

ORIGINAL ARTICLE



***In vitro* Evaluation of Antibiotic Resistance Patterns Among *Bacillus* Species**

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Antimicrobial resistance is currently one of the most public health serious concerns because of the overuse and unsuitable use of antibiotics. Therefore, this study was conducted to evaluate the resistance patterns of five *Bacillus* species including *B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. simplex* and *B. tequilensis* in vitro towards antibiotic on NA media. The inhibition of *Bacillus* growth was detected after 72 hours of incubation. The obtained data indicated that *Bacillus* resistance to antibiotic was species-related as *B. subtilis*, (N = 19; % 94.74), *B. amyloliquefaciens*, (N = 11; % 90.91), and *B. atrophaeus* (N = 3; % 0), *B. simplex* (N = 2; % 0) and for *B. tequilensis* (N = 3; % 66.6) towards Cefazolin. Most of the *Bacillus* strains were resistant against Cefazolin (79%), Tobramycin (16%), Pefloxacin (11%), and Gentamycin (11%), whereas no resistance was shown against Levofloxacin and Nitrofurantoin. The percentage of zone inhibition of the *Bacillus* ssp. colonies (in mm diameter) ranged from 0 to 60 mm, compared to the untreated control. The present work has demonstrated species-specific differences in the antimicrobial susceptibility of *Bacillus* spp. and observation *Bacillus* resistant strains is of alarm about how this resistance could spread to other bacteria, and then to humans.

Key words: Antimicrobial resistance, *Bacillus* species, inhibition zone

Antibiotic-resistant bacteria are one of the most important problems affecting public health at the current century, since the natural environment plays a key role in the wider spread of this phenomenon (Bhullar *et al.*, 2012; Aslam *et al.*, 2021). Selection of resistant organisms in nature might result from natural creation of antibiotics by soil organisms, from animals or human's wastes or animal feed or crops runoff (Ben *et al.*, 2019). However, although soils are the largest repositories of microbial diversity, the procedures that determine their microbial community dynamics are not completely understood. Increasing our understanding of the antibiotic resistance levels in soils is extremely important to protect public health, since it has been found that the harmless soil bacteria could be the original source of some antibiotic resistance genes identified in hospitals (Forsberg *et al.*, 2012).

It has been reported that agricultural soils have multidrug resistant bacteria and have the highest level of resistance to three major antibiotic classes (Popowska *et al.*, 2012). Therefore, more research on soil-associated antibiotic-resistant bacteria, from the ecological reservoir to humans, is urgently needed. Recently, the emergence of antibiotic resistance *Bacillus* has increased and its likely public health consequences have resulted in many countries (Lee *et al.*, 2019; Cha *et al.*, 2023). To inhibit the spread of Antimicrobial resistance, it is essential to evaluate *Bacillus* spp. and their antimicrobial resistance profile. Some strains of *Bacillus* spp. are becoming increasingly resistant to antibiotics, allowing for the acquisition and emergence of new antimicrobial resistance strains (Lee *et al.*, 2019).

Although *Bacillus* species have been used as food microbial supplements, information obtainable on the profiles of antimicrobial susceptibility is fairly limited. Even less evidence is presently accessible on the antimicrobial susceptibility of *Bacillus* spp. isolated from soils; therefore, the purpose of this study was to identify the antibiotic resistance of local *Bacillus* spp. isolated from different soil sites in Syria.

MATERIALS AND METHODS

Bacterial strains

In this study, out of 525 *Bacillus* isolates, 38 strains consisting of *B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. simplex* and *B. tequilensis* were used (Table 1). They were collected from soil samples of different Syrian regions (Ammoun *et al.*, 2011; Harba *et al.*, 2020). The strains were grown on nutrient broth (NB) culture and the colonies of prospective *Bacillus* spp. were identified according to Wulff *et al.* (2002) (Table 1).

In vitro susceptibility to antibiotic

Six antibiotics; Cefazolin, Tobramycin, Pefloxacin, Gentamycin, Levofloxacin and Nitrofurantoin were tested in this study. To evaluate susceptibility to each antibiotic, *Bacillus* spp. strains were grown on NB media. Plates were inoculated (2×10^8 CFU/ml) and allowed to dry for 5 minutes, before adding the antibiotic disk and incubated at 25°C for 72 h. Plates without antibiotic were served as controls. Susceptibility to antibiotic was tested using the inhibition zone diameter (Masood and Aslam, 2010) which is classified as follows; ≥ 21 mm (susceptible: +), 20-14 mm (intermediate resistant: ++) and >13 mm or no zone of inhibition (resistant: +++).

Data analysis

All experiments were achieved three times with three Petri dishes per replicate, for each bacterium- antibiotic *in vitro* evaluation. Presence of a clear zone around *Bacillus* colonies was considered as an index of the antibiotic sensitivity which were analyzed descriptively and presented here using the diameter of colonies.

RESULTS AND DISCUSSION

In the present work, the susceptibilities of 38 *Bacillus* strains comprising five *Bacillus* spp. (*B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. simplex* and *B. tequilensis*) against 6 antibiotics were determined on NA media. Susceptibility of *Bacillus* spp. was concluded and validated by the inhibition zone towards six antibiotics comparing with the control 72 hours post incubation (Fig. 1). The obtained data indicated that *Bacillus* resistance to antibiotic was species-related as *B. subtilis*, (N = 19; % 94.74), *B. amyloliquefaciens*, (N = 11; % 90.91), and *B. atrophaeus* (N = 3; % 0), *B. simplex* (N = 2; % 0) and for *B. tequilensis* (N = 3; %

66.6) towards Cefazolin. Most of the *Bacillus* strains were resistant against Cefazolin (79%), Tobramycin (16%), Pefloxacin (11%), and Gentamycin (11%),

whereas no resistance were shown against Levofloxacin and Nitrofurantoin (Table2; Fig. 2).

Table 1: *Bacillus* species used in the study.

<i>Bacillus</i> Species	Number of strains	Morphology
<i>atrophaeus</i>	3	brown-black, opaque, smooth, circular
<i>amyloliquefaciens</i>	11	creamy white with irregular margins
<i>simplex</i>	2	small, creamy, smooth, glossym, circular, sticky texture
<i>subtilis</i>	19	fuzzy white, opaque, rough, with jagged edges
<i>tequilensis</i>	3	yellowish, opaque, smooth, circular

Table 2: Antibiotic sensitivity of *Bacillus* species

No.	<i>Bacillus</i> spp.	Nitrofurantoin	Gentamycin	Tobramycin	Levofloxacin	Cefazolin	Pefloxacin
<i>amyloliquefaciens</i>							
1	82.c	+	+++	++	+	+	++
2	96.c	+	++	++	+	+++	+
3	96.e	+	++	+++	+	+++	+
4	123.a	+	+++	++	+	+++	+
5	128.b	+	+++	+++	+	+++	+
6	134.c	+	++	++	+	+++	+
7	159.d	+	++	++	+	+++	+
8	177.c	+	+	++	+	+++	+
9	185.c	+	+	+	+	+++	+
10	190.d	+	+	+	+	+++	+
11	200.d	+	++	+	+	+++	+
<i>atrophaeus</i>							
12	15.b	+	++	++	+	+	++
13	63.e	+	++	++	+	+	+++
14	199.a	+	++	++	+	+	+
<i>simplex</i>							
15	30.a	+	++	++	+	+	+++
16	198.b	+	++	++	+	+	+++
<i>subtilis</i>							
17	35.a	+	+	++	+	+++	+
18	41.b	+	++	++	+	+++	+
19	44.a	+	++	+++	+	+++	+
20	60.a	+	++	++	+	+++	+
21	73.b	+	++	++	+	+++	+
22	113.c	+	++	++	+	+++	+
23	116.c	+	++	++	+	+++	+
24	118.c	+	++	++	+	+++	+
25	124.b	+	++	++	+	+	+
26	130.d	+	++	+++	+	+++	+
27	132.c	+	++	++	+	+++	+
28	132.e	+	++	++	+	+++	+
29	133	+	++	++	+	+++	+
30	134.d	+	++	++	+	+++	+
31	135.d	+	+++	++	+	+++	+
32	139.d	+	++	+++	+	+++	+
33	151.c	+	++	++	+	+++	+
34	168.c	+	++	++	+	+++	+
35	190.e	+	++	++	+	+++	+
<i>tequilensis</i>							
36	69.a	+	++	++	+	+	+++
37	145.d	+	++	+++	+	+++	+
38	150.d	+	++	++	+	+++	+

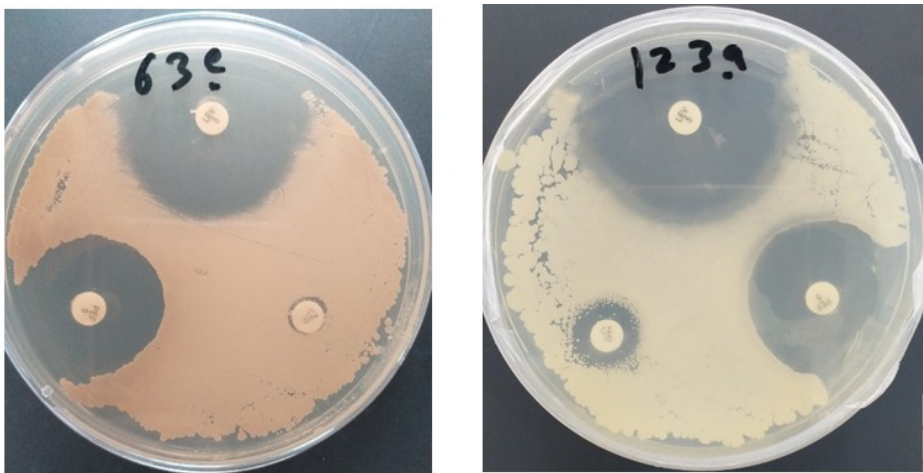


Figure 1. Testing of antimicrobial resistance *in vitro*. These petri dishes contain *Bacillus* (creamy yellow and creamy red) cultured on NB media; *B. atrophaeus* (63e) and *B. amyloliquefaciens* (123a). The white discs each contain antibiotic. Where clear zones appear around the discs, bacterial growth has been prevented by the antibiotic.

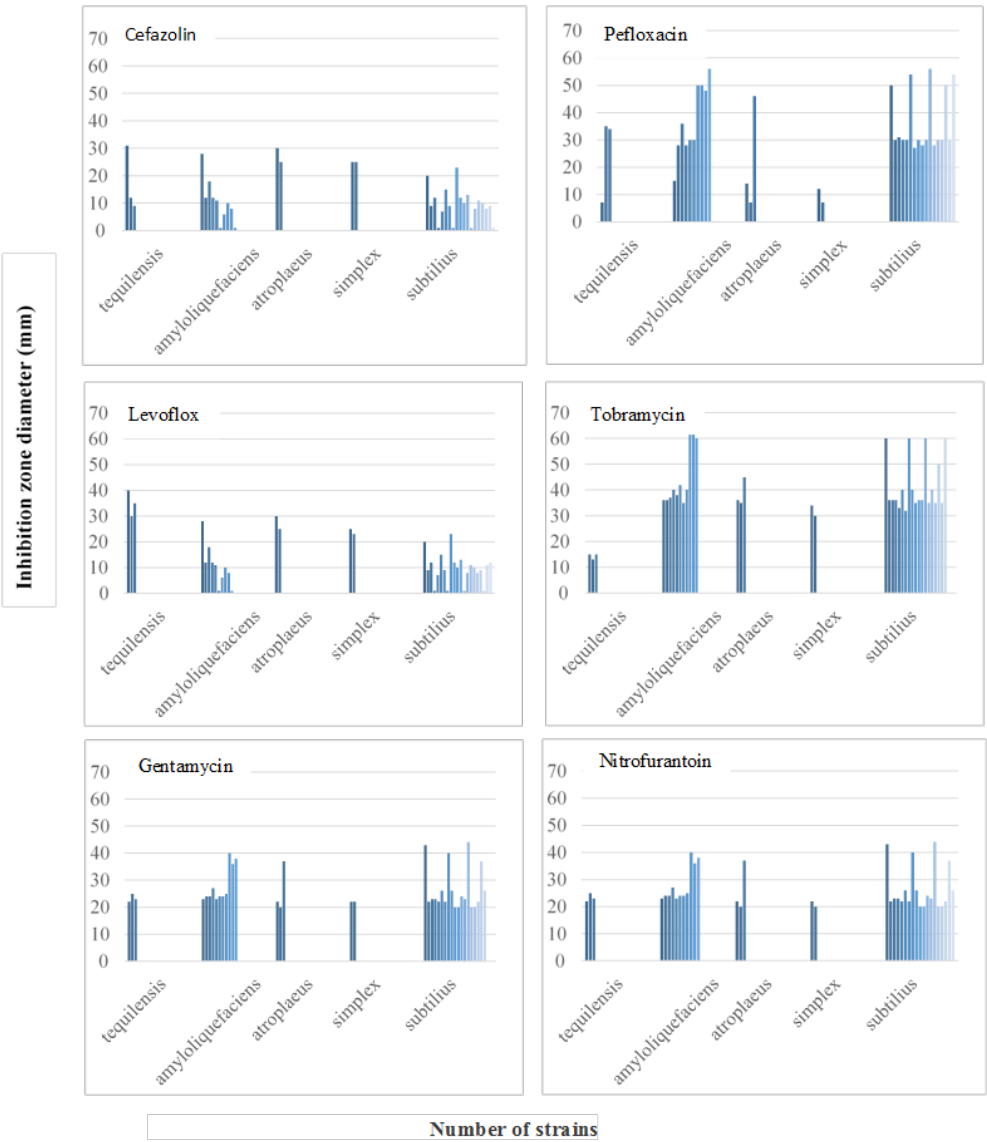


Figure 2. Distribution of inhibition zone diameter in the *in vitro* six antibiotic resistance test among *Bacillus* species

The percentage of zone inhibition of the *Bacillus* spp. colonies (in mm diameter) ranged from 0 to 60 mm, compared to the untreated control, suggesting the existence of potential risk for *Bacillus* spp. infections (Fig. 1). These results therefore demonstrated the presence of species-specific variations in the antimicrobial susceptibility of *Bacillus* spp., parallel to the remarks of other workers (Reva *et al.*, 1995; Lee *et al.*, 2019).

Since *Bacillus* spp. have clinical importance, defining their resistance to antibiotics is necessary for treatment during outbreaks. Our results demonstrated that some *Bacillus* spp. Such as *B. amyloliquefaciens*, *B. atrophaeus* and *B. tequilensis* were highly resistant towards Cefazolin (Fig. 2), and in low resistance levels against Tobramycin, Pefloxacin and Gentamycin, and these are in line with other studies that found such resistance of *Bacillus* spp. to multi-antibiotic treatments in several sources, including foods (Spyridaki *et al.*, 2000; Cha *et al.*, 2023).

Different mechanisms of bacitracin resistance among bacteria have been reported (Bernard *et al.*, 2005; Halawa *et al.*, 2024). However, little is known about the mechanisms of this resistance in *Bacillus* spp. Podlesek *et al.* (2000) observed further that deletion of the *bcrA* or the *bcrC* gene severely impaired bacitracin resistance. However, nothing is known about other possible mechanisms of resistance to quinolones, namely the elimination of antibiotics through an active efflux system and/or decreased outer membrane permeability.

On the other hand, horizontal gene transfers are the main mechanisms through which antibiotic resistance genes are exchanged among bacteria of diverse origin including environmental, non-pathogenic, human pathogenic, gram- positive and negative via mobile DNA elements such as plasmids and transposons – either with or without mobile integrons (Pruden *et al.*, 2006). This might explain the antibiotic resistance among *Bacillus* species detected in this study.

CONCLUSION

In conclusion, our study demonstrated that *Bacillus* strains were highly susceptible to Levofloxacin and

Nitrofurantoin. Species-specific variations in the patterns of resistance to Cefazolin, Tobramycin, Pefloxacin, Gentamycin were observed. We found also that *B. subtilis*, (N = 19; % 94.74), *B. amyloliquefaciens*, (N = 11; % 90.91) and for *B. tequilensis* (N = 3; % 66.6) were sensitive to Cefazolin, and in low resistance levels against Tobramycin, Pefloxacin and Gentamycin, suggesting the existence of potential risk for *Bacillus* spp. infections. Therefore, antimicrobial susceptibility testing is required as routine microbiological analyses of soils in Syria.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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