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Antagonistic Activities of *Brevibacillus brevis* OZF6 and *Pseudomonas chlororaphis* UFB2 on Microbes Isolated from *Rattus norvegicus* Foot-path Soil and *Trichophyton mentagrophytes* (*interdigitale*) [C.P. Robin.] Sabour.

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOO designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors MKO and DJA managed the analyses of the study. Authors OAA, OMO and OOO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aims to evaluate the antagonistic activities of *Brevibacillus brevis* and *Pseudomonas chlororaphis* isolated from *Rattus norvegicus* (Brown rat) nest against *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from the brown rat's foot-path soil sample and also on *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9533 as a test organism in order to ascertain their antagonistic activities.

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Place and Duration of Study: This research was carried out in the Department of Microbiology, The Federal University of Technology, Akure, Ondo State, Nigeria between November 2015 and May 2016.
Methodology: The isolates *B. brevis* and *P. chlororaphis* were isolated and characterized from *Rattus norvegicus* (Brown rat) nests using standard microbiological techniques. The pure cultures of the isolates were screened for antagonism against the following test organisms: *S. aureus*, *P. aeruginosa* obtained from foot-path soil samples and *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9533 using *in vitro* antagonistic assay co-culture technique. Physicochemical analyses of *R. norvegicus* nest and soil samples were accomplished according to standard analytical methods. Analysis of variance (anova) was performed followed by Duncan multiple range post hoc tests, considered the value p =.05.
Results: The isolate *B. brevis* exhibited strong antagonism against *S. aureus* and *P. aeruginosa* with inhibition zones of 22.13±0.32 mm and 18.20±0.10 mm but had no antagonistic effect on *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9533. However, *P. chlororaphis* showed antagonism against *S. aureus* and *T. mentagrophytes* (*interdigitale*) ATCC 9533 with inhibition zones of 17.50±0.17 mm and 15.13±0.20 mm but had no inhibition against *P. aeruginosa*. The results of this study revealed the antagonistic activities of *B. brevis* and *P. chlororaphis* on the test organisms and the findings from the physicochemical assay showed that the temperature and pH of the Brown rat nest supports optimum growth of microorganisms.
Conclusion: Findings from this research revealed the antagonistic potentials of *B. brevis* and *P. chlororaphis* against the test organisms and that Brown rat nests from which these organisms were isolated serve as good reservoirs for microorganisms.

Keywords: Antagostic activities; Brevibacillus brevis; Pseudomonas chlororaphis; Rattus norvegicus; Trichophyton mentagrophytes (interdigitale) ATCC 9533.

1. INTRODUCTION

The persistent increase in levels of resistance of bacteria to antibiotics is making scientists focus more on the discovery of new natural products derived therapeutics with better antibacterial efficacy and less toxicity to humans, animals and the environment [1].

Brevibacillus brevis (formally known as *Bacillus brevis*) is a Gram-positive, aerobic, spore-forming bacillus commonly found in soil and decaying matter and rarely associated with infectious diseases [2]. Certain strains of *B. brevis* can synthesize antibiotics gramicidin and tyrocidine [3] while *Pseudomonas chlororaphis* have been used as soil inoculant in agriculture and is reported to act as a bio-control agent against certain fungal pathogens [4]. *P. chlororaphis* lends its name within the genus *Pseudomonas* and subgroups *P. aurantiaca*, *P. aureofaciens*, *P. fragi*, *P. lundensis*, and *P. taetrolens* have been identified based on 16S rRNA analysis [5].

Rattus norvegicus is a brown or grey rodent with body length 25 cm and a short tail; the male weighs an average of 350 g and the female 250 g. It is commonly known as brown rat, “emóigbó”, or Norway rat [6]. *R. norvegicus* make their nests with plant materials from *Bidens pilosa* and *Tridax procumbens* leaves usually woven repeatedly hidden under Tree sheds and Mud hills. The brown rat derives protection from predators and pathogenic microbes in their nests [6].

Following the significant differences in the diversity of microbes isolated and characterized from *R. norvegicus* nest and the foot-path soil region of the brown rat which was in close proximity to each other as evaluated in this study instigated the need to ascertain the factor(s) responsible for this observation. Thus, the present study was carried out to investigate the antagonistic activities of *B. brevis* and *P. chlororaphis* isolated from *R. norvegicus* (Brown rat) nest against *S. aureus* and *P. aeruginosa* isolated from soil samples and *T. mentagrophytes* ATCC 9533.

2. MATERIALS AND METHODS

2.1 Determination of *Rattus norvegicus* Nest Temperature

The clinical thermometer was used to determine the average temperature of brown rat nests. The thermometer was placed in an undulating position at different intervals and the mean temperature reading was recorded. This was done to ascertain the environmental effect of the

nest on the brown rat and the surrounding microbiome.

2.2 Determination of *Rattus norvegicus* Tunnel soil Sample pH

This test was carried out in order to ascertain the pH of the soil sample and thus determine the microbes that can survive and grow in such environmental condition. A 1.0 g of the soil sample was dissolved in 100 mL of deionized water and the pH was determined using a pH meter (Kent EIL 7055) [7].

2.3 Measurement of Distance (cm)

The distance between the site where *R. norvegicus* nest was positioned and its foot-path soil were measured using tape rule calibrated in centimetre (cm).

2.4 Preparation of Culture Media

Mueller-Hinton agar (Oxoid) was prepared by measuring 38 g of the agar with electronic weighing balance (Metlar electronic balance; Model: MT – 301) and subsequently suspending it into 1000 mL of sterile distilled water. Nutrient agar (Oxoid) was prepared by suspending 28 g of the agar into 1000 mL of sterile distilled water. A suspension was formed which on heating with occasional swirling of the flask dissolved. A clean cotton wool was used to plug the conical flask and wrapped carefully with aluminum foil. This was autoclaved at 121°C for 15 min using Floor *Model* AA13 Autoclave. Sabouraud's glucose agar (Difco) was prepared according to manufacturers' specifications. After sterilization, the medium was allowed to cool and 1% chloramphenicol was added to inhibit bacteria that might be present in it.

2.5 Isolation of Microorganisms Associated with Brown Rat Soil Samples and Nests

The isolation of bacteria and fungi were carried out using nutrient agar and Sabouraud's glucose agar using standard methods. Soil samples beneath *R. norvegicus* nests were collected with the aid of a soil auger. The soil samples were carefully transferred into air tight plastic bags and immediately transferred to the laboratory for different analysis within 6hr of collection.

A 1 g of the soil samples was weighed using electronic weighing balance (Metlar electronic balance; Model: MT – 301) and subsequently prepared with using the serial dilution procedure until the dilutions 10^{-4} and 10^{-5} were obtained for each sample. These two dilutions were cultured on nutrient agar and Sabouraud's glucose agar via pour plate technique.

2.6 Identification of Bacterial Isolates

The identification of bacterial isolates was accomplished by morphological, biochemical characterizations and molecular characterizations. The isolates were identified with reference to the Bergey's Manual of Systematic Bacteriology [8], the Advanced Bacterial Identification Software (ABIS) online Encyclopedia [9] and molecular technique via whole genome sequencing. The bacterial DNA of the isolates obtained from *R. norvegicus* nest (*B. brevis* and *P. chlororaphis*) were extracted and amplified in polymerase chain reaction as described by Ologun et al. [10]. The amplified DNA sequencing of *B. brevis* strain OZF6 was further identified by 16S rRNA.The rRNA sequencing of strain OZF6 was carried out by Macrogen Inc., Amsterdam, Netherlands using 785F (5′CCAGCAGCCGCGGTAATACG3′) and 907R (5′TACCAGGGTATCTAATCC3′) primers. Similar sequences were correlated and identified using the nBLAST/ NCBI for further identification of OZF6 strain.

2.7 Preparation and Maintenance of Pure Cultures

Isolates of different bacterial colonies and fungi were sub-cultured by streaking on nutrient and Sabouraud's glucose agar until pure cultures were obtained. Isolates were inoculated into nutrient agar slants, incubated for 24 h and then kept as stock cultures in the refrigerator at 4^{o}C. Pure fungal isolates were also maintained on Sabouraud's glucose agar slants in the refrigerator at the same temperature.

2.8 Standardization of inoculums for Antagonistic Activities

The method described by Owoyemi and Oladunmoye was employed in the standardization of the inocula before antagonistic assays were carried out on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and

Trichophyton mentagrophytes (*interdigitale*) ATCC 9533 [11].

2.9 Preparation of Culture Supernatant

A 48 hours broth culture of isolates were obtained as cell-free solution through centrifuging at 12000 rpm for 12 minutes and followed by filtration of the supernatant with Whatmann filter paper 1. The filtrate obtained was then stored at 4°C [12].

2.10 Antagonistic Activities of *Brevibacillus brevis* OZF6 and *Pseudomonas chlororaphis* UFB2 on Selected Isolates and Test Organism

The method of Oyeniran (2015) was adopted with modifications for the antagonistic assays of the microbial isolates on test organisms [13]. A single streak was made from 24 h old culture of the isolates, 23 mm away from the center of the petridish used. The plates were then incubated for 24 h at 37°C after which a single streak of the test pathogens was placed perpendicularly to the point of isolate inoculation. The set up was incubated at 37°C for 24 hrs for the bacteria and 28°C for 7 days for the fungi test; the control experiment was carried out and horizontal zones of inhibitions were measured.

2.11 Statistical Analysis

Data obtained are presented as mean ± SE (standard error), treatment groups were analyzed using one-way analysis of variance (ANOVA) and data means were compared with Duncan's New multiple range tests at the level of $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Physico-chemical Parameters of Soil Samples and Microbial Load

The results of the physicochemical parameters of samples are presented in Table 1. The Table 1 showed the temperature, pH, colour and texture of soil samples and the nests evaluated. The temperature ranged from 36.5 to 36.6°C and the pH range was between 6.6 and 6.9. The microbial load of the soil samples and the nest is shown in Table 2. The foot path soil sample had the highest colony forming units (4.6×10^6) followed by the nest soil (1.2×10^6) while the nest itself had the lowest (1.0×10^6 cfu/mL) for the bacteria. However, the highest spore forming units was also observed on the foot path soil sample of 2.7×10^5 followed by the nest soil of 1.4×10^6 and the least was observed on the nest with 1.3×10^6 sfu/mL.

3.2 Microbiological and Biochemical Characteristics of Bacterial Isolates

The bacteria isolated from *R. norvegicus* nest comprising the soil sample beneath its nest, foot-path (passage) and from the nest were characterized and identified as belonging to a total of five (5) genera which include: *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium sporogens*, *Bacillus cereus* and *Brevibacillus brevis*. The details of their morphological and biochemical characteristics are shown in Tables 3 and 4 respectively.

Table 1. Physico-chemical parameters of *Rattus norvegicus* nest location

Parameter	NS	FS	N
Temperature	36.5°C	37°C	36.6^{O}C
pH	6.9	6.6	
Colour	Brown	Dark brown	Greenish
Texture	Smooth	Smooth	Smooth
Size	Small	Small	Medium

Key: NS= Nest soil, FS= Foot path soil, N= Nest

Table 2. Microbial loads of *Rattus norvegicus* soil samples and nest

Sample source	Mean bacterial count (cfu/mL)	Mean fungal count (sfu/mL)
NS	1.2×10^6	1.4×10^6
FS	4.6×10^6	2.7×10^5
N	1.0×10^6	1.3×10^6

Key: NS= Nest soil, FS= Foot path soil, N= Nest

3.3 Measurement of Distance (cm)

The distance between the site where *R. norvegicus* nest was positioned and its footh-path soil measured was 50.5 cm.

3.4 Antagonistic Activities of *B. brevis* OZF6 and *P. chlororaphis* UFB2 Isolated from *R. norvegicus* Nest on Selected Organisms Isolated from *R. norvegicus* Foot Path-soil Sample and Test Organism

The antagonistic activities of the organisms isolated from *R. norvegicus* nest (*B. brevis* and

P. chlororaphis) were tested against the selected isolated organisms from *R. norvegicus* foot path-soil sample which includes: *S. aureus* and *P. aeruginosa* and also against *T. mentagrophyte* (*interdigitale*) ATCC 9533. The antagonistic activities of *B. brevis* and *P. chloraphis* after 24 hours of incubation shown in Fig. 1 revealed that the metabolites obtained from the isolated organisms from the brown rat nest had inhibitory effect against some of the isolates from the soil samples. The metabolite from *B. brevis* demonstrated good antibacterial activities against *S. aureus* and *P. aeruginosa* with the zones of inhibition of 22.13±0.32 mm and 18.20±0.10 mm respectively but, does not have inhibitory effect on *T. mentagrophyte* (*interdigitale*) ATCC 9533. However, *P. chlororaphis* had inhibitory effect on *S. aureus* and *T. mentagrophyte* (*interdigitale*) ATCC 9533 with the zones of inhibition of 17.50±0.17 mm and 15.13±0.20 mm respectively but, had no antibacterial effect on *Pseudomonas aeruginosa*.

Newly born *Rattus norvegicus* are prone to infection because they are without fur and their immune system is still very immature, but the woven nests made by the female adult rat helps the new born brown rats to survive infections. It has been observed that apparently healthy young and adult rat skins are always tender, devoid of physical lesions and these attributes are to be traceable to the antimicrobial activities of the plant materials used by *Rattus norvegicus* in the construction of its nest and other possible factors [6].

In light of these findings, investigations on the microbial diversity of the microbes isolated from *R. norvegicus* nest and its foot-path showed the need to evaluate antagonistic activities of the isolates obtained from brown rat nests against the pathogens isolated from its immediate surroundings such as foot-path soils. This is because the microbes present in the rat nests are different from those found on the surrounding foot-path soil despite their proximity.

The rat nest temperature and pH evaluated in the study provide useful information on the environmental factors which may aid the growth of microbes since pH of sample sites was neutral to slightly acidic. The microbial load obtained in the results also revealed that there was reduction in microbes isolated in the region where the nest was situated compared to those in the rat foot-path soils. This could be as a result of the antimicrobial activities of the plant materials used in the construction of the nest [6,11] and the antagonistic activities of the microbes on the rat nests.

Findings from this research have found *B. brevis* isolated from *R. norvegicus* nest to possess antagonistic activities against *S. aureus* and *P. aeruginosa*. This observation is in conformity with the findings of Agarry [14] who linked the antagonistic activities of *Bacillus* species to the production of endospores and their ability to adapt better to environmental extremes than other pathogens. *Bacillus* strains have also been described by Gao [15] as promising metabolite producers of carotenoids, cholic acid derivatives and organic acids. These abilities may account for the antagonistic activity of *B. brevis* from the brown rat nest against the test pathogens evaluated in this study. Furthermore, *Bacillus* spp may have developed several molecular based adaptive mechanisms of survival in an extreme environment and their ability to produce extracellular compounds which are toxic to other pathogens does not only ensure their survival in such hostile environment but also revealed

Table 3. Macroscopic and colonial morphology of bacteria isolated from *R. norvegicus* nest and soil samples

Isolates	Colony shape	Elevation	Edge	Optical characteristics	Colony surface	Pigmentation
FS1	Circular	Raised	Entire	Translucent	Smooth	Cream
FS2	Circular	Convex	Entire	Opaque	Rough	Cream-yellow
FS3	Irregular	Raised	Undulate	Opaque	Dull	Cream
FS4	Circular	Flat	Entire	Translucent	Smooth	Green
NS1	Circular	Raised	Rhizoid	Opaque	Smooth	Yellowish-gray
NS2	Irregular	Flat	Entire	Opaque	Smooth	Cream
N1	Circular	Flat	Entire	Fluorescent	Smooth	Green
N2	Irregular	Flat	Entire	Opaque	Dull	Pale Yellow

Key: FS= Foot path soil, NS= Nest soil and N= Nest

Table 4. Microscopic and biochemical characteristics of bacteria isolated from *R. norvegicus* nest and soil samples

Biochemical characteristic	FS1	FS2	FS3	FS4	NS1	NS2	N1	NN2
Gram Reaction	-	+	+	-	+	+	-	+
Shape	Rod	Cocci	Rod	Rod	Rod	Rod	Rod	Rod
Spore	-	-	+	-	+	+	-	+
Catalase	+	+	+	+		+	+	+
Starch hydrolysis	-	+	+	-	-	+	-	-
Oxidase Reaction	-	-	-	+	-	-	+	-
Citrate Utilization	-	-	+	+		-	+	-
Motility	+	-	+	+	+	+	+	+
Coagulase	-	+	-	-		-		+
KIA Test								
H_2S Utilization	-	-	-	-	+	-		-
Glucose fermentation	+	+	+	+	+	+	+	+
Lactose fermentation	+	+	-	-	-	-	+	-
Sugar fermentation								
Mannitol	AG^-	AG	AG	AG-	A^-G-	A^-G		AG
Lactose	AG	AG	A^-	A^-G	A^-G-	A^-G		A^-G^-
Sucrose	A^-G^-	AG	AG	A^-G-	A^-G^-	A^-G		AG
Fructose	A^-G^-	AG	AG	AG-		AG		AG
Glucose	AG	AG	AG	AG	AG	AG	AG	AG
Maltose	A^-G^-	AG	AG	A^-G-	AG	AG		AG
Arabinose	AG^-	A^-G	A^-	A^-G-		A^-G-		A^-G^-
Gas production	+	+	+	_	_	_	+	+
Methyl Red test	+	_		_	_	_		
Tentatively Identified Microorganisms	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Clostridium sporogenes*	*Bacillus Cereus*	*Ps. Chl.*	*B.brevis*

Key: FS = Foot path soil sample, NS = Nest soil sample and N = Nest, A^- = No acid production, AG^- = Production of acid but not gas, A^-G^- = Negative for both acid and gas, AG = Produce acid and gas, Ps. chl. = Pseudomonas chlororaphis

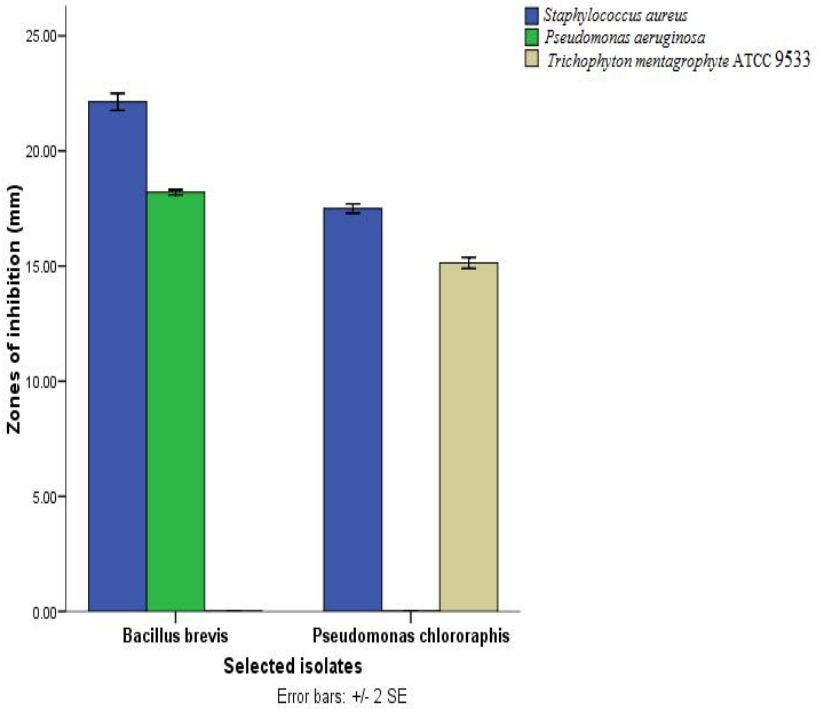

Fig 1. Antagonistic activities of *Bacillus brevis* (*Brevibacillus brevis*) and *Pseudomonas chlororaphis* from brown rat's nest on selected organisms isolated from *Rattus- norvegicus* foot path-soil sample and *Trichophyton mentagrophytes* (*interdigitale*) ATCC 9533

the potential usage of their products (metabolites) in the pharmaceutical industry [13]. The production of toxic compounds by the specie of *B. brevis* isolated from the rat nest evaluated in this study could justify the absence of *S. aureus* and *P. aeruginosa* in such territory. Coupled with the antimicrobial efficacy of the plant materials used in the construction of *R. norvegicus* nest as reported by Owoyemi and Oladunmoye [6,11], the presence of these microbes in the brown's rat nest might also prevent the microbes in the foot-path region of the brown's rat from colonizing the skin of the young rats without fur and thus setting them free of various infections most especially the skin-related ones.

Moreover, the result of this study also revealed the significant antagonistic activities of *P. chlororaphis* isolated from *R. norvegicus* nest on both *S. aureus* and *T. mentagrophytes* (*interdigitale*) ATCC 9533. The antagonistic efficacy of *P. chlororaphis* could be as a result of compounds secreted by it and this corroborates the findings of Ranjbariyan [16] who reported that *P. chlororaphis* secretes a phenazine-like compound which has overt antifungal effect against pathogenic dermatophytes; his study also concluded that *P. chlororaphis* may be considered a rich source of useful metabolites with potential application in antifungal drug discovery.

4. CONCLUSION

The antagonistic potentials observed in *B. brevis* and *P. chlororaphis* isolated from *R. norvegicus* nest against its foot-path soil isolates (*S. aureus* and *P. aeruginosa*) and *T. mentagrophytes* (*interdigitale*) ATCC 9533 [test organism] was proven in this study. However, studies on identification of the active ingredients present in the cultured supernatants (metabolites) of *B. brevis* and *P. chlororaphis* is recommended for control of infections caused by these pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Trichophyton mentagrophytes*) evaluated in this study.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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