Original Research Article

The Impact of Diameter of Inhibition in Crowded Plates on the *In-vitro* Inhibitory Strength of Potential Antibiotic-Producing Bacteria isolated from some natural habitats

Abstract

Antimicrobial resistance is a global problem, and efforts to replace failing antibiotics are crucial. We investigated the impact of the diameter of inhibition (mm) in crowded plates of potential antibiotic-producing bacteria on their *in-vitro* inhibitory strength. Our previous research identified 12 potential antibiotic-producing bacteria from natural habitats and obtained their diameters of inhibition in preliminary crowded plates. The potential candidates were further challenged with some pathogenic microbes by the Agar-well diffusion technique to test their strength. The results showed that the diameter of inhibition of the potential antibiotic-producing isolates obtained at the crowded plate technique level was not a predictor of the potency of potential antibiotic-producing bacteria (r_s = 0.52, P = 0.08). In conclusionIn summary, the selection of potent candidate microbes for the development of new antimicrobial compounds against multidrug-resistant pathogens cannot be determined based on the diameter of inhibition in a preliminary investigation using the crowded plate technique. Further studiesinvolvingcomplementary techniques are necessary to determine the best potential candidate organisms for the development of novel antimicrobial compounds.

Keywords: Crowded plate technique, antibiotic-producing bacteria, diameter of inhibition, Agar-well Diffusion method, Natural habitats.

UNDER PEER REVIEW

1.0 Introduction

Antibiotic resistance has become an existential threat considering the recurring treatment failures of infectious diseases, including the sluggish discovery and development of antibiotics. Resistance to conventional antimicrobial agents attributable to antibiotic overuse, misuse, extensive agricultural applications, and the acquisition of antibiotic-resistant genes (horizontal gene transfer) (Evans *et al.,* 2020; Nadeem *et al.,* 2020) has prompted the need for their replacement.

Soil, among other sources, is considered a veritable reservoir for novel antibiotic-producing microbes (Rafiq *et al.*, 2018), given that two-thirds of antibiotics currently in use are of microbial origin (Tawiah *et al.*, 2012).Microbes have populated the earth for over 3.5 billion years,as the estimated population of prokaryotic cells in the biosphere is 4-6 x 10^{30}(Whitman *et al.,* 1998),

thereby complicating the soil microbial community. The antibiotic-producing traits of some soil microbes aretraceable to the development of survival mechanisms and dominance over other competing bacteria in their natural ecosystems(Dwivedi and Sisodia, 2019; Martínez *et al*., 2015).

The crowded plate technique (CPT) is one of the basic screening assays of choice for the detection of antibiotic-producing microbes within complex microbial populations (300-400 colonies). This technique detects microorganisms that display inhibitory activity against other microorganisms. This involves the preparation of serial dilutions from the stock sample, followed by culturing on agar plates. Ahalo or clear zone of inhibition around a culture is an indicator of a potentialantibiotic-producing microbe in the confluent population of the culture plate (Waksman andLechevalier, 1962). This is followed by further screening,whereby the pure-cultured potential candidate is challenged with pathogenic strains to determine the potency of their antibiotic production. The application of CPTin the search for antibiotics-producing microbes is well documented (Hussaini*et al*., 2021; Ibnouf*et al*., 2022).

To the best of our knowledge, no study has sought to understand the influence of the diameter of inhibition of potential antibiotic-producing bacteria determined using the Crowded plate technique on their antagonistic activity of the potential candidate isolates. We investigated the potency of the antibiotic-producing bacteria obtained from the crowded plate technique (CPT). To better understand this, we assessed the strength of the potential candidate by comparing the diameter of the inhibition (DI) *insitu* from CPT with the number of susceptible test pathogenic microbes.

2.0 Materials and Methods:

2.1 Screening for potential antibiotic-producing microorganisms

Potential antibiotic-producing bacteria were isolated from soil samples collected from natural habitats in Abuja, Nigeria, following CPT, according to Bavishi*et al.* (2017). The selected habitats included termite mounds, riverbanks and plant rhizospheres, such as:*Anacardium occidentale* L. (cashew tree), *Gmelina arborea* Roxb. ex Sm. (beechwood), and *Ageratum conyzoides* L. (goat weed). The assay was performed at the Department of Microbiology and

UNDER PEER REVIEW

Biotechnology, National Institute for Pharmaceutical Research and Development, Abuja (NIPRD).

Briefly, 1 g of each soil sample was weighed into a 9 mL sterile normal saline (NS) (0.85% sodium chloride solution) tube to prepare the stock sample. A serial dilution of the stock was prepared by briefvortexing and aseptically transferring 1 mL aliquot from it into a 9 mL NS tube to obtain $1:10^1$. The serial dilutions continued till $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$dilutions were achieved. Using the spread plate technique, a 0.1 mL aliquot of the inoculum from $1:10^2$ dilutions and $1:10^4$ dilutions was inoculated on nutrient agar plates (HiMedia) seeded with nystatin (50 µg/mL). Inoculated plates were incubated at 37 °C for 24 h in an inverted position. Culturing was performed in triplicate. Colonies that were antagonists against other bacteria were identified by halos or clear zones of inhibition around them, which were measured in millimeters. Potential candidates were selected, subcultured, and stock cultures of pure colonies were stored on slants at 4 °C for further testing.

2.2 Isolation of the bioactive secondary metabolites

The pure cultures were inoculated into sterile 10 mL nutrient broth (NB) and kept in a shaker incubator at 120 rpm at 37°C for 96 h. Thereafter, the tubes were centrifuged at 6000 rpm for 10 minutes to remove the supernatant from the cell sludge. Cell-freesupernatant was obtained after filtration with a 0.45 µm membrane filter unit (Millipore brand, Massachusetts, USA).

2.3 Standardization of the test microbes

For the standardization of the test bacteria,24-hour culture plateswere suspended in sterilized 2 mL of nutrient broth and incubated at 37°C for 2 h. The microbial suspensions were adjusted to 0.5 McFarland turbidity standard (corresponding to1 x 10^8 CFU/mL for bacteria)equivalent toan optical density of 0.08-0.13 for bacteria and 0.05 for yeast at a wavelength of 600 nm using a spectrophotometer (Jenway 6405 UV/VIS, UK).The bacteria suspensions were diluted 1:100 in nutrient broth to obtain 1 x 10^6 CFU/mL (CLSI, 2016). The yeast (*C. albicans*) suspension concentration was 1 x 10^6 CFU/mL equivalent to 0.5 McFarland standard (CLSI, 2008).

2.3.1 Antimicrobial susceptibility testing

Antibiotic-producing strength of the cell-free filtrates was carried out by the Agar-well diffusion technique as described by Rafiq *et al.* (2018). The standardized test pathogenic clinical and

UNDER PEER REVIEW

reference strains were*Bacillus subtilis*, *Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi, Candida albicans,Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922).The diameter of inhibition (DI)in the culture plates was measured in millimeters (mm) after incubationat 37ºC for 24 h, and the number of susceptible test isolates recorded to determine the potency of the potential antibiotic-producing isolates.

2.4 Statistical Analysis

All experiments were performed in triplicates. Thecorrelations between the diameters of inhibition of the antibiotic-producing bacteria obtained from the CPT and the inhibited pathogenic microbes were tested with Spearman's rank correlation coefficient using the SPSS (version 25).

3.0 Results

A total of 12 potential antibiotic-producing bacteria were isolated from natural habitats by the CPT (Balogun *et al.,* 2022). Figure 1 illustrates the number of potential candidates isolated from the selected natural habitats. Briefly, a total of 9 (9/12: 75%) were isolated from the rhizosphere of plants [*Anacardium occidentale* L. (cashew tree), *Gmelina arborea*Roxb. ex Sm. (beechwood) and *Ageratum conyzoides* L. (goat weed)]; two (2/12: 16.7%) from the river banks and 1 (1/12: 8.3%) was isolated from the termite mounds.

The potential antibiotic-producing bacteria coded AAH02 to ARS03/01 and their zones of inhibition at the preliminary identification stage in the crowded plates were as follows (Figure 2): (a) AAH02: 21 mm. (b) ACR01: 6 mm. (c) AMR06/01: 5 mm. (d) AMR06/02: 16 mm. (e) AMR06/03: 6 mm. (f) AMP03/01: 7 mm. (g) AMP03/02: 7 mm. (h) AMP03/03: 4 mm. (i) AMP03/04: 3 mm. (j) AMP03/05: 8 mm. (k) ASS02/01:22 mm. (l) ARS03/01: 16 mm.

Table 1 and Figure 2 show their inhibitory activities against some selected pathogenic strains. Briefly, the potential antibiotic-producing bacteria inhibited the total numbers of pathogenic strains as follows: AAH02: 4,ACR01: 0, AMR06/01: 1, AMR06/02: 0, AMR06/03: 0,

UNDER PEER REVIEW

AMP03/01: 0, AMP03/02: 4, AMP03/03: 0, AMP03/04: 0, AMP03/05: 3, ASS02/01: 7, andARS03/01: 0. In summary, only 5 of the candidates showed activity against at least 1 test pathogenic strain. The filtrate of AMP03/02isolated from the rhizosphere of *Ageratum conyzoides*L. (goat weed) showed inhibitory activities against *B. subtilis* (12.2±0.4 mm), *S. aureus* (10.3±0.3 mm), *P. aeruginosa* (10.1±0.4 mm) and *S. typhi* (9.4±0.4 mm) respectively?.AMP03/05also isolated fromthe rhizosphere of *Ageratum conyzoides*L. (goat weed)showed inhibitory activities against*B. subtilis* (10.0±0.4 mm), *S. aureus* (9.1±0.4 mm) and *K. pneumonia*e (10.0±0.4 mm) respectively. The filtrate of AAH02 isolated from the termite mound showed inhibitory activities against *B. subtilis* (12.1±0.4 mm), *C. albicans* (9.3±0.4 mm), *P. aeruginosa* (9.4±0.6 mm) and *S. typhi* (11.1±0.4 mm) respectively?.The filtrate obtained from AMR06/01obtainedfrom the rhizosphere of*Gmelina arborea* Roxb. ex Sm. (beechwood)inhibited only *B. subtilis* (9.3±0.4 mm) among the tested pathogens.ASS02/01isolated from the river bankshowed antimicrobial activities against 7 test isolates namely *Streptococcus pneumonia* (11.4±0.4 mm), *Salmonella typhi* (10.2±0.2 mm), *Escherichia coli* (11.0±0.0 mm), *Staphylococcus aureus* (10.3±0.4 mm), *Bacillus subtilis* (9.5±0.4 mm), *Pseudomonas aeruginosa* (8.4±0.4 mm) and *Candida albicans* (9.3±0.3 mm).

Overall, the *in-vitro* diameters of inhibition of the potential antibiotic-producing bacteria determined by the CPT and the number of test pathogenic microbes they inhibited were not significantly correlated (Spearman rank correlation r_s= 0.52, P = 0.08). However, some potential candidates like AAH02 and ASS02/01 with higher DI of 21and 22 mm showed inhibitions against a total of 4 and 7 pathogenic strains, respectively.

UNDER PEER REVIEW

4.0 Discussion

Given the occurring delays in the antibiotic discovery pipeline (WHO, 2017), the search for potent antibiotic-producing bacteria has become crucial. For this purpose, bacteria are prioritizedconsidering that they easily propagate within a short period.I think this paragraph should be moved to the introduction section to justify doing this work.

CPT was explored to identify antibiotic-producing bacteria, which were subsequently challenged with some pathogenic microbes. Overall, there was no significant correlation between the DI of

the potential antibiotic-producing bacteria determined by CPT and the number of test pathogenic microbes they inhibited (r_s= 0.52, P = 0.08), suggesting the need for alternative or more comprehensive methods to determine the potency of antibiotic-producing bacteria identified by CPT¿What comprehensive methods are necessary?. This clarifies the inconsistencies observed between the DIs and the number of inhibited test pathogens for some of the potential candidates in this study. For example, potential candidates AMR06/02 and ARS03/01 each with 16 mm as the DI in the crowded plates showed no *in-vitro* activity against all the test pathogenic strains; however, another potential candidate AMP03/02 with a lower DI of 7 mm inhibited four test pathogenic strains. The findings suggest that the DIs of the potential antibiotic-producing bacteria obtained from CPT are not markers for selecting the most suitable potential candidate organism (Figure 2). For example, ASS02/01 and AAH02 with zones of inhibition of 22 mm and 21 mm from crowded plates inhibited seven and four test microbes, respectively. Others such asAMP03/02 (DI: 7 mm from the crowded plates) inhibited four test microbes and AMP03/05 (DI: 8 mm) inhibited three test isolates. AMR06/01, with a DI of 5 mm, inhibited only one (1) of the test microbes.

Despite the appreciable DI displayed by some potential candidates from the crowded plates, they showed no activity against any of the tested pathogenic microbes. These includedACR01 (DI: 6 mm), AMR06/02 (DI:16 mm), AMR06/03 (DI: 6 mm), AMP03/01(DI:7 mm), AMP03/03(DI: 4 mm), AMP03/04(DI: 3 mm), and ARS03/01 (DI: 16 mm). The unpredictability of the inhibitory strength in relation to the preliminary DI from the crowded plates could be a result of the strength of other competing microbes encountered by the antibiotic-producing bacteria during the competition for survival within and between microbial species in their natural habitats. Competition for survival is a rule of microbial communities (Butaitė*et al.,* 2017; Granato*et al.,* 2019). Studies have shown that inter- and intra-microbial species compete favorably in the microbial community depending on their ability to develop dynamic survival mechanisms (Bottery*et al.*, 2021). Such mechanisms often involve the release of molecules into the microbial environment (BaishyaandWakeman, 2019), the potency of which depends on the strength of other competing microbes.

The highest number of antibiotic-producing bacteria (75%) recorded in the rhizospheres (Figure 1) is consistent with other studies (Ali *et al*., 2018; Saraf *et al*., 2014), suggesting that the

UNDER PEER REVIEW

rhizosphere could be an excellent source of antibiotic-producing microbes. Rhizospheres are a rich source of nutrients and energy compared with bulk soil, considering the abundance of net fixed carbon (11%) released to it by plants (Babalola, 2010; Dennis *et al.,* 2010). Prashar*et al.* (2014)reported that the elevated concentration of nutrients in the rhizosphere could be responsible for more microbes in this region. Competition for survival among the microbes in the rhizosphere could have primed some (microorganisms) to evolve into the release of antimicrobial compounds (inhibitory compounds) to thrive in the environment.

The five potential candidate strains,AAH02, AMR06/01, AMP03/02, AMP03/05and ASS02/01, were molecularly identified as *Bacillus licheniformis* strain SCDB 34, *Bacillus lentus* strain NCTC4824, *Bacillus cereus* strain A1, *Bacillus brevis* strain NCTC2611, and *Bacillus subtilis* subsp. *subtilis* 168, respectively (Balogun *et al.*, 2022). Table 1 illustrates*Bacillus subtilis* subsp. *subtilis* 168 as the strongest antibiotic-producer haven displayed a broad spectrum of inhibitory activity against the Gram-positives, negatives and yeast, suggesting that it could be a source for the lead compounds toremedy the challenges of multidrug-resistant pathogens.Drug-resistant Gram-positive and Gram-negative bacteria, including yeasts have been a major global therapeutic challenge (Alexander *et al.*, 2014; Ernst *etal.*, 2018; Holmes *et al.,* 2021). I think this paragraph should be moved to the introduction section to justify doing this work.

To the best of our knowledge, this is the first study to report the correlation between the diameters of inhibition of antibiotic-producing bacteria obtained in crowded plates and the inhibitory strength against the test pathogenic microbes.Few findings are reported from this study

Conclusion

Diameters of inhibition of potential antibiotic-producing bacteria from the preliminary Crowded plate investigation is not a marker for the choice of potent potential candidate strain(r_s= 0.52, P = 0.08), suggesting that further complementary screenings of the potential candidates (regardless of the DIs at the CPT level), is recommended to determine the strongest candidate suitable for the development of novel antimicrobial compounds. Further studies on this work are in progress.

UNDER PEER REVIEW

Ethical approval: Not applicable

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UNDER PEER REVIEW

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UNDER PEER REVIEW

Table 1: Summary of the inhibitory activity of the potential antibiotic-producing bacteria.

	Gram positive pathogen			Gram negative pathogen				Yeast
Potential antibiotics Producing isolates	*S. aureus* (ATCC 25923)	*B. subtilis*	*E. coli* (ATCC 25922)	*S. typhi*	*P. mirabilis*	*P. aeruginosa* (ATCC 27853)	*K. pneumonia*	*C. albicans*
AAH02	-	+	-	+	-	+	-	+
ACR01	-	-	-	-	-	-	-	-
AMR06/01	-	+	-	-	-	-	-	-
AMR06/02	-	-	-	-	-	-	-	-
AMR06/03	-	-	-	-	-	-	-	-
AMP03/01	-	-	-	-	-	-	-	-
AMP03/02	+	+	-	+	-	+	-	-
AMP03/03	-	-	-	-	-	-	-	-
AMP03/04	-	-	-	-	-	-	-	-
AMP03/05	+	+	-	-	-	-	+	-
ASS02/01	+	+	+	+	-	+	+	+
ARS03/01	-	-	-	-	-	-	-	-

+: Antimicrobial activity; -: No activity

UNDER PEER REVIEW

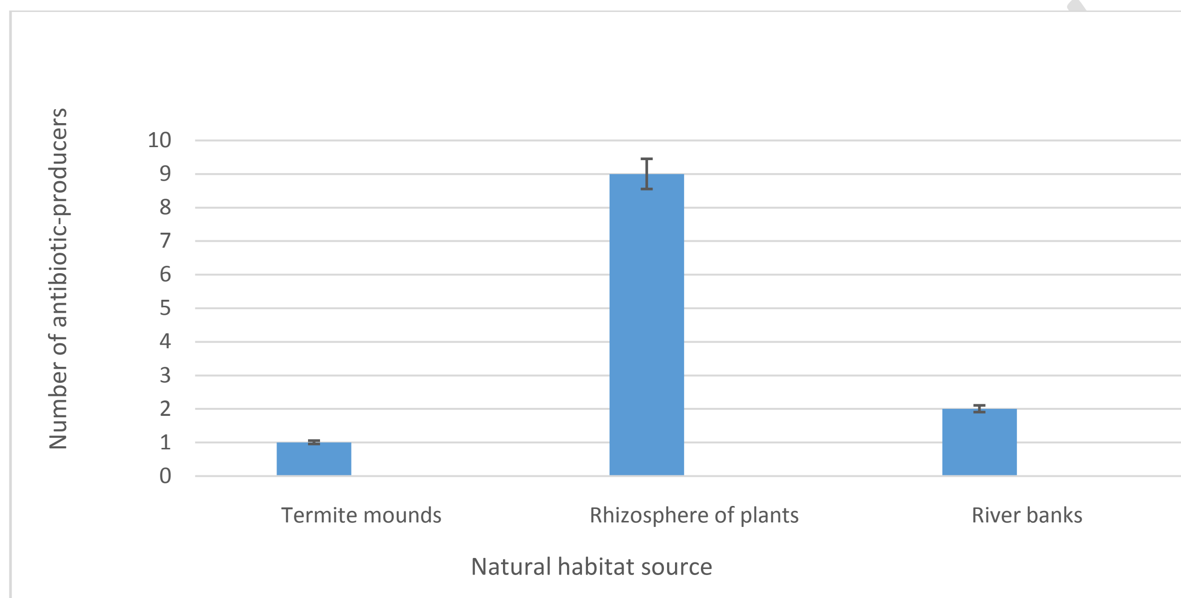

Figure 1: Potential antibiotic-producing bacteria from the natural habitats.

UNDER PEER

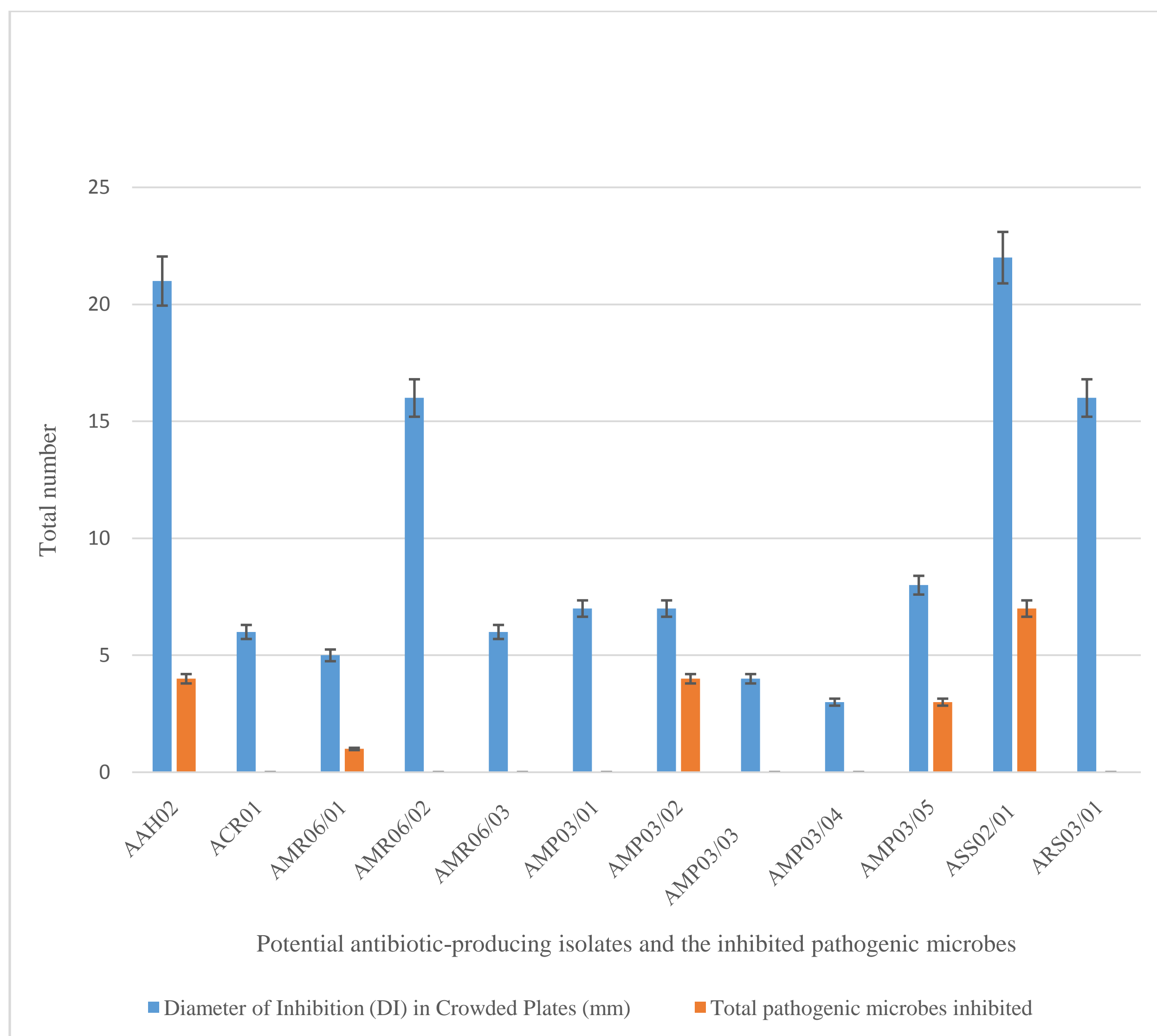

Figure 2:The diameters of inhibition of the potential antibiotic-producing isolates in the crowded plates versus the total test microbes