OD_{620nm} using a Microplate reader (Thermo Fisher-Scientific, Shanghai, China) with 96 wells, and viability was assessed by plating at each established time point [22].

2.5.5. Salt tolerance

MRS broth was prepared with different concentrations of NaCl (2, 4, 6, 8, and 10%). Subsequently, it was inoculated with 2% (v/v) of the BF2A1 strain (adjusted to OD 0.1) and incubated at 37°C for 24 h. The growth was determined by measuring the OD_{620nm} at 24 h using a UV–VIS Spectrophotometer (Model VE-5100 Company Scientiphyca Vela Quin Mexico City, Mexico) [21].

2.5.6. Antagonistic activity

The antagonistic activity was performed using the disk diffusion method [21] and microplate-based with OD_{620nm} readings conducted in a Microplate reader (Thermo Fisher-Scientific Shanghai, China).

To perform the disk diffusion antibiogram, the pathogenic strains were adjusted to an inoculum of 0.5 OD_{620nm}. Sterile filter paper disks loaded (diameter 3 mm) with 10 µl of BF2 cell culture were placed on the Muller-Hinton agar plate (Becton Dickinson-Bioxon, Mexico City, Mexico) that had previously been inoculated with each pathogenic strain. These plates were then incubated for 24 h at 37°C. The inhibition zone was reported as sensitivity values (S: sensitive, SN: no sensitivity, SL: limited sensitivity, Sm: medium sensitivity, and SV: very sensitive).

For the microplate inhibition assay, filtered supernatant of the BF2A1 strain was obtained using a Millex-HV filter 0.45 µm (MERCK, Massachusetts, USA) and Laclei and Lbp served as the controls. To determine the inhibitory effect, 10 µl, 20 µl, and 30 µl of supernatant were used with each pathogen. The microplates were incubated at 37°C for 24 h, and OD_{620nm} was measured. The inhibition percentage (%I) was calculated as **Eq. (1)**:

$$\%I = \frac{ODcontrol}{ODassay} \times 100 \quad (1)$$

This percentage was reported for each test performed in comparison to the control and each corresponding pathogenic strain.

2.6. Simulated gastric digestion

2.6.1. Culture conditions

To evaluate the survival of probiotic bacteria under gastrointestinal conditions, the techniques by the Ortakci and Sert [23], and Gayoso et al. [24] were used with some modifications. The strains Laclei and Lbp were used as controls, alongside BF2A1 strain. The three strains were cultured in MRS medium with an initial a $DO_{620nm} \geq 0.5$. The evaluation of bacterial survival occurred in the oral phase (OP), gastric phase (GP), and intestinal phase (IP).

2.6.2. In vitro digestion

For the OP, 5 mL of each culture was extracted and mixed with 4 mL of saline solution (pH 6.5) in a Falcon tube. To this mixture, 125 µl of α-amylase solution (10 units/mg, Sigma-Aldrich, San Luis Misuri, USA) solution (1.3 mg/mL $CaCl_2$ 1 mM) was added, adjusting the pH to 6.5 with 1 M $NaHCO_3$. This was incubated at 37°C for 5 min at 100 rpm. The GP was performed in the same tube, adding 9 mL of gastric fluid composed of pepsin (lyophilized pepsin, salt-free, 2500 units/mg protein, Sigma-Aldrich, San Luis Misuri, USA), 3 mg/mL, 0.85% NaCl and pH adjustment to 2.0 with 1 M HCl. It

was then incubated at 37°C for 2 h at 100 rpm. For the IP, 9 mL of a bile juice solution consisting of pancreatin (4 x UPS specifications CAS-Number 8049-47-6, Sigma-Aldrich, San Luis Misuri, USA) 1.9 mg/mL, 1.2% bile salts (BB0225, BioBasic, Ontario, Canada) and pH adjustment to 7.0 with 1 M $NaHCO_3$ was added and incubated at 37°C for 2 h at 100 rpm. Following each phase, the survival assay was performed in triplicate, and logCFU/mL values were reported. **Eq. (2)** was used to calculate the percentage of survival (%S):

$$\%S = \frac{\text{logCFU/mL}\,final - \text{logCFU/mL initial}}{\text{logCFU/mL}\,initial} \times 100 \quad (2)$$

2.7. Statistical analysis

Data analysis was conducted using GraphPad Prism software (version 8.0.1. licensed to UPAEP). One-way ANOVA with Dunnett's multiple comparisons test was employed to determine significant differences between means, with a significance level of $\alpha = 0.05$. The presented data correspond to the mean values of three replicates per experiment.

3. Results

3.1. Macroscopic morphology of BF2A1 isolated from aguamiel

Macroscopic morphology of BF2A1 on TSA medium exhibits to colonies with a matte beige color, circular shape, umbonate elevation, eroded margin, rough surface, opaque density, and soft consistency coincides with other authors [14,25,26,27]. On MRS agar, colonies are yellow with filamentous form, umbonate elevation, filamentous margin, granular surface, opaque density, hard consistency, and white pigment. On Salt and Mannitol Agar, colonies are matte beige, circular, convex elevation, eroded margin, rough surface, opaque density, soft consistency, and variable mucosity (**Fig. S1**). BF2A1 is a short Gram-positive Bacillus, spore-forming, with central spore position [28].

3.2. Molecular and Spectrometry identification

The PCR product obtained from the amplification of the 16SrRNA using primers 27F and 1492R was approximately 1500 bp. The alignment using the BLAST algorithm of NCBI showed that the BF2A1 strain has a 100% sequence similarity with *B. mojavensis* IFO 15718, corroborating the results obtained by MALDI-TOF MS. A phylogenetic analysis was performed with 16SrRNA data corresponding to *B. mojavensis* reported in NCBI. BF2A1 is phylogenetically close to the *B. subtilis, Bacillus tequilensis,* and *Bacillus halotolerans* strains compared with the rest of species in *B. subtilis* group (**Fig. S2**). Taxonomic identification by Mass Spectrometry demonstrated that the BF2A1 strain isolated from aguamiel corresponds to the species *B. mojavensis* with a score of 2.241.

3.3. Biochemical profile

The biochemical tests conducted to BF2A1 (Table 1) showed the results consisted with the biochemical profiles reported by Gatson et al. [14], Roberts et al. [25], Choi et al. [26], and Liu et al. [27]. BF2A1 is a short, gram-positive *Bacillus* with a central spore and exhibits positive results for catalase, citrate, γ-hemolytic, ornithine, acetoin, and halotolerat to 7.5% NaCl. It tests negative for mobility, indole production, and methyl red. Additionally, it assimilates D-Glycerol, D-Glucose, D-Fructose, D-Xylose, Sucrose, Maltose, and Inulin, but not Lactose, and does not produce gas, in accordance with Bergey's Manual [28].

3.4. Probiotic activity tests

The results of the probiotic activity tests for BF2A1 and Laclei are presented in Table 2. The pH tolerance assay shows no significant difference between BF2A1 (OD_{620nm} 0.289 ± 0.012) and Laclei (OD_{620nm} 0.274 ± 0.039). For the bile salt tolerance test, BF2A1 (OD_{620nm} 0.103 ± 0.089) exhibited significant differences when compared to Laclei (OD_{620nm} 1.47 ± 0.479) at 0.3. In the NaCl tolerance assay, the significant difference of BF2A1 at an 8% concentration (OD_{620nm} 0.254 ± 0.096) was significantly higher than Laclei (OD_{620nm} 0.00 ± 0.0). In the lysozyme resistance assay, BF2A1 (OD_{620nm} 0.995 ± 0.048) showed similar values to Laclei (OD_{620nm} 1.062 ± 0.038) at 2% concentration.

Table 1
Biochemical profile obtained from *B. mojavesis* BF2A1 compared to profiles reported by various authors.

Assay	BF2A1	Roberts et al. [25]	Gatson et al. [14]	Choi et al. [26]	Liu et al. [27]
Gram	+	+	+	+	+
Spore position	C	C/P	C/P	C/P	C
Motility	-	+	+	+	+
Hemolysis	+	NR	+	NR	NR
Catalase	+	+	+	+	NR
Indole	-	-	-	NR	-
Ornithine	+	NR	-	NR	NR
Citrate	+	+	+	+	+
Acetoin	+	+	+	+	+
Methyl red	-	NR	NR	NR	-
Salt tolerance (7.5%)	+	+	+	+	+
D-Glycerol	+	+	+	+	NR
D-Glucose	+	+	NR	+	+
D-Fructose	+	NR	NR	+	NR
D-Xylose	+	+	NR	NR	NR
D-Mannitol	+	+	NR	+	+
Lactose	-	-	-	-	NR
Sacarose	+	NR	NR	+	+
Maltose	+	NR	NR	+	NR
Inulin	+	NR	NR	+	NR
Gas production	-	-	-	-	-

(+): positive reaction; (-): negative reaction; C: central; P: paracentral; NR: not reported.

Table 2
Results of probiotic challenges applied for *L. leichmannii* ATCC® 7830TM (Laclei) and *B. mojavensis* BF2A1.

pH	Strains	
	Laclei (OD_{620nm})	BF2A1 (OD_{620nm})
6.5	0.258 ± 0.041	0.293 ± 0.014
5	0.270 ± 0.043	0.269 ± 0.003
3	0.253 ± 0.055	0.282 ± 0.009
2	0.274 ± 0.039	0.289 ± 0.012
Bile salt (%)		
0	0.7 ± 0.01	0.3 ± 0.015
0.05	2.2 ± 0.007**	0.912 ± 0.163**
0.1	2.26 ± 0.191**	0.752 ± 0.203*
0.15	2.024 ± 0.023*	0.401 ± 0.046
0.3	1.47 ± 0.479	0.103 ± 0.089
NaCl (%)		
0	0.7 ± 0.01	0.7 ± 0.02
2	0.063 ± 0.020**	0.702 ± 0.097
4	0.024 ± 0.027**	0.641 ± 0.048
6	0.00 ± 0.00**	0.339 ± 0.085**
8	0.00 ± 0.00**	0.254 ± 0.096**
10	0.010 ± 0.010**	0.026 ± 0.036**
Lysozime (%)		
0	0.118 ± 0.005	0.300 ± 0.02
0.5	1.338 ± 0.240**	1.399 ± 0.078**
1	1.135 ± 0.096**	1.342 ± 0.078**
2	1.062 ± 0.038**	0.995 ± 0.048**
3	1.116 ± 0.148**	1.018 ± 0.119**

OD_{620nm} mean and SD of triplicate assays, with one-way ANOVA analysis ($\alpha = 0.05$) and Dunnett's multiple comparisons. Corresponding significant difference and *P* value are reported (*$P = 0.01$, **$P = 0.001$, ***$P < 0.0001$).

Table 3
Antagonistic activity against pathogenic strains by the disk diffusion method [21] for *L. leichmannii* ATCC® 7830TM (Laclei) and *B. mojavensis* BF2A1.

Pathogen	Laclei (mm)	BF2A1 (mm)
E. coli ATCC 25922	7.75 ± 10.9^{SN}	5.50 ± 7.78^{SN}
S. aureus ATCC 25923	5.5 ± 7.78^{SN}	14.70 ± 1.77^{Sm}
K. pneumoneae ATCC 10031	8.50 ± 0.707^{SL}	4.00 ± 5.66^{SN}
P. aeruginosa ATCC 27853	3.50 ± 4.95^{SN}	6.50 ± 0.71^{SN}
C. albicans ATCC 60193	11.00 ± 1.414^{SL}	8.00 ± 1.41^{SN}

Inhibition zone (mm) determined in duplicate for each assay is reported. Sensitivity values are as follows: S: sensitive, SN: no sensitivity, SL: limited sensitivity, Sm: medium sensitivity, and SV: very sensitive. Values SN $\leq$ 8 mm, SL 9–14 mm, Sm 15–19 mm, SV $\geq$ 20 mm.

Regarding the antagonistic activity determined by the disk diffusion method, the supernatant from BF2A1 culture exhibited sensitivity toward *S. aureus* (S: 15–19 mm) (Table 3). In the microplate antagonism assay, BF2A1 demonstrated inhibition on *C. albicans* (65%), *E. coli* (25%) and *S. aureus* (8%) (Table 4).

3.5. Gastric simulation assay

In the gastric simulation assay, no statistically significant differences were observed in the initial and OP phases for the samples of Laclei, Lbp and BF2A1 (Table 5). However, significant differences were noted in GP and IP when comparing the three strains ($P < 0.0001$). Laclei and Lbp exhibited a reduction of at least a 2 Log CFU/mL in the IP, while BF2A1 showed a Log 1 reduction in CFU/mL (Fig. 1). To validate these results, the CFU/mL count was determined at pH 2, revealing that BF2A1 maintained viability 2 h after inoculation under these conditions, compared to the control strain Lbp (**Fig. S3**).

4. Discussion

BF2A1 exhibited different macroscopic morphologies in various media, as shown in **Fig. S1**, showcasing the strain's adaptability to these environments. The growth observed in Salt and Mannitol Agar, where *Bacillus sp.* growth has not been previously reported, underscores BF2A1's tolerance to halophilic conditions. This trait is atypical for LAB, highlighting its significance when using media with high salt content.

The microscopic morphology exhibited by BF2A1 aligns with the characteristic features as documented in Bergey's Manual. It is a Gram-positive, spore-forming bacterium. The biotyping test, amplification of 16SrRNA gen, and sequencing confirm that BF2A1 belongs to the species *B. mojavensis*. Phylogenetic analysis based on 16SrRNA [8] reveals that BF2A1 is closely related to *B. subtilis, B. tequilensis and B. halotolerance* (**Fig. S2**). While there is a phylogenetic relationship among these strains, BF2A1 is considered of plant origin since it was isolated from aguamiel, which can be associated with the Agave plant from which nectar is obtained. It is possible that BF2A1 is part of the native microbiota of the plant. Escalante et al. [3] reported the presence of *B. licheniformis* in Pulque, but until now, no other species of *Bacillus spp* had been reported. Upon sequencing and alignment using BLAST, BF2A1 shows 100% similarity with *B. mojavensis* IFO 15718. The *B. subtilis* clade exhibits very close phylogenetic relationships (98–100%), Patel et al. [8] recommend using additional molecular markers to differentiate between species, such as *gyrA* and *rpoA* genes. These markers can provide more specific and accurate identification, aiding in the distinction of one species from another within the same genus. This study has demonstrated the presence of *B. mojavensis*, as an important species that had not been reported before in aguamiel. Jezewska-Frackowiak et al. [19] demonstrated that *B. mojavensis* spores are used as a supplement when analyzing commercial products, signifying the applicability of this microorganism in the food industry.

The biochemical tests are applied to BF2A1, as shown in Table 1, yielding conclusive results. However, some data presented by Gatson et al. [14], Roberts et al. [25], Choi et al. [26], and Li et al. [27] differ in specific aspects. In this regard, BF2A1 exhibits hemolytic activity, assimilation of ornithine, and does not produce acids. On the other hand, the assimilation of carbon sources such as fructose and inulin suggests its potential to utilize prebiotics, indicating its suitability for probiotic applications.

In the probiotic activity results, BF2A1 demonstrates tolerance to low pH (2), bile salts (0.3%), NaCl (8%), and lysozyme (3%). Elshaghabee et al. [6] mention that when comparing *Bacillus* genus to the *Lactobacillus* genus, *Bacillus* genus exhibits important advantages as probiotics. One of these advantages is the spore-forming ability of *Bacillus* genus, which confers stress resistance when exposed to gastrointestinal tract (GIT) conditions. This enables *Bacillus* to survive the harsh conditions of the GIT and reach the intestine where it can colonize. Under optimal intestinal conditions, the spores germinate, giving rise to vegetative cells that can exert beneficial probiotic effects in the host [13].

The results of antagonistic activity (Table 4) show the effect on *E. coli, S. aureus*, and *C. albicans.* Caulier et al. [29] describe that *Bacillus* genus produces two types of antagonistic molecules known as ribosomal peptides (RPs), and non-ribosomal peptides (NRPs). Lipopeptides are a type of NRP produced by the genus, including iturin, surfactin, fengycin, and kurstakins as antimicrobial and antifungal agents. Youcef-Ali et al. [30] report that *B. mojavensis* shows antifungal activity against the pathogenic yeast *C. albicans*, while Caulier et al. [29] report that some of these antagonistic substances affect *E. coli, S. aureus, K. pneumoniae,* and *C. albicans*. The antagonistic effect exerted by the BF2A1 strain may be

Table 4
Antagonistic activity in microplate with different volumes of supernatant of *L. leichmannii* ATCC® 7830TM (Laclei), *B. mojavensis* BF2A1, and pathogen strains *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *K. pneumoneae* ATCC 1003 1, and *C. albicans* ATCC 60193.

Pathogen	**Volume supernatant Laclei**					
E. coli	30 µl	%I	20 µl	% I	10 µl	% I
OD_{620nm} 0.885 ± 0.299	OD_{620nm} 0.065 ± 0.07**	93	OD_{620nm} 0.262 ± 0.056**	70.3	OD_{620nm} 0.936 ± 0.128	5.76
S. aureus 0.50 ± 0.082	0.146 ± 0.028**	70.8	0.224 ± 0.024**	55.2	0.319 ± 0.044**	36.2
K. pneumoneae 0.752 ± 0.032	0.710 ± 0.030	5.56	1.079 ± 0.045***	ND	1.237 ± 0.043***	ND
C. albicans 0.1540 ± 0.001	0.356 ± 0.040	ND	0.390 ± 0.066	ND	0.998 ± 0.538*	2.0
Pathogen	**Volume supernatant BF2A1**					
E. coli	30 µl	% I	20 µl	% I	10 µl	% I
OD_{620nm} 0.700 ± 0.01	OD_{620nm} 0.521 ± 0.040**	25.5	OD_{620nm} 0.688 ± 0.271	1.71	OD_{620nm} 1.152 ± 0.081**	ND
S. aureus 0.600 ± 0.02[a]	0.547 ± 0.005	8	0.651 ± 0.022	ND	1.318 ± 0.058***	ND
K. pneumoneae 1.000 ± 0.002	1.216 ± 0.098*	ND	1.178 ± 0.107	ND	1.307 ± 0.094*	ND
C. albicans 0.700 ± 0.01	0.245 ± 0.039	65	0.497 ± 0.275	29	1.00 ± 0.000**	ND

OD_{620nm} mean and SD of triplicate assays are shown. The percentage of inhibition (%I) and one-way ANOVA analysis ($\alpha = 0.05$) with Dunnett's multiple comparisons are reported. Corresponding significant difference is reported (*P = 0.01, **P = 0.001, ***P < 0.0001), ND not detected.

Table 5
Results obtained in each phase of the gastric simulation: oral phase (OP), gastric phase (GP), and intestinal phase (IP) for *L. leichmannii* ATCC7830TM (Laclei), *L. plantarum* (Lbp) and *B. mojavensis* BF2A1. Mean and SD of triplicate assays are shown, expressed in logCFU/mL. One-way NOVA analysis ($\alpha = 0.05$) with Dunnett's multiple comparisons is reported. Corresponding significant difference is reported. (*P = 0.01, **P = 0.001, ***P < 0.0001) and survival percentage (%S).

Strain	**Control LogCFU/mL**	**OP LogCFU/mL**	**%S**	**GP LogCFU/mL**	**%S**	**IP LogCFU/mL**	**%S**
Laclei	9.8 ± 0.10	9.7 ± 0.17	98.9	8.8 ± 0.10***	89.8	7.7 ± 0.07***	78.6
Lbp	9.5 ± 0.09	9.4 ± 0.11	98.9	8.7 ± 0.058***	91.6	7.6 ± 0.06***	80
BF2A1	9.6 ± 0.11	9.7 ± 0.10	100	9.3 ± 0.20***	95.9	8.1 ± 0.09***	84.4

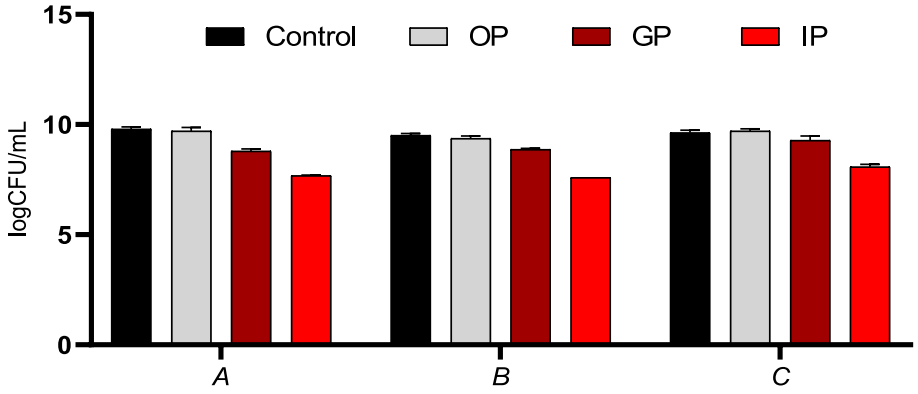

Fig. 1. Gastric simulation of oral phase (OP), gastric phase (GP), and intestinal phase (IP), performed on strains A) *L. leichmannii* ATCC 7830TM (Laclei), B) *L. plantarum* (Lbp), and C) *B. mojavensis* BF2A1. Triplicate assays are shown, with LogCFU/mL values presented. One-way ANOVA analysis ($\alpha = 0.05$) with Dunnett's multiple comparisons is reported.

related to the production of these metabolites, but at present, we cannot ensure that the study strain produces more than one metabolite with this inhibitory effect. Fanaei et al. [31,32] report that *B. mojavensis* is capable of producing lipopeptides and their different isoforms, presenting antagonistic activity against pathogens and does not generate toxicity in PC12 and PBMC cells, considering that this type of molecule has pharmacological applications.

In the gastric simulation assay showed in Table 5 and Fig. 1, BF2A1 did not show significant differences in OP and GP, with values of 9 Log CFU/mL. In contrast, Laclei and Lbp showed a reduction of 1 and 2 log CFU/mL in PG and IP, respectively. The percentage of survival for BF2A1 was 95.9% in GP, and 84.4% in IP, which were higher values compared to the control strains (Table 5).

Studies of Jezewska-Frackowiak et al. [19] have shown that *Bacillus* sp. can grow under extreme and pH extremes. These advantages for bimodal existence in the environment and gastrointestinal tract suggest potential biotechnological applications. The survival and stability of the structure against the gastric barrier demonstrate that the *Bacillus* genus can be considered a potential food additive and can also be included as intestinal commensals that perform biological functions in hosts. While tolerance to gas-

trointestinal conditions is a determining factor in achieving beneficial effects in hosts, it merely demonstrates the microbial adaptability. Another advantage of probiotic consumption is the immunomodulatory benefits in the host [33].

In the human gut microbiote (HGM), a complex microbial community with dynamic mutualistic interactions is responsible for the digestion and absorption of dietary components takes place. The consumption of specific food such as prebiotics can modulate the HGM by providing fermentable substrate to produce bacterial metabolites with beneficial effects on host health. These molecules like short-chain fatty acids, tryptophan and organic acids have shown positive effects on controlling pathogenic bacteria, improving mineral absorption, regulating weight control and obesity, modulating immune response homeostasis, enhancing gut barrier function modulating, brain function and exhibiting anticancer activity. This suggests that the beneficial effects may be attributed to the metabolic capacities of the gut microbiota and the subsequent synthesis of specific metabolites with functional properties beneficial to the host [34].

Studies conducted by Kim et al. [35] *in vitro* suggest that the culture supernatant *B. mojavensis* KJS-3 should be safe for consumption as a probiotic, and further testing is warranted to confirm this *in vivo*. Cytotoxicity and genotoxicity assay with Chinese hamster lung cells (CHL) showed no clastogenicity of culture supernatant fermented by *B. mojavensis* KJS-3. The cytotoxic effects of BF2A1 in mammalian cell lines and murine models are currently being analyzed to identify immunomodulatory effects and cytokine induction when using BF2A1; these results are not yet published.

The elucidation of the antagonistic properties of BF2A1 against pathogenic microorganisms suggests an alternative to the use of antibiotics. It proposes the use of BF2A1 as a possible probiotic therapy, like LAB bacteria. This complements our study by demonstrating physicochemical properties such as adhesion, hydrophobicity, self-aggregation, and co-aggregation against various microbial pathogens.

During this research, work has been done to determine the production of secondary metabolites with antagonistic effects produced by BF2A1, like what has been reported with *B. mojavensis* by Fanaei et al. [31,32]. Caulier et al. [29] showed that *Bacillus* genus produces different metabolites of important biological activity such as ribosomal peptides (bacteriocins and enzymes) (RPs), the polyketides (PKs), non-ribosomal peptides (NRPs) and volatile compounds which have been associated with antimicrobial, antifungal, antitumor and immunomodulatory activity.

The study of the *Bacillus* genus continues to hold much scientific potential. For example, species belonging to the clade *B. subtilis* have been associated with the production of γ-aminobutyric acid (GABA) as a secondary metabolite. It would be interesting to explore this in BF2A1, as GABA plays a major role as an inhibitory neurotransmitter in the central nervous system. Several neurological disorders such as anxiety, depression and sleeping disorder are closely associated with the presence of low level of GABA in the brain [35].

5. Conclusions

Agave-derived aguamiel is an agroindustrial product of nutritional importance due to the diversity of microorganisms with probiotic potential. Among these, non-acid lactic bacteria such as the *Bacillus* genus stands out. The strain isolated from aguamiel named BF2A1 was identified as *B. mojavensis*. Based on the results obtained in the probiotic activity tests, these microorganisms can be considered to have probiotic potential.

In this study, we demonstrated that the BF2A1 strain exhibits probiotic properties. When compared to previously studied probiotic strains such as Laclei and Lbp, the BF2A1 shows distinct biological typical of the *Bacillus* genus. It also displays significant differences in probiotic activity tests, including resistance to passage through the gastrointestinal tract demonstrated in the gastric simulation test.

As it was isolated from aguamiel, BF2A1 can be considered a microorganism naturally present in *Agave salmiana* forming part of the native microbiota. Unlike other identified species, *B. mojavensis* has not been reported as a probiotic microorganism. Its demonstrated antagonistic capacity against bacterial and fungal pathogens can be channeled into the development of functional foods and medical-pharmaceutical applications, where its benefits could impact various areas of biotechnology.

It is important to consider toxicity studies to ensure its safety, such as cytotoxic and genotoxic studies, or tests in murine models to rule out any adverse effects. The results of this research provide a foundation for future targeted metabolomics studies to evaluate antimicrobial, antiproliferative, anti-inflammatory and immunomodulatory effects.

The probiotic capacity identified in BF2A1 can be further explored with assays focusing on adhesion, hydrophobicity, self-aggregation, and co-aggregation against microbial pathogens. Additionally, investigating whether BF2A1 produces GABA and its role as an inhibitory neurotransmitter in the central nervous system is an exciting avenue for future research.

Author contributions

- Study conception and design: Martínez-Ortiz VM, Trujillo-López MA, Pérez-Armendáriz B.
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Supplementary material

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