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Antimicrobial activity of *Bacillus* species isolated from Yemeni soils against some human pathogens

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Abstract

Bacillus species represent one of the most dominant soil bacteria and are capable of producing diverse metabolites with antimicrobial properties. This study aimed to isolate *Bacillus* spp. from Yemeni soils and evaluate their antimicrobial activity. Thirty soil samples were collected, and the isolates were characterized using phenotypic and biochemical tests. Their antimicrobial potential was assessed against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sp., *Salmonella* sp., and *Candida albicans* using agar disc and well diffusion methods. In agar disc assays, the strongest inhibitory effects were observed against *Salmonella* sp. (13–32.5 mm), followed by *E. coli* (13–25.4 mm), *C. albicans* (10–18.5 mm), and *S. aureus* (10–15.5 mm). Five isolates exhibited antibacterial activity against *Salmonella* sp. in well diffusion assay. These results highlight the significant antimicrobial potential of *Bacillus* isolates derived from Yemeni soils. Further studies are recommended to identify bioactive metabolites and explore their suitability for the development of novel therapeutic agents.

Article Info

Keywords:
Soil , *Bacillus* spp., Antimicrobial activity, Human pathogens.

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1. INTRODUCTION

The prevalence of infections has significantly increased over the past 30 years, and this pattern is anticipated to persist due to the ongoing evolution of certain microorganisms towards multidrug resistance [1]. *S. aureus* is a highly virulent pathogen responsible for a wide range of conditions, including food-borne illnesses, food poisoning, skin and soft tissue infections, abscesses, mastitis, and bacteremia [2]. *Salmonella* spp. remain a major cause of acute diarrheal illness worldwide, with clinical manifestations ranging from mild gastroenteritis to severe enteric fever [3]. The *Pseudomonas* genus includes important plant pathogenic agents of food spoilage and opportunistic pathogens in animals and humans [4, 5]. Certain strains of *E. coli* can cause diverse human diseases, ranging from intestinal to extraintestinal infections [6]. *C. albicans* is the major causative agent of superficial and invasive candidiasis, particularly among immunocompromised individuals globally [7]. The management of infectious diseases attributable to pathogenic bacterial and fungal strains has historically represented one of the most enduring challenges in the clinical field [8–10]. This has prompted researchers to develop innovative and more efficacious inhibitory agents [11, 12] to combat these challenges. To achieve this objective, the initial phase of the identification of novel antibiotic agents involves screening bacterial and fungal strains capable of producing inhibitory compounds [13]. *Bacillus*, recognized as one of the most prevalent bacterial strains in soil, represents a significant microbial group for the biological control of pathogens. *Bacillus* species, which produce a diverse array of metabolites exhibiting antimicrobial properties, have found extensive applications in both medicine and the pharmaceutical industry. They possess the ability to inhibit the growth and function of various cellular organisms, including bacteria, fungi, insects, nematodes, and acellular entities such as viruses [14–17]. Diverse strains of *Bacillus* have been identified to produce certain antibiotics, such *B. polymyxa*, *B. subtilis*, *B. lichenformis*, *B. pumulus*, *B. brevis*. The antibi-

otics produced include gramicidin, polymyxin, fengycin, iturin, subtilin, surfactin, macrolactin, lichenysin and difficidin [18, 19].

Yemen is generally characterized by extremely diverse soil, physiographies, topography, and climate. Soil ranges from arid plains to fertile highlands. This environmental variability contributes to a wide range of soil physicochemical properties that may influence microbial diversity and metabolic potential [20, 21].

Investigating *Bacillus* species not only enhances our understanding of regional microbial biodiversity but also contributes to the development of sustainable land management strategies suited to Yemen's unique environmental conditions. Although numerous studies worldwide have reported the antimicrobial potential of *Bacillus* species isolated from soil, particularly against plant pathogens, there is still a notable research gap in Yemen. To the best of our knowledge, no studies have been conducted in Yemen to evaluate the antimicrobial activity of *Bacillus* spp. against human pathogenic microorganisms. This gap underscores the importance of further investigations to explore the antimicrobial potential of *Bacillus* spp. against clinically relevant human pathogens, thereby supporting their possible applications in the medical and pharmaceutical fields within Yemen.

2. MATERIALS AND METHODS

2.1. Collection of soil samples

Thirty soil samples were obtained from eight locations in Sana'a City, Yemen (Al-Rawda, Saref, Heziaz, Wadi Dhahr, Thahban, Shumylah, Al-sabeen, and khawlan). Soil samples were collected from 2 to 5 cm below the surface with a sterile spatula in sterile containers. The samples were labeled and immediately transported to the laboratory and stored at room temperature until microbiological examination.

2.2. Isolation and identification of *Bacillus* spp.

2.2.1. Isolation of *Bacillus* spp. from soil samples

A soil suspension was prepared by dissolving 1 g of soil in 9 mL of sterile distilled water and agitated vigorously for 2 min. Soil samples were heated in a water bath for 30 min. The suspension was then serially diluted in sterile distilled water from 10^{-1} to 10^{6}. After that, 0.1 ml of soil suspension was spread over nutrient agar plates. The inoculated plates were incubated at 37℃ for 24-48 h. The plates were examined after the incubation period for rough and abundant colonies with waxy growth and irregular spreading edge [22, 23]. *Bacillus* isolates were purified by streaking onto nutrient agar (NA) plates.

2.2.2. Identification of *Bacillus* spp.

Identification of *Bacillus* spp. based on phenotypic characteristics and biochemical tests. The isolated *Bacillus* species were identified according to Bergey's Manual of Systematic Bacteriology (1984) [24].

2.3. Biosafety and ethical considerations

B. cereus is a potentially pathogenic bacterium capable of producing emetic or diarrheal toxins, that can cause foodborne illnesses and opportunistic infections in clinical settings. Therefore, it is classified as a Risk Group 2 (RG-2) organism and must be handled under Biosafety Level 2 (BSL-2) laboratory conditions to ensure safe containment and minimize exposure risk [25].

2.4. Antimicrobial activity of *Bacillus* spp. against pathogenic microorganisms

i. Test microorganisms

S. aureus, E. coli, Pseudomonas sp., and *C. albicans* were collected from Military Hospital, Sana'a- Yemen. *Salmonella* sp. was collected from the Microbiology Laboratory of the Faculty of Science, Sana'a University. All *Bacillus* isolates were tested for their antimicrobial activity against various human pathogenic microorganisms using two methods:

ii.Agar Disc Diffusion Method (ADD)

Pathogenic bacteria were streaked on NA and incubated at 37℃ for 24-48 h. *C. albicans* was streaked on Sabouraud Dextrose agar (SDA) and incubated at 28℃ for 48 h. NA plates inoculated with each *Bacillus* isolate were cut using a cork borer (6 mm). and transferred to the surface of plates seeded with pathogenic microorganisms under aseptic conditions. These plates were kept for 1 h in a refrigerator and then incubated at 37℃ for bacteria and 28℃ for *C. albicans*. The experiments were conducted in triplicates. The antimicrobial activity of *Bacillus* was recorded in terms of the inhibition zone of pathogenic microorganism's growth around the agar disc [26].

iii. Agar Well Diffusion Method (AWD)

NA and SDA (20 ml) were poured into sterile Petri dishes. Suspension (100 μm) of cultured target microorganisms were spread on the plates, and wells of 6 mm diameter were punched in the agar with a sterile cork borer. All *Bacillus* isolates were cultured in Nutrient Broth (NB) and incubated at 37℃ for 24-48 h. *Bacillus* cultures were centrifuged at 6000 g for 15 min to remove cell debris. After centrifugation, each sample (100 μl) was added to the wells of agar plates inoculated with the target microorganisms. The dishes were placed in a refrigerator for 1hour to facilitate diffusion and incubated for 24 h at 37℃ for bacteria and 28℃ for *C. albicans* [27]. There were

3 replicates for each treatment. The antimicrobial potential of *Bacillus* was assessed based on the diameter of the clear inhibition zones surrounding the agar discs.

2.5. Statistical analyses

The antagonistic activity of *Bacillus* isolates against pathogenic microorganisms was determined in triplicate and subjected to statistical analysis; the data are presented as the mean of triplicates ± standard deviation by IBM SPSS Statistics, version 27. Homogeneity of variances was evaluated using Tukey's test and one-way ANOVAs, and differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *Bacillus* spp. isolated from soil

Among the 30 soil samples, 15 isolates of *Bacillus* were isolated, identified, and classified into four species: six (40%) *B. cereus*, five (33.33%) *B. azotoformans*, three (20%) *B. subtilis* and one (6.67%) *B. globisporus.* Phenotypic characterization showed that the colony on nutrient agar medium formed circular or irregular edges, rough, opaque, fuzzy white or cream- colored colonies, gram-positive, and spore-forming bacteria (Figure 1). The biochemical characterization of *Bacillus* spp. is presented in (Table, 1).

In a similar study conducted by Amin et al. [28], only 30 strains of *Bacillus* spp. were isolated from 50 soil samples. These bacteria were classified into four species including *B. cereus* (86.6%), *B. subtilis* (6.6%), *B. thuringiensis* (3.3%), and *B. pumilus* (3.3%). Dangol *et al.* [29] isolated 41 isolates from the soil and screened them for antibiotic production. Among the 13 isolates that demonstrated antibiotic-producing capabilities, two main frequency groups were observed. Isolates occurring at a frequency of 7.69% included *B. licheniformis*, *B. thuringiensis*, *B. subtilis*, *B. brevis*, *B. alvei*, *B. megaterium*, and *B. coagulans*. In contrast, the isolates detected at a higher frequency (15.81%) included *B. laterosporus*, *B. firmus*, and *B. larvae*. These findings indicate that multiple *Bacillus* species contribute to antimicrobial activity, with certain species appearing more prevalent among antibiotic-producing isolates. Koilybayeva et al. [30] isolated and identified 19 bacterial strains using biochemical and phylogenetic analyses. Phylogenetic analysis revealed that three gram-positive bacterial isolates, BSS11, BSS17, and BSS19, showed 99% nucleotide sequence similarity with *B. subtilis* O-3, *B. subtilis* Md1-42, and *B. subtilis* Khozestan 2. In a study by Wafula et al. [31], phylogenetic analysis of isolates D61, D19, and, D51, suggested that they were closely related to *B. cereus* with 89-96% rDNA sequence similarity, whereas isolates D5, D16, S31, D70, and D2 were closely related to *B. thuringiensis* with 83-96% rDNA sequence similarity. Isolate S30 was closely related to *B. subtilis* with 99% rDNA sequence similarity, and isolate D29 was closely related to *B. mycoides* with 97% rDNA sequence similarity. Hellany et al. [32] isolated seven *Bacillus* isolates, and based on the MALDI-TOF results, BC01, BC02, BC06, and BC07 were identified as *B. subtilis*, while the isolates BC03, BC04, and BC05 were classified as *B. cereus*. Risanti et al. [33] isolated and characterized *Bacillus* species from the rhizospheres of vegetable crops. Thirteen isolates were isolated, and four *Bacillus* isolates were identified through morphological, biochemical, and 16S rRNA gene analyses as *B. safensis*, *B. altitudinis*, and *Bacillus* sp.

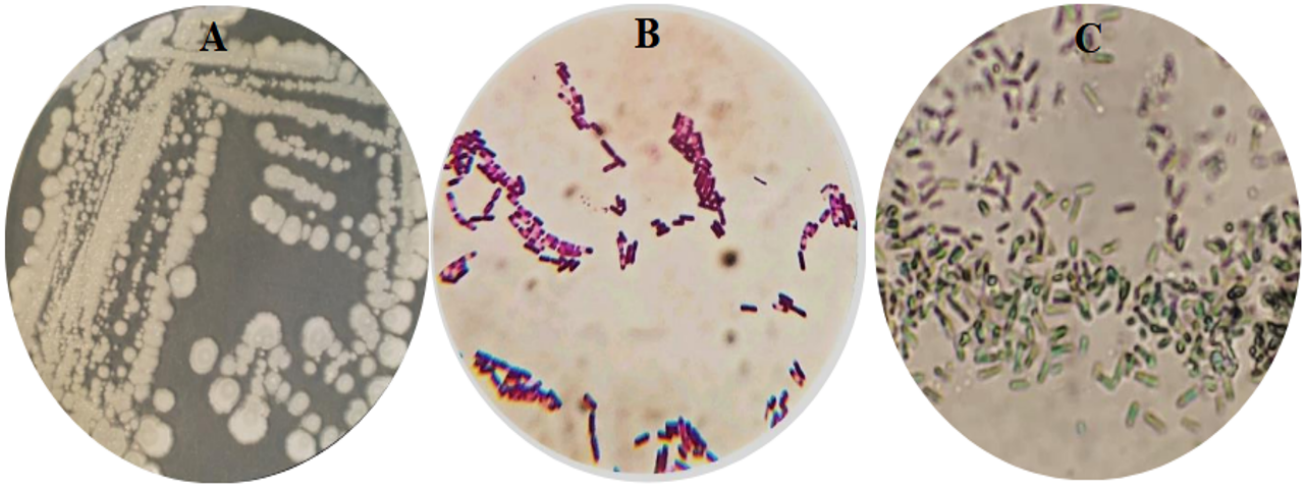

Figure 1. Phenotypic characterization of *Bacillus* spp. a. *Bacillus* sp. growth on NA b. Gram stain (100× magnification), c. Malachite green staining shows endospore.

Table 1. Biochemical characterization of *Bacillus* spp.

NO.	*Bacillus* spp.	Catalase	Oxidase	Citrate test	Indole test	L/G fermentation	Urease	Endospore Formation
B1	*B. subtilis*	+	+	-	-	A/A	+	+
B2	*B. subtilis*	+	+	-	-	A/A	+	+
B3	*B. azotoformans*	-	+	+	-	K/A	-	+
B4	*B. subtilis*	+	+	-	-	A/A	+	+
B5	*B. cereus*	+	-	+	-	K/A	-	+
B6	*B. azotoformans*	-	+	+	-	K/A	-	+
B7	*B. cereus*	+	-	+	-	K/A	-	+
B8	*B. cereus*	+	+	-	-	A/A	+	+
B9	*B. cereus*	+	-	+	-	K/A	-	+
B10	*B. cereus*	+	-	+	-	K/A	-	+
B11	*B. azotoformans*	-	+	+	-	K/A	-	+
B12	*B. globisporus*	+	+	-	-	A/A	+	+
B13	*B. cereus*	+	-	+	-	K/A	-	+
B14	*B. azotoformans*	-	+	+	-	K/A	-	+
B15	*B. azotoformans*	-	+	+	-	K/A	-	+

A/A: Acid/Acid, **K/A:** Alkaline/Acid

3.2. Antimicrobial activity of *Bacillus* spp. against some pathogenic microorganisms

3.2.1. Agar Disc Diffusion Method

The antagonistic test showed that *Bacillus* spp. had varying degrees of inhibitory activity against the test microorganisms (Table, 2). Out of the 15 isolates, nine (60%) showed activity against *Salmonella* sp. In contrast, eight isolates of *Bacillus* (53.33%) showed inhibitory activity against *S. aureus*, six isolates (40%) showed inhibitory activity against *E. coli*, and four isolates showed activity against *C. albicans* (26.66%). No efficacy of *Bacillus* isolates against *Pseudomonas* sp. has been demonstrated. In a similar study by Amin et al. [28], two strains of *B. cereus* showed antimicrobial activity against *S. aureus*, *E. coli*, *Salmonella typhi*, *Corynebacterium diphtheriae* and *Shigella dysenteriae*. The *Bacillus* isolates exhibited variable antimicrobial activity, with the highest inhibition observed against *Salmonella* sp., ranging from 13 ± 0.30 mm to 32.5 ± 0.17 mm. Inhibition zones against *E. coli* ranged from 13 ± 1.00 mm to 24.5 ± 1.00 mm, while activity against *S. aureus* varied between 10 ± 0.20 mm and 15.5 ± 1.50 mm. The isolates also demonstrated inhibitory effects on *C. albicans*, with inhibition zones ranging from 10 ± 1.50 mm to 18.5 ± 1.00 mm. In our study the antagonistic activity of *Bacillus* spp. was subjected to a one-way ANOVA, which confirmed a highly significant difference in susceptibility among the tested pathogenic microorganisms ($p < 0.001$). This is broadly consistent with the work of Palacios-Rodríguez et al. [34], who reported a *B. amyloliquefaciens* BS4 strain that inhibited *E. coli* with a zone of 20.50 ± 0.70 mm (culture) and 19.67 ± 0.58 mm (supernatant). In contrast, Gharieb et al. [35] demonstrated that *B. subtilis* exhibited an inhibitory efficacy ranging from 33.5 to 44.4% against 15 *Candida* isolates.

The variability in antimicrobial activity among *Bacillus* isolates is likely driven by strain-specific genetic differences, particularly in the presence and regulation of biosynthetic gene clusters responsible for secondary metabolite production [36]. The *Bacillus* genus is highly diverse, with more than 169 reported antibiotic secondary metabolites, and *B. subtilis* alone produces approximately 68 of them [37]. Consequently, the antimicrobial performance of each isolate depends largely on its specific metabolites. Differences in the type and composition of bioactive compounds, such as bacitracin, bacilysin, and subtilin, and their associated mechanisms of action further explain the heterogeneity observed across species and strains [38, 39].

3.2.2. Agar well Diffusion method

In general, *Bacillus* spp. did not show antimicrobial activity against most of the tested microorganisms, except *Salmonella* sp. Notably, five isolates, three identified as *B. subtilis* (B1, B2, and B8) and two identified as *B. cereus* (B5 and B13), demonstrated measurable inhibitory effects against *Salmonella* sp. Among these, isolate B8 displayed the strongest activity, producing an inhibition zone of 41 mm (Table 3 and Figure 2). These results agree with those of Yilmaz et al. [40], who examined the antimicrobial activity of 29 *Bacillus* spp. strains against the tested bacteria.

Some isolates (five *Bacillus* isolates) showed antimicrobial activity against (*S. aureus* ATCC 25923, *P. fluorescens* RSKK 240, *Micrococcus flavus*, *B. megaterium*

Table 2. Antimicrobial activity of *Bacillus* spp. against pathogenic microorganisms using ADD

No.	*Bacillus* spp.	*Salmonella* sp.	*S. aureus*	*Pseudomonas* sp.	*E. coli*	*C. albicans*
Inhibition zone (mm.)						
1	***B. subtilis***	18 ± 0.17	-	-	24.5 ± 1.00	-
2	***B. subtilis***	13 ± 0.50	-	-	13 ± 1.00	-
3	***B. azotoformans***	-	15.5 ± 1.50	-	-	-
4	***B. subtilis***	-	11 ± 0.31	-	-	16 ± 1.50
5	***B. cereus***	-	10 ± 0.20	-	15 ± 1.00	-
6	***B. azotoformans***	-	14 ± 0.50	-	-	18.5 ± 1.00
7	***B. cereus***	-	-	-	18 ± 0.29	13 ± 0.20
8	***B. cereus***	14 ± 2.00	13 ± 0.35	-	-	10 ± 1.50
9	***B. cereus***	19.5 ± 1.00	-	-	18 ± 2.00	-
10	***B. cereus***	21 ± 0.20	-	-	-	-
11	***B. azotoformans***	32.5 ± 0.17	11 ± 0.35	-	-	-
12	***B. globisporus***	20 ± 1.50	-	-	-	-
13	***B. cereus***	13 ± 0.30	10 ± 0.30	-	-	-
14	***B. azotoformans***	-	-	-	-	-
15	***B. azotoformans***	15 ± 2.00	13 ± 0.30	-	14 ± 0.30	-
p value < 0.001						

Each value represents mean (n=3) ± standard deviation.

RSKK 578, *B. thuringiensis* RSKK 380, *B. cereus* F2) by the agar well diffusion method, but other isolates did not show antimicrobial activity. Abdulkadir and Waliyu [41] isolated *B. lentus* and *B. alvei* from the soil that showed antibacterial activity against *S. aureus* whereas *B. pumilus* showed only a slight zone of inhibition of *Proteus* spp.

The antibacterial efficacy of *Bacillus* isolates against *Salmonella* spp. is attributed to their ability to synthesize various antimicrobial agents. These agents can inhibit the growth of *Salmonella* sp. and even kill bacteria [42]. None of the *Bacillus* isolates demonstrated antibacterial efficacy against *Pseudomonas* sp. This could be because *Pseudomonas* sp. has been shown to possess a high level of inherent resistance to a majority of antibiotics, which is facilitated by mechanisms such as diminished outer membrane permeability, efflux systems that extrude antibiotics from the cytoplasm, and the production of antibiotics. Antibiotic-inactivating enzymes, including β-lactamases, and the formation of biofilms act as diffusion barriers that restrict the access of secondary metabolites to bacterial cells [43, 44].

In our study, the antimicrobial activity observed in the liquid media was notably lower than that obtained using the agar disc diffusion method. This indicates that antimicrobial agents may exhibit enhanced efficacy in inhibiting microbial growth when concentrated within the agar disc rather than when dispersed in liquid medium. Several factors related to diffusion dynamics and the solubility profile of the agent may account for this phenomenon. In liquid media, the antimicrobial agent is uniformly distributed throughout the medium, resulting in a reduced effective concentration compared with the localized area adjacent to the disc in the agar method. This lower concentration may be inadequate to effectively inhibit microbial growth, even if the agent is effective at higher concentrations [45].

Although *Bacillus* isolates from Yemeni soils exhibited promising antimicrobial activity, this study was limited by its reliance on phenotypic and biochemical characterization without genomic analysis of biosynthetic gene clusters. Additionally, specific antimicrobial metabolites were not chemically identified, leaving their bioactive components and mechanisms of action unknown. Future studies integrating genomic and metabolomic analyses are needed to elucidate the antimicrobial potential and practical applications of these isolates.

4. CONCLUSION

This study revealed that isolated *Bacillus* isolates from Yemeni soils exhibit regional antimicrobial significance in Yemen's unique soil ecosystems as promising reservoirs for discovering new antimicrobial agents. Future research involving genomic and metabolomic characterization is essential to identify the specific metabolites responsible for this activity and to assess their potential for pharmaceutical or biotechnological applications.

Author contributions

N.A.A.M. wrote the main manuscript text. All authors contributed to the study conception and design.

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Competing interests

Table 3. Antimicrobial activity of *Bacillus* species against pathogenic microorganisms by AWD.

No.	*Bacillus* spp.	*Salmonella* sp.	*S. aureus*	*Pseudomonas* sp.	*E. coli*	*C. albicans*
			Inhibition zone (mm.)			
1	***B. subtilis***	10 ± 1.00	-	-	-	-
2	***B. subtilis***	11 ± 0.50	-	-	-	-
3	***B. azotoformans***	-	-	-	-	-
4	***B. subtilis***	-	-	-	-	-
5	***B. cereus***	19 ± 0.87	-	-	-	-
6	***B. azotoformans***	-	-	-	-	-
7	***B. cereus***	-	-	-	-	-
8	***B. cereus***	41 ± 0.87	-	-	-	-
9	***B. cereus***	-	-	-	-	-
10	***B. cereus***	-	-	-	-	-
11	***B. azotoformans***	-	-	-	-	-
12	***B. globisporus***	-	-	-	-	-
13	***B. cereus***	27 ± 1.0	-	-	-	-
14	***B. azotoformans***	-	-	-	-	-
15	***B. azotoformans***	-	-	-	-	-
			p value < 0.001			

Each value represents mean (n=3) ± standard deviation.

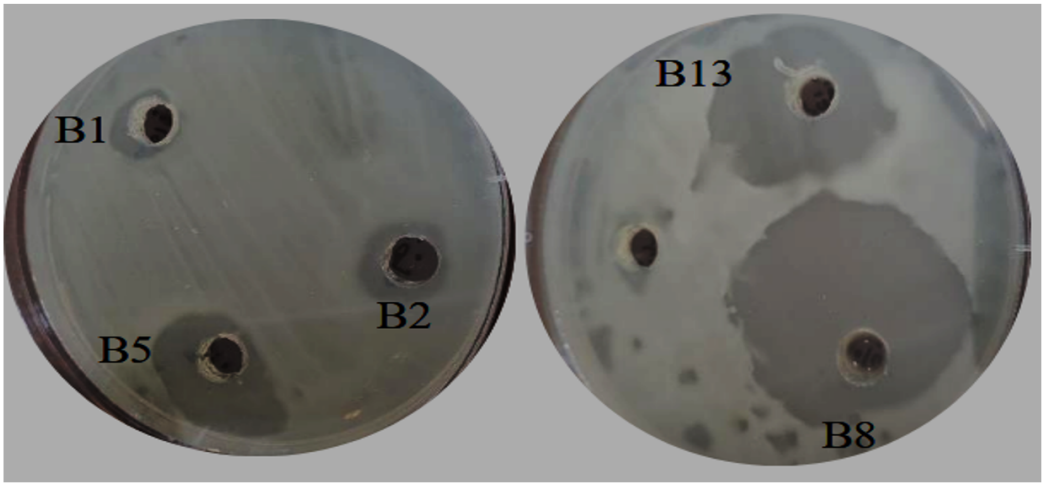

Figure 2. Antimicrobial activity of *Bacillus* spp. against *Salmonella* sp. by AWD.

The authors declare no competing interests.

Data Availability Statement

All relevant data supporting the findings of this study are included within the manuscript and its supporting information files

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