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Evaluation of *in vitro* antibacterial activity and *in vivo Caenorhabditis elegans* assays of *Bacillus velezensis* AU1 and AU2

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ABSTRACT

Alkaliphilic bacteria, including *Bacillus* species, represent a promising but underexplored source of bioactive compounds with potential antibacterial applications. The growing concern over antibiotic resistance highlights the need for discovering new antibacterial agents. This study aimed to evaluate the *in vitro* antibacterial activity of *Bacillus velezensis* strain AU1 and AU2 isolated from Montañeza Waterfalls and assess their *in vivo* efficacy using *Caenorhabditis elegans* as a model organism. AU1 and AU2 isolates were subjected to bacterial fermentation, followed by liquid-liquid extraction using ethyl acetate as a solvent to yield crude extracts. AU1 and AU2 extracts exhibited antibacterial activity against Gram-positive bacteria, *Bacillus subtilis,* and Gram-negative *Escherichia coli,* with zones of inhibition ranging from 12.40−15.63 mm and 8.47−14.10 mm, respectively. AU1 increased the lifespan of the worms, improved locomotion, facilitated higher reproduction rates, and was strongly preferred by the worms over *E. coli* OP50 control or AU2. Furthermore, worms treated with AU1 were also able to survive during Gram-positive bacterial infection, indicating that AU1 may offer superior nutritional benefits that contribute to these positive outcomes. Further investigation is warranted to isolate and characterize bioactive compounds from crude extracts as potential sources for new or novel antimicrobial drugs.

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INTRODUCTION

The growing prevalence of antimicrobial-resistant pathogens poses an urgent global health threat, necessitating the discovery of new antimicrobial agents (Maglangit et al. 2021). Extremophiles, particularly alkaliphilic microorganisms, thrive in unique and hostile niches with high pH conditions, producing bioactive molecules as adaptive mechanisms for survival (Sarethy et al. 2011; Maglangit et al. 2023). These metabolites often possess potent antimicrobial properties, positioning alkaliphilic isolates as promising candidates for the development of new antimicrobials. Recent reports have shown that bacterial isolates from alkaline environments exhibit bioactivity against a variety of drug-resistant pathogens, offering new avenues for drug discovery (Sarethy et al. 2011; Khalikova et al. 2020).

KEYWORDS

Natural products, secondary metabolites, *Bacillus* sp., *C. elegans*

Furthermore, alkaliphilic bacteria produce unique and stable enzymes, such as alkaline proteases, alkaline cellulases, and Cyclodextrin Glucanotransferase (CGTase), which remain stable and active at high pH levels, making them valuable in the food industry, bioremediation, and other industrial applications (Bosito et al. 2023; Joshi et al. 2008; Preiss et al. 2015; Horikoshi 1999). These bacteria are often isolated from rare alkaline habitats, such as soda lakes (Borkar 2015; Sultanpuram et al. 2015), which provide selective pressure for unique biological traits with potential biotechnological applications.

While recent studies have highlighted the antibacterial potential of alkaliphilic bacteria, research focusing specifically on isolates from alkaline waterfalls remains scarce (Grant et al. 1990; Hudson, 2013). In this study, we investigate the antibacterial potential of two alkaliphilic isolates, *Bacillus velezensis* strain AU1 and AU2, sourced from Montañeza Waterfalls (Mainit Hot Springs) in Malabuyoc, Cebu. Beyond antimicrobial activity, we also evaluate their physiological effects on the model organism *Caenorhabditis elegans* (*i.e.*, survival, lifespan, locomotion, reproductive health, chemotaxis, anti-infective response), which is essential for assessing their safety and potential applications. *C. elegans* serves as an excellent *in vivo* model in the study of natural product extracts due to its genetic and biological simplicity, rapid lifecycle, and cost-effectiveness (Ha et al., 2022). *C. elegans* exhibits 60−80% genomic similarity to humans, including those involved in aging, metabolism, stress response, and cell death, all of which are critical to human health. Unlike higher vertebrate models, experiments involving *C. elegans* raise no ethical concerns (Ermolaeva and Schumacher 2014).

To the best of our knowledge, this is the first study to report on the effects of bacterial extracts from alkaliphilic isolates collected from alkaline waterfalls in the Philippines on *C. elegans*. This research provides valuable insights into the antibacterial properties of AU1 and AU2 isolates, contributing to the expanding field of natural product drug discovery from rare habitats.

MATERIALS AND METHODS

Reagents, Chemicals, and Media
All chemicals and reagents used in the experiments were of analytical grade and sourced from the local supplier, Harnwell Chemicals Corporation (Duksan, Korea). Unless otherwise stated, all media were obtained from HiMedia (India).

Bacterial Isolation
The two bacterial strains, *Bacillus velezensis* strain AU1 and AU2, in this study were isolated from water samples collected from the Montañeza Waterfalls (Mainit Hot Spring), Malabuyoc, Cebu, Philippines (coordinates 9°40'59.9" N 123°20'35.2" E). The Montañeza Falls is surrounded by massive limestone boulders, which form the walls of a towering canyon that guides the water to its main drop.

A 100-mL water sample was collected at a depth of 15−20 cm from the river floor of Montañeza Waterfalls (Luo et al. 2018). Collected samples were stored in sterile tubes and placed in an insulated container to maintain the temperature during transportation to the laboratory. The samples were processed immediately after collection. The pH, water temperature, water conductivity, and total dissolved solids (TDS) were also measured on-site during sample collection using a multifunctional meter (Mikroelectron, Jordan).

The water sample (100 µL) was inoculated onto nutrient agar and adjusted to pH 10.0. The NaOH solution (6.0 M) was used to adjust the pH of the media to 10.0, simulating their alkaline habitat. The plates were incubated for seven days at 30 °C (Ibrahim et al. 2006) and observed daily for colony growth. The bacterial strain was further purified using the streaking plate and sub-culturing techniques until a single observable colony of AU1 and AU2 was detected. Pure isolates were stored in 20 % glycerol (v/v) at -80 °C for preservation and culture collection.

Phenotypic characterization
AU1 and AU2 isolates were subjected to cellular and morphological characterization based on their colony shape, margin, color, and Gram reaction (Li et al. 2016). The strains were also tested for various biochemical properties, including salt tolerance, and their ability to produce catalase, amylase, and oxidase using 18- to 24-hour bacterial cultures (Tindall et al. 2014).

16S rRNA sequencing
The DNA of AU1 and AU2 was extracted using HiPurA® Bacterial Genome DNA Purification kit following the manufacturer's instructions. Genomic DNA samples were sent to the Philippine Genome Center, Quezon City, Philippines, for 16S rRNA gene sequencing.

The 16S rRNA sequence was trimmed using A Plasmid Editor (ApE) v.3.1.3 (Davis and Jorgensen 2022) to remove undefined peaks. A Basic Local Alignment Search Tool (BLAST) search was conducted using the nucleotide sequences at NCBI with default parameters. The sequences of the ten species exhibiting the highest percentage of identity, excluding uncultured species, were retrieved from the GenBank database. These sequences were imported into the Molecular Evolutionary Genetics Analysis (MEGA) software version 11.0.1 and utilized to estimate the phylogenetic relationships of each strain using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) alignment (Tamura et al. 2021). The phylogeny of the isolates was estimated using the Maximum-likelihood method and General Time Reversible model with 1000 bootstrap replicates (Nei and Kumar 2000; Tamura et al. 2021).

Bacterial extraction
AU1 and AU2 strains were separately cultivated in a 50 mL nutrient broth (pH 10) and incubated in a rotary shaker for seven days at 120 rpm and 30 °C to facilitate bacterial growth and metabolite production (Jiang et al., 2016). The secondary metabolites from the culture broths were extracted twice with ethyl acetate (EtOAc) via a liquid-liquid partition method (Huo et al., 2020; Mostafa et al., 2018). The organic layer was collected and evaporated to dryness using a rotary evaporator at 40 °C and 120 rpm. The AU1 and AU2 extracts were then diluted with DMSO, transferred to an amber vial, and lyophilized using a freeze-dryer for three days. The weight of the dry crude extracts was then determined.

In vitro antimicrobial bioassay
The crude extracts were subjected to Kirby-Bauer disc diffusion assays following the standard procedure by the Clinical and Laboratory Standards Institute (CLSI 2012) using *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 as the test organisms. The test organisms were standardized to 0.5 McFarland (approximately 1.5×10^8 CFU / mL) (Gonelimali et al. 2018; Smith et al. 2008). The standardized bacterial suspension was streaked onto the MH agar using a sterile cotton swab by rotation and streaking technique, starting from the uppermost part and proceeding downwards while cautiously swabbing from one side to the other to the bottom of the plate. This process was repeated thrice after every rotation (60°) of the plate to ensure uniform distribution of the inoculum across the entire surface of the MHA plate (Jorgensen et al. 2009).

Susceptibility discs (6 mm) were impregnated with the test extracts (1 mg) and placed onto the surface of the MHA plates inoculated with the test organisms. The positive and negative controls were standard susceptibility gentamicin discs (Oxoid™, 10 µg) and DMSO (10 µL), respectively. The plates were incubated for 18−24 hours at 37 °C, and then the zones of inhibition (ZOI) were measured using a calibrated ruler (Jorgensen et al. 2009). Three replicates were performed.

Caenorhabditis elegans and its growth condition

Wildtype N2 strains of *C. elegans* were maintained in a glycerol stock solution and cultured on a nematode growth medium (NGM) (3 g of NaCl, 2.5 g of peptone, and 20 g of agar in 975 mL of H_2O) supplemented with 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M (pH 6.0) KPO_4 buffer. The food source of the worms, *E. coli* OP50, was cultured in a Tryptic Soy Broth (TSB) and then inoculated onto the NGM plates, which were subsequently incubated at 20 °C overnight. After 5 days of growth, the worms matured into hermaphrodite adults carrying eggs. They were then subjected to a synchronization process using M9 buffer (KH_2PO_4—3 g, Na_2HPO_4—6 g, NaCl—5 g, 1 M $MgSO_4$—1 mL, and distilled water—1,000 mL) and sodium hypochlorite/sodium hydroxide solution (Ali et al. 2023). Eggs were extracted from the NGM plates using 3 mL of M9 buffer and centrifuged at 1000 rpm for 3 minutes. The resulting supernatant was discarded, and a bleach solution (sodium hydroxide, 300 µL sodium hypochlorite, and 5 mL of distilled water) was then added to the tube. The bleach solution dissolved the outer layers of the adult worms, releasing the eggs. The solution was then centrifuged and washed with M9 buffer thrice, discarding the supernatant in each wash to ensure the remaining bleach was removed. The final pellet of worms was suspended in 3 mL of M9 buffer and was incubated at 25 °C. After 25.5 hours, the worms were centrifuged at 1,000 rpm for a minute and then transplanted on new NGM petri plates with 10 µL of *E. coli* OP50 lawn. L4 stage worms were then collected after two days.

Lifespan assay

Ten synchronized L4 stage worms were placed into each dish of a 24-well tissue culture plate with NGM seeded with 10 µL of *E. coli* OP50. The worms were transferred to the plate using a sterile 0.2 mm copper wire worm picker. Different concentrations of AU1 and AU2 extracts (50 µg, 100 µg, 150 µg, 200 µg), 10 µL each, were then added to the plates with worms. The plate was then incubated at 25 °C for 24 days (Ali et al. 2023; Moy et al. 2009). In this assay, *C. elegans* on *E. coli* OP50 lawn served as the control group, and (*C. elegans* + AU1) and (*C. elegans* + AU2) were the test groups. The number of living worms was monitored and counted daily, with deaths recorded if the worms did not respond to gentle prodding by the worm picker after five (5) seconds of observation. Meanwhile, worms that underwent vulval bursts because of internal hatching were deemed censored and excluded from the study (Ali et al. 2023). All observations were conducted using a dissecting microscope set at 10× magnification, with results displayed on a monitor. The mean lifespan (MLS) of the worms was calculated using equation 1:

$$MLS = \frac{1}{N}\sum_{j}\frac{X_j + X_{j+1}}{2}d_j$$

(Equation 1)

Where N represents the total number of worms and d_j represents the worms that have died in the age range (X_J *to* X_{J+1}). The standard error was also measured. Meanwhile, the survival increase percentage of the worms under the different treatments was obtained using equation 2 (Donato et al. 2017):

$$Survival\ Increase\ (\%) = \frac{MLS\ (Treatment) - MLS\ (Control)}{MLS\ (Control)} x\ 100\%$$

(Equation 2)

Locomotion assessment

L4 stage worms were transferred into a 35-mm NGM petri plate with 10 µL of *E. coli* OP50. Following the results of the lifespan assay, we selected the extracts that demonstrated the most significant extension of *C. elegans* lifespan for further testing. Specifically, 100 µg of AU1 and 150 µg of AU2 extracts (10 µL) were chosen based on their superior performance in the initial assay. The plates were incubated at 25 °C.

The movements of the worms were recorded throughout their lifespan every 24 hours for 21 days, following locomotion categories with modifications (Ali et al. 2023). 'Class A' is when worms move vigorously and spontaneously without prodding; 'Class B' is when the movement is sluggish and incoherent; 'Class C' is when the worm moves only its head and tail; 'Class D' is when there is little movement in response to prodding; and 'Class E' is when worms are found dead and do not respond to prodding. The motility of the worms was observed under the 10× magnification of a dissecting microscope with a monitor.

Brood size measurement

Two new L4 stage worms were transferred into a 35-mm NGM petri plate. This assay was performed using AU1 and AU2 extracts as the test groups and *E. coli* OP50 as the control-fed group. Ten microliters of AU1 (50 µg), AU2 (50 µg), and *E. coli* OP50 were independently seeded to each plate. Ten synchronized hermaphrodites in each setup were observed until they laid eggs to determine the total number of offspring induced by the different treatments. The assay was performed in five replicates. The brood size of the worms was counted under the 10× magnification of a dissecting microscope equipped with a monitor.

Chemotaxis assay

A 55-mm NGM petri plate was divided into three regions comprising the top part represented by area A, the middle part as the starting point with ten L4 stage worms, and the bottom part as area B (McDiarmid et al. 2018; Queirós et al. 2021). Ten microliters of the extracts (AU1 at 100 µg or AU2 at 150 µg) were seeded into the respective areas. This assay included (AU1 vs *E. coli* OP50), (AU2 vs *E. coli* OP50), and (AU1 vs AU2). The worms were incubated at 25 °C and observed after 40 minutes and 120 minutes using a dissecting microscope with a monitor (10× magnification). The chemotaxis index was determined using Equation 3 (Queirós et al. 2021).

$$Chemotactic\ Index\ (CI) = \frac{(A - B)}{(A + B)}$$

(Equation 3)

Where A represents 'Area A' and B 'Area B'.

The worms that did not move to any of the designated areas were classified as having no choice and were not included in the calculation of the chemotaxis index (Ali *et al.* 2023). A positive CI indicates attraction to the test extract, while a negative CI indicates repulsion (McDiarmid et al. 2018).

Survival assay with Gram-positive bacteria exposure
Ten L4 stage worms were transferred into a 55-mm Nutrient Agar (NA) petri plate using a sterile 0.2 mm copper wire worm picker. Gram-positive bacteria *Staphylococcus aureus* ATCC 29213 and *B. subtilis* were cultured into Mueller-Hinton (MH) Broth in a rotary shaker at 120 rpm and 37 °C overnight. Each bacterial culture, with an optical density (OD) of 1.0 at 600 nm, was inoculated into the NA plates to expose the worms to the bacteria following the protocol described by Angusamy et al. (2023). Six different set-ups were employed, consisting of the control group (unseeded NGM) and the test groups (*C. elegans* + 50 µg vancomycin; *C. elegans* + 50 µg vancomycin + 100 µg AU1; *C. elegans* + 50 µg vancomycin + 150 µg AU2; *C. elegans* + 100 µg AU1; and *C. elegans* + 150 µg AU1).

The percentage of worm survival was calculated using Equation 4.

$$Survival\ (\%) = \frac{Test}{Control}\ x\ 100\% \quad \text{(Equation 4)}$$

The survival assay was performed using three replicates per setup. The worms were observed for 72 hours at 6-hour intervals. The survival of the worms was monitored using the 10× magnification of a dissecting microscope with a monitor.

Statistical Analysis
The Kaplan-Meier method was used to analyze the longevity of the worms under the different treatments with the Mantel-Cox log-rank test and Gehan-Breslow-Wilcoxon test to compare the significant difference between the survival curves using GraphPad Prism 10.2.1. Meanwhile, the survival difference of the treatments was analyzed using the 'survival' package with Chi-square statistics analysis, wherein the survival curves were plotted using the 'survminer' package in the R Studio version 4.3.3 (Therneau, 2024). The differences in the brood sizes between the treatments were analyzed using the Mann-Whitney U test and Hodges-Lehmann difference between medians. The assessment for locomotion and the chemotactic index, presented in means with the standard error, were performed using R Studio version 4.3.3. Brown-Forsythe test was performed to check the homoscedasticity of the results of the survival percentage of the worms in the survival assay. Kruskal-Wallis test was used to find the statistical differences between the treatments of the worms under gram-positive bacteria exposure, and Dunn's multiple comparisons test was used as a post-hoc analysis. The statistical significance of each test was set at $p < 0.05$.

RESULT AND DISCUSSION

The Montañeza Waterfalls exhibit a pH range between 9.7 and 10.0, conducive to the proliferation of alkaliphilic bacteria (Atanasov et al. 2021). The falls are surrounded by large limestone boulders, which likely contribute to the alkaline nature of the water. Composed mainly of calcium carbonate ($CaCO_3$), limestone gradually dissolves as water flows over and through it, releasing calcium ions and bicarbonates that elevate the pH. The continuous interaction between the water and these mineral-rich boulders creates an environment suitable for diverse alkaliphilic microorganisms to thrive (Borkar 2015; Olsson et al. 2014). The water temperature ranges from 25 °C to 30 °C, optimal for their enzymatic functions to maintain sufficient metabolic activity (Almeida and Marana 2019). The electrical conductivity and total dissolved solids (TDS) of the waters were 474 µS/cm and 240 mg/L, respectively, suggesting a rich concentration of dissolved minerals and nutrients that alkaliphiles require for growth.

Phenotypic and biochemical characterization of isolates
AU1 and AU2 isolates exhibited robust growth across a pH range of 7.0 to 11.0 (Table 1). Optimal growth conditions were observed at pH 9.0 for AU1 and AU2. AU1 demonstrated resilience to salinity, tolerating a maximum of 5% NaCl, while AU2 could not tolerate salt. This tolerance suggests that these microorganisms have adapted to the high TDS and electrical conductivity present in their environment.

Table 1: Phenotypic and growth characteristics of AU1 and AU2 isolates from Montañeza Waterfalls

Characteristic	AU1	AU2
Cell morphology	Rod, single	Rod, single
Colony morphology	White, rhizoid, irregular	White, round, entire
Gram stain	+	+
pH range (optimum pH)	7.0−10.0 (9.0)	7.0−10.0 (9.0)
% NaCl tolerance	0−5	0
Catalase test	+	+
Starch hydrolysis test	+	+
Oxidase test	+	-

Gram staining revealed that AU1 and AU2 were Gram-positive (G+) rod-shaped single-celled bacteria, indicated by their deep, purple-colored cells when viewed under a compound microscope at 1000× magnification. The Gram-positive strains exhibited the same colony morphology, characterized by colored colonies with a round shape and irregular margins. These characteristics are consistent with the description of bacteria belonging to the genus *Bacillus* (Barka et al. 2015; LU et al. 2018; Tasaki et al. 2017; Turnbull 1996). Moreover, AU1 and AU2 exhibited catalase enzyme production and showed the ability to hydrolyze starch. Only AU1 could produce oxidase enzymes.

Phylogeny
The identity of AU1 and AU2 was determined based on 16S rRNA gene sequence analysis (Figure 1). Based on the results obtained from a BLAST analysis, the strains have been identified to belong to the genus *Bacillus*. AU1 and AU2 are related species under the same clade with 98% bootstrap value. However, *Bacillus velezensis* AU1 is more likely to be similar to *B. velezensis* strain FZB42 than *B. velezensis* AU2 with 77% bootstrap. This finding underscores the genetic similarity and evolutionary relationship of these strains with other members of the *Bacillus* genus.

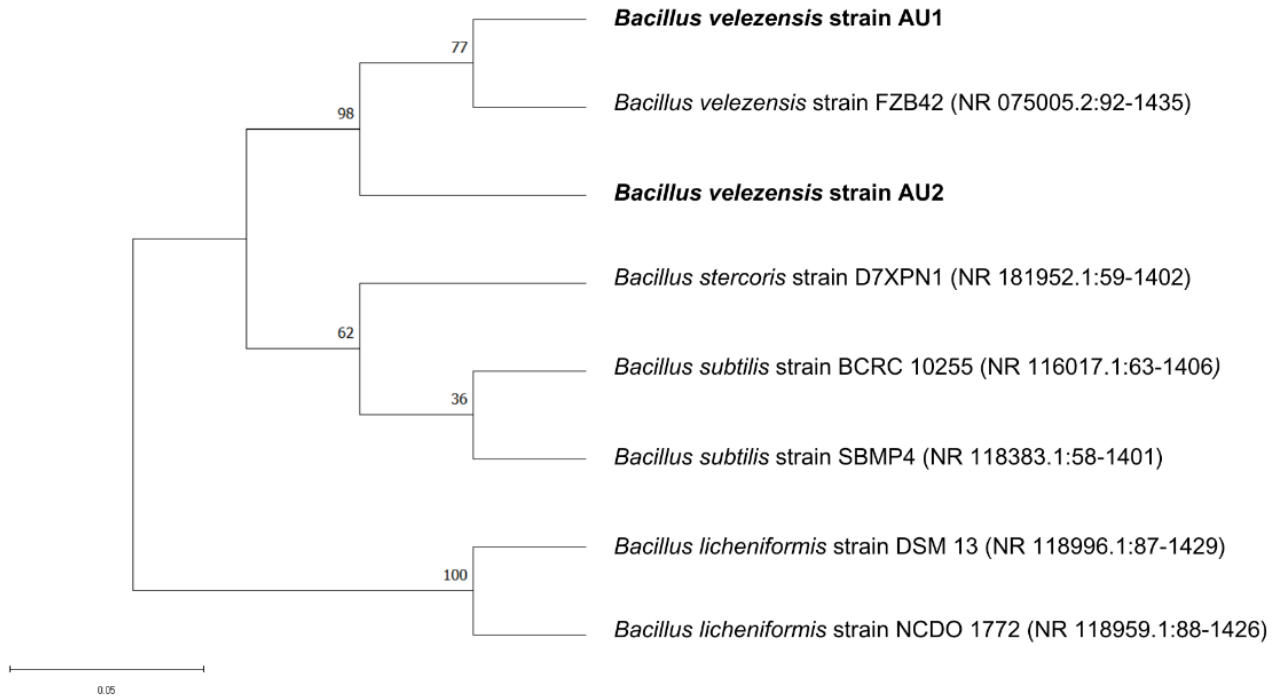

Figure 1: Maximum Likelihood tree showing the phylogenetic relationships of *Bacillus velezensis* strain AU1 and AU2 with closely related *Bacillus* species 16s rRNA gene sequences from the GenBank database. Percentage bootstrap values from 1000 resamplings are indicated at the nodes.

Bacillus velezensis strains, closely related to the isolates identified in the study, are well-known for their ability to adapt to extreme environmental conditions, such as high salinity and elevated pH (Ersoy Omeroglu et al. 2021), which aligns with the conditions observed in Montañeza Falls. For instance, *B. velezensis* strains (QH03-23 and JS39D) isolated from alkaline Lake Bogoria demonstrate optimal growth at temperatures between 30−35 °C and can tolerate salinity levels up to 0.5 M NaCl (Wekesa et al. 2022). Moreover, enzymes produced by *B. velezensis* exhibited robust activity under alkaline conditions and thermal stability, suggesting that the isolates from the waterfall likely produce bioactive molecules that remain functional under such conditions (Pavlović et al. 2024).

In recent years, *B. velezensis* has been recognized as a valuable source of biocontrol agents due to its ability to produce a wide array of secondary metabolites, including lipopeptides, polyketides, siderophores, bacteriocins, and lanthipeptides. These metabolites have been shown to suppress several phytopathogenic microbes (Rabee et al., 2023; Chen et al., 2007), suggesting that *B. velezensis* AU1 and *B. velezensis* AU2 isolated from Montañeza Falls may also produce bioactive compounds that could serve as biocontrol agents in agriculture.

Antimicrobial bioassay

AU1 and AU2 extracts exhibited notable bioactivity in both Gram-positive *B. subtilis* and Gram-negative *E. coli* (Table 2). AU1 and AU2 displayed a ZOI of 15.63 ± 2.21 mm and 12.40 ± 5.16 mm, respectively, against *B. subtilis*, with AU1 showing no significant difference when compared with gentamicin control (ZOI = 22.17 ± 2.49 mm) (two-way ANOVA, $p > 0.05$). The results suggest that the AU1 extract has strong antibacterial properties against *B. subtilis* comparable to those of the established antibiotic, making it a promising candidate for further investigation as a potential antimicrobial agent.

Meanwhile, AU1 and AU2 extracts exhibited inhibitory effects against the Gram-negative bacterium *E. coli*, with ZOIs of 14.10 ± 2.11 mm and 8.47 ± 0.87 mm, respectively. AU1 did not significantly differ with gentamicin control (ZOI = 20.17 ± 0.21 mm), indicating no significant difference in antimicrobial activity (two-way ANOVA, $p > 0.05$). While these inhibition zones indicate some antimicrobial activity, the overall potency of AU2 was significantly lower than that of the control antibiotic gentamicin (ZOI = 20.17 ± 0.21; $p > 0.05$). This suggests that while AU2 extracts can hinder bacterial growth, the efficacy is not statistically comparable to the well-established antibiotic.

Meanwhile, AU1 and AU2 extracts exhibited inhibitory effects against the Gram-negative bacterium *E. coli*, with inhibition zones of 14.10 ± 2.11 mm and 8.47 ± 0.87 mm, respectively. AU1 did not significantly differ with gentamicin control (ZOI = 20.17 ± 0.21 mm), indicating no significant difference in antimicrobial activity (two-way ANOVA, $p > 0.05$). While the inhibition zones of AU2 indicate some antimicrobial activity, the overall potency of AU2 was significantly lower than that of the control antibiotic gentamicin (two-way ANOVA; $p < 0.05$). This suggests that AU1 has strong antibacterial properties comparable to an established antibiotic, whereas AU2 inhibits bacterial growth but is significantly less effective than the established antibiotic.

Table 2: Zone of inhibition measurements of AU1 and AU2 extracts and controls.

Sample	Conc (µg)	Zone of Inhibition (mm)*	
		***B. subtilis* ATCC 6633**	***E. coli* ATCC 2592**
AU1	1000	$15.63 \pm 2.21^{a,b}$	$14.10 \pm 2.11^{a,c,d}$
AU2	1000	12.40 ± 5.16^{a}	8.47 ± 0.87^{c}
GEN**	10	22.17 ± 2.49^{b}	$20.17 \pm 0.21^{b,d}$
DMSO	-	0	0

*mean ± SD; n = 3; ** GEN – gentamicin 10 µg (Oxoid™), positive control; DMSO – dimethylsulfoxide, negative control, Different letters in a column ($^{a, b, c}$) indicate significant differences at $p < 0.05$.

Comparing the effect of different extracts against the two test organisms, extracts showed a stronger inhibitory effect against Gram-positive bacteria than against Gram-negative bacteria. However, there is no significant difference in their antimicrobial activity, as determined by the two-way ANOVA ($F(3,1) = 3.889$, $p > 0.05$). The double membrane structure in Gram-negative bacteria presents a formidable barrier to the penetration of antibiotics, making infections more challenging to treat (Kurnianto et al. 2020). AU1 and AU2 extracts show significant promise, demonstrating the ability to inhibit both types of pathogens. This characteristic highlights its potential as a valuable antimicrobial agent, warranting further research to isolate and characterize the bioactive components responsible for the activity.

Survival and longevity of *C. elegans*

Varying AU1 and AU2 concentrations showed distinct effects on the worms' lifespan compared to the control. Worms in the control setup had a mean lifespan of approximately 14 days (Figure 2). AU1 extracts at concentrations of 100 µg and 150 µg exhibited mean lifespans higher than the control, reaching 19.67 days (42% survival percentage increase) and 15.17 days (10% survival percentage increase) (Figure 2A). Meanwhile, extremely low (50 µg) and extremely high concentrations (200 µg) exhibited a lifespan shorter than the control, lasting 13 days and 10 days, respectively. AU2 extract (150 µg concentration) exhibited a mean lifespan higher than the control, reaching 14.33 days (2% increase) (Figure 2B). Meanwhile, other concentrations (50 µg, 100 µg, 200 µg) showed a shorter mean lifespan of the worms compared to the control.

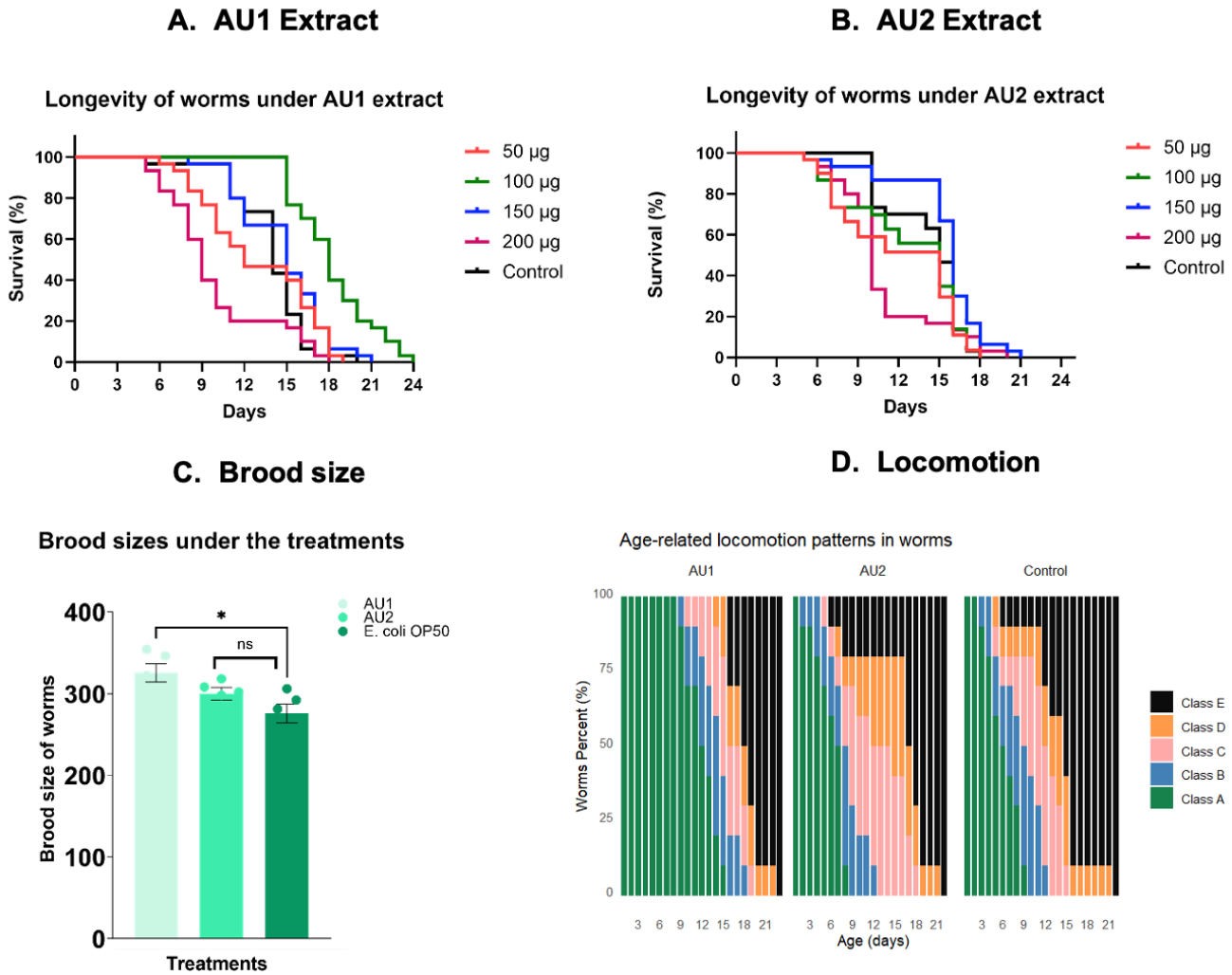

Figure 2: (A) Lifespan of worms across the different concentrations of AU1 extract. (B) Lifespan of worms across the different concentrations of AU2 extract. (C) Comparison of brood sizes of worms (n = 10). Asterisk (*, $p < 0.05$) indicates a statistically significant difference from the control-fed group; ns = not significantly different. (D) Comparison of worm locomotion under AU1 (100 µg) and AU2 (150 µg) across their lifespan (21 days).

The aging process has implications for the reproduction and locomotor functions of worms. As worms age, energy allocation prioritizes maintenance over these functions, typically leading to reduced brood size and movement (Jenkins et al. 2004; Qian and

Chia 1992). Worms exposed to AU1 extract produced an average brood size of 325.4 eggs (Figure 2C), a significant increase from the control group (275.6 eggs) (Mann Whitney test, $p < 0.05$). Meanwhile, worms exposed to AU2 extract had a mean brood size of 299.6 eggs, which did not significantly differ from the control group (Mann Whitney test, $p > 0.05$). The results suggest that AU1 and AU2 extracts may contain bioactive components that serve as additional nutrients for *C. elegans*, enhancing their overall health, fitness, and reproductive capacity. Improved nutrition can boost fertility, resulting in more eggs and larger broods (Ali et al. 2023). An earlier study revealed that *C. elegans* exposed to ethanol extracts of *Citri Reticulatae* Semen (CRSE), a wildly used traditional Chinese medicine, promoted the lifespan and reproductive output of the nematodes by enhancing autophagy—a process critical for cellular maintenance and energy efficiency (Long et al. 2022). The CRSE extracts likely provided the essential nutrients, such as sterol in Drosophila (Zanco et al., 2021), potentially supporting increased metabolic efficiency for yolk production and higher offspring yield (Long et al. 2022).

Worms experienced a decline in motor activity as they got older compared to their early stages (Figure 2D). This is evident in the congruent decrease of all movement classes (A-D) across control and test groups. The locomotion scores of the worms that were fed with AU1 (100 µg) were consistently higher than the worms fed with AU2 (150 µg) or the control. One hundred (100%) worms under AU1 were still categorized as Class A, exhibiting spontaneous or vigorous movement on the 8th day of observation, higher than the control (25%) and AU2 (10%). Worm deaths, categorized as Class E, started to appear on the 6th day in the control and AU2. Meanwhile, worms under AU1 did not experience any deaths until after the 16th day of observation.

The findings suggest that the extracts AU1 and AU2 may have anti-aging benefits and extend the lifespan of *C. elegans* by modulating key biological pathways. The extracts might activate the insulin/insulin-like growth factor signaling (IIS) pathway, which plays a crucial role in regulating aging and stress responses in *C. elegans*, similar to its role in humans. A key component of this pathway is the DAF-16 transcription factor, which is crucial for promoting longevity. The extracts may boost the expression and activity of the *daf-16* gene so it can withstand oxidative stress and other environmental factors, thereby slowing down the aging of worms (Okoro et al. 2021). However, the interaction between AU1 or AU2 extracts with DAF-16 remains to be investigated. Understanding this interaction will be vital to unravel the mechanism through which the extracts confer their potential anti-aging benefits.

Chemotaxis index

The chemotactic response of worms was evaluated by observing their movement towards the treatments (AU1 vs *E. coli* OP50), (AU2 vs *E. coli* OP50), and (AU1 vs AU2) after 40 minutes and 120 minutes. More than 80% of the worms across all the setups showed no choice during the first 40 minutes of observation (Figure 3). After 120 minutes, 67% of the worms preferred AU1 (Figure 3A), and 60% favored AU2 over the food source (Figure 3B). However, worms showed a stronger preference for AU1 compared to AU2 (CI = 0.81) (Figure 3C). These results indicated that the extracts may contain compounds or nutrients that are more appealing or beneficial to the worms, thereby enhancing their movement towards the extracts over *E. coli* OP50 (Luo et al. 2018; Mori and Ohshima 1997). This enhanced attraction could indicate the extracts' potential health-promoting effects, which warrant further investigation into their specific components and mechanisms of action.

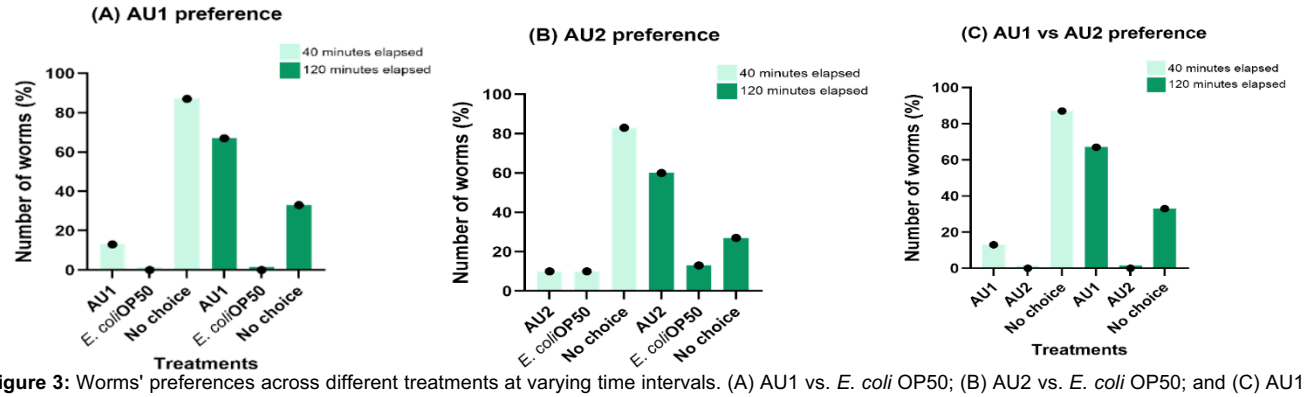

Figure 3: Worms' preferences across different treatments at varying time intervals. (A) AU1 vs. *E. coli* OP50; (B) AU2 vs. *E. coli* OP50; and (C) AU1 vs. AU2

Anti-infective screening

The potential protective effects of AU1 and AU2 extracts against bacterial infections were evaluated by exposing the worms to Gram-positive bacteria, *S. aureus* and *B. subtilis* (Figure 4). Treated worms have a significantly higher survival percentage, more than 50% survival increase, compared to non-treated worms (Kruskal-Wallis test, $p < 0.05$). AU1 extract displayed significant protective effects, maintaining 100% worm survival until the 30th hour and over 60% survival for the entire duration of the experiment. When combined with vancomycin, worm survival increased by approximately 90% at the 72nd hour of observation. Similarly, combining AU2 with vancomycin resulted in a 50% increase in worm survival at the same time point. On the other hand, worms in the control group (without extracts) exhibited a decreased survival rate, plummeting to less than 20% survival by the 72nd hour. These findings suggest that both extracts, particularly when used in conjunction with vancomycin, significantly improve the worms' resilience to bacterial infections over an extended period (Irazoqui and Ausubel, 2010; Sifri et al. 2005), opening an avenue for potential therapeutic strategies.

Conversely, the presence of *B. subtilis* did not result in worm mortality but promoted longevity through biofilm formation. The worms had 100% survival across all setups (Figure 4B), which is consistent with an earlier study by Donato et al. (2017). Biofilm-proficient *B. subtilis* strains were observed to colonize the gut of the worms, resulting in an extended lifespan. This longevity-promoting effect was attributed to two molecules produced by *B. subtilis,* which are the quorum-sensing pentapeptide Competence Stimulating Factor (CSF) and nitric oxide (NO) (Donato et al. 2017). The *B. subtilis* quorum-sensing

pentapeptide CSF has been previously shown to contribute to intestinal homeostasis. It achieves this by activating key survival pathways in the host, such as the p38 MAP kinase and protein kinase B, and by inducing the production of cytoprotective heat shock proteins (HSPs) (Fujiya et al. 2007; Williams 2007).

Taken together, while both AU1 and AU2 confer significant health benefits to *C. elegans*, AU1 consistently outperforms AU2 and the control in various key areas, making it more effective for enhancing the worms' overall health and longevity.

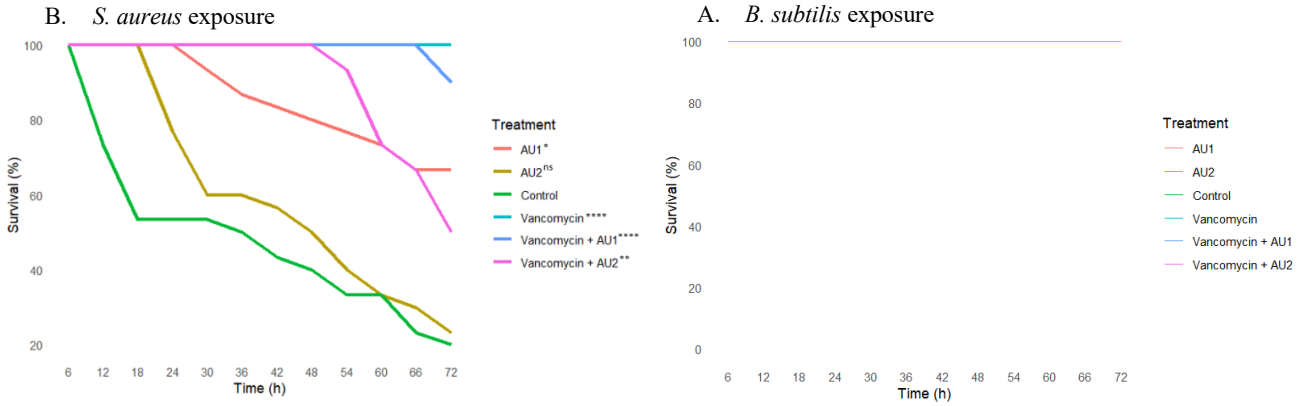

Figure 4: (A) Survival of worms exposed to *S. aureus* under the different treatments (n = 10). Asterisk (*, p < 0.05); **, p < 0.01; ****) indicate statistically significant differences from the control group; ns = not significantly different. (B) Survival of worms exposed to *B. subtilis* under the different treatments (n = 10).

CONCLUSION

This study highlights the Montañeza Waterfalls as a promising hotspot for the discovery of antibacterial agents from alkaliphilic isolates. *Bacillus velezensis* strains AU1 and AU2 exhibited inhibitory effects against both Gram-positive and Gram-negative pathogens. AU1 and AU2 also displayed a significant lifespan-enhancing potential and higher mean brood size compared to the control group, indicating enhanced vitality and reproductive health. When exposed to *S. aureus*, which induced mortality, both AU1 and AU2 rescued the worms from the infection's lethal effects. This highlights the protective properties of AU1 and AU2 against bacterial infections, with AU1 showing superior overall benefits in terms of lifespan, reproduction, locomotion, and infection resistance. Further investigation is warranted to isolate and characterize bioactive compounds from crude extracts as potential sources for new or novel antimicrobial drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceptualization–FM; Methodology and investigation – ASBU, LMA, NGG, FM, MIRP; Data analysis – ASBU, LMA, NGG, FM; Writing Original Draft – ASBU, LMA, NGG, FM; Review and Editing – ASBU, LMA, NGG, FM, MIRP; Supervision – FM; Project administration – FM Funding acquisition – FM

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SUPPLEMENTARY INFORMATION

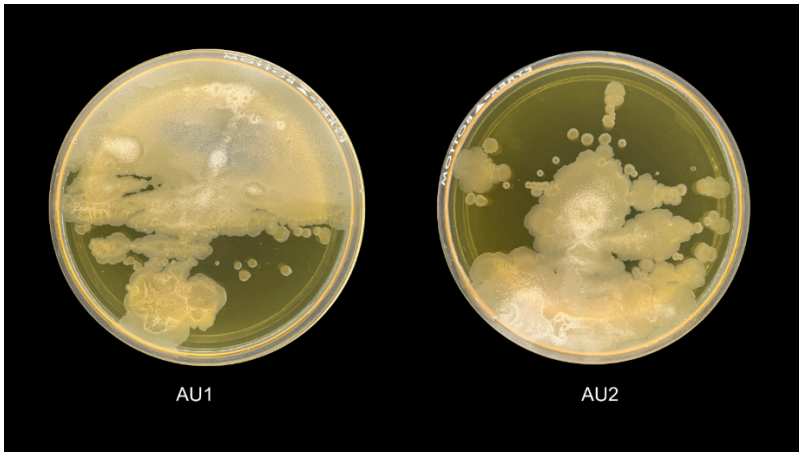

Figure S1: *Bacillus velezensis* AU1 and AU2bacterial strains isolated from Montañeza Waterfalls

Table S1: Zone of inhibition of AU1 and AU2 extracts against *B. subtilis* ATCC 6633

Extract	*B. subtilis* ATCC 6633				
	Zone of inhibition (mm)				
	Replicate 1	Replicate 2	Replicate 3	Mean	SD
AU1	13.20	16.20	17.50	15.63	2.21
AU2	8.7	10.20	18.30	12.40	5.16
Genta*	20.3	21.2	25.00	22.17	2.49
DMSO	0	0	0	0	0

*Genta – Gentamicin (Oxoid™; 10 µg); SD – standard deviation

Table S2: Zone of inhibition of AU1 and AU2 extracts against *E. coli* ATCC 2592

Extract	*E. coli* ATCC 2592				
	Zone of inhibition (mm)				
	Replicate 1	Replicate 2	Replicate 3	Mean	SD
AU1	16.30	12.10	13.90	14.10	2.11
AU2	9.20	7.50	8.70	8.47	0.87
Genta*	20.00	20.40	20.10	20.17	0.21
DMSO	0	0	0	0	0

*Genta – Gentamicin (Oxoid™; 10 µg); SD – standard deviation

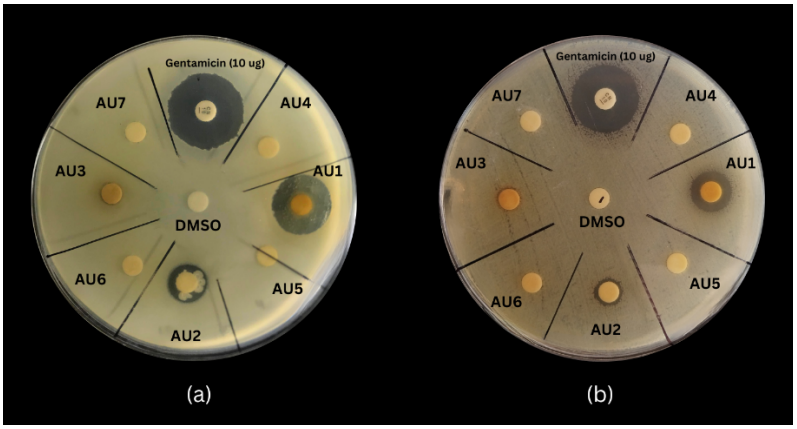

Figure S2: Representative of Antimicrobial disc diffusion test of AU1-AU7 extracts against (a) *B. subtilis* ATCC 6633 (b) *E. coli* ATCC 2592.