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Impact of Bat Guano Fertilizer on Soil Bacteria Community Structure and Antibiogram of Associated Bacteria: An Alert to Food Insecurity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the impact of Bat guano fertilization on soil microbial community structure and antibiotic resistance pattern of recovered isolates.
Study Design: Soil experiment with various Bat guano fertilized farmland soils.
Place and Duration of Study: Department of Microbiology, Renaissance University, Enugu State, Nigeria, between May, 2021 and July, 2021.
Methodology: Physicochemical and microbiological analyses of test soil samples were done following standard methods. Bacterial isolates were identified *via* an analytical profile index (API 20E) test kit, antibiotic resistance pattern of the bacterial species was ascertained using the Kirby Bauer disc diffusion method.
Results: The highest total culturable heterotrophic bacteria count recorded was from bat guano-fertilized soil (8.0×10^5 CFU/g) relative to control (1.09×10^5 CFU/g). Cultured isolates from bat guano-fertilized soils belonged to the genera *Escherichia, Klebsiella, Enterobacter, Hafnia,*

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Staphylococcus, Salmonella, Pleisiomonas, Pseudomonas and *Aeromonas*, relative to the control which had *Aeromonas* and *Staphylococcus*. *Enterobacter* spp. and *Staphylococcus* spp. had the highest frequency of occurrence (18.4%) across the bat guano-fertilized soils. Bat guano also impacted the microbial structure of the soil, introducing potential enteric pathogens, pathogenic bacteria implicated in human and animal diseases and multi-drug resistant bacterial pathogens. Antibiotic susceptibility test revealed that four of the bacterial isolates (*Hafnia alvei*, *Salmonella typhimurium*, *Pleisomonas* sp., and *Klebsiella* spp.) expressed multi-antibiotic resistance to Gentamycin, Cefuroxime, Chloramphenicol, Augmentin, Streptomycin, Septrin, Ofloxacin, Amoxicillin and Ampiclox. Multi-antibiotic resistance indexes of these bacteria were greater than the 0.2 threshold, suggesting the species originated from a potentially dangerous source (i.e. bat guano) and were likely introduced into the soils via faecal contamination (i.e. guano fertilization of soils).

Conclusion: The use of bat guano as organic fertilizer in agricultural lands pose health risks to farmers and consumers of foods (especially those eaten raw or slightly cooked) cultivated with them.This thus, alerts scientific community on the insecurity of food and human health posed by the use of bat guano fertilizer.

Keywords: Antibiotic resistance; bacteria; bat guano; food insecurity; organic fertilizer; soil structure.

1. INTRODUCTION

Among the diverse habitats for microorganisms in nature, the soil ecosystem is one of the major habitats for microbes, where they function significantly in elemental nutrient cycling and transformation of organic matter, carbon, phosphorus, sulfur, and nitrogen, as well as involve in the organic matter breakdown [1]. Guano (Spanish from Quechua: wanu) is the term for the pile of seabirds or bats excreta. Bat guano is an extremely efficient biological (organic) fertilizer, owing to its richness in mineral matter, phosphate and nitrogen, as such, it has been sourced centuries past by agriculturists in several regions of the world, to upscale plant productivity and soil structure [2]. However, in regions where farmers chiefly rely upon bat guano as organic manure, both farmers and consumers of food crops cultivated with the bat guano are at risk of being exposed to guano-inhabiting food-borne pathogens capable of contaminating food crops or infecting livestock which may feed on the crops [3].

In spite of the fact that bat guano harbour beneficial soil microorganisms which offer invaluable services to the ecosystem, bats are recognized as a natural host for diverse mammalian pathogens, viruses inclusive (such as numerous coronaviruses, Ebola, and henipaviruses, that are responsible for several outbreaks including the ongoing COVID-19 pandemic), bacteria (such as *Salmonella* spp., *Escherichia*, *Klebsiella*, and others) and fungi (such as pathogenic yeasts) and these are also recovered from their excreta [3,4]. Balance in the diversity of microorganisms reflects the integrity of a community of microbes and thus could forecast the trend of changes in soil nutrient conditions and soil quality. That is why microbial diversity is recognized as the most sensitive class of bio-indicators [5].

The ongoing COVID-19 pandemic requires concern about the adverse effects posed by anthropogenic activities on ecosystems, which heightens interaction between humans and animals. The World Health Organization's concept of "One Health" in 2017, "to articulate the interrelationship and connection between animal and human health to the health and safety of the environments in which they coexist", further emphasizes this point.

The ease with which pathogenic microbes spread in the environment by contact with other animals, consumption of uncooked foods or drinking of contaminated water, and likely human infection, has led to a quest for knowing the microbiome of bat guano, as these communities may allow for the spread and emergence of new zoonotic disease occurrence. In addition, bacteria resistant to antibiotics and multi-antibiotic resistance have been reported in bat isolates, suggesting the likelihood that bats are environmental hosts for antibiotic resistance [6,7,8].

The multi-antibiotic resistance (MAR) indexing is identified as an efficient and inexpensive technique to track the source of bacteria otherwise known as "bacteria source tracking" [9]. Consequently, the MAR index is a useful marker to determine the life-threatening danger of

pollution [10]. MAR index value above 0.2 is indicative of a high-risk source of contamination where antibiotics are frequently in use.

The majority of studies are chiefly centred on investigating microbial communities living in bats and bat guano. There is a paucity of publications on the effect of bat guano on soil microbial community structure in farmlands and on farmers. There is also limited knowledge of the antimicrobial resistance profile of microbial species recovered from bat guano-impacted soils.

This article seeks to address this knowledge gap by identifying the bacterial communities present in the various bat guano fertilized soils, assessing the impact of the fertilizer on soil bacterial communities, and determining the antibiogram of the associated bacterial species.

2. MATERIALS AND METHODS

2.1 Study Area

This research was conducted at Department of Microbiology, Renaissance University (RNU), Ugbawka, Enugu State, Nigeria which is situated at coordinates: 6.310° N, 7.557° E.

2.2 Collection and Preparation of Soil Samples

Three sets of soil samples were used in this study;

- Farmland soil freshly fertilized with bat guano.
- Aged bat guano fertilized farmland soil.
- Pristine farmland soil (control).

The sample codes and site descriptions are displayed in Table 1.

Sample collection was done according to the method of Parajuli and Duffy [11]. Both aged and freshly fertilized farmland topsoil samples were obtained from diverse locations (Table 1) in RNU, with a sterilized hand trowel, placed in sterile polyethylene bags and labeled samples A, B, C_1, and C_2. Pristine farmland topsoil sample was also collected from a location North of RNU, with a sterilized hand trowel, placed in a sterile polyethylene and labeled “Control”. These samples were taken to the laboratory for more analysis.

2.3 Analysis of the Physico-chemical Characteristics of Soil

Soil particle size was determined following the modified hydrometer method of Andres et al. [12]. The pH of the soil samples was measured using a pH meter (Metler Toledo Seven compact series).

Soil moisture content was measured by the dry oven technique [13]. Five grams (5g) of soil was measured into an already-weighed Petridish (a). The sample was transferred in a Petridish into the oven and allowed to stand for 1 hour at 105^{o}C. After this, the sample was cooled in a desiccator and weighed (b). Percentage moisture is given as:

$$\text{Percent moisture} = \frac{(a - b)}{(\text{Sample weight})} \times 100$$

Where; a = sample weight (wet weight)
b = weight after drying

Soil electrical conductivity (EC) was determined by submerging a calibrated conductivity meter in the samples.

Table 1. Sample codes and description

Sample code	Location	Site Identification
A	RNU	Aged (4 months old) bat guano fertilized farmland soil far East of RNU, farming activity (Yam).
B	RNU	Aged (2 months old) bat guano fertilized farmland soil East of RNU, farming activities [maize, groundnuts, black beans, red pepper, vegetables (fluted pumpkin, waterleaf)]
C_1	RNU	Farmland soil freshly fertilized with bat guano East of RNU, farming activities (groundnuts, black beans, red pepper, fluted pumpkin)
C_2	RNU	Farmland soil freshly fertilized with bat guano North of RNU, farming activity (cassava)
Control	RNU	Pristine farmland soil North of RNU, farming activity (cassava cultivation)

Soil percentage carbon (SOC) and organic matter (SOM) were measured following the [14] approach. The former (SOC) was evaluated by oxidizing with potassium dichromate and titrating with ferrous sulphate using the indicator diphenylamine. Percentage SOM was estimated from SOC via the equation: percentage SOM = percent organic carbon (OC) × 1.724. At end point of the titration, the colour of diphenylamine changed from violet to green. Soil total nitrogen and phosphorus were ascertained following the technique of Samira et al. [15].

2.4 Culture-dependent Analysis

2.4.1 Isolation and enumeration of bacterial species

Bacterial species were isolated and enumerated adopting the technique of Newman et al. [16]. Ten grams (10 g) of each of the bat guano fertilized soil samples were homogenized with 90 ml of sterile distilled water and serial dilutions (10^{-1} to 10^{-6}) were prepared. An aliquot (0.1 ml) from each dilution (10^{-4}, 10^{-5},10^{-6}) was spread-plated on nutrient agar (NA) (Titan Biotech, India), blood agar (BA) (Titan Biotech, India), salmonella-shigella agar (SSA) (Titan Biotech, India) and eosin methylene blue agar (EMB) (Titan Biotech, India). Inoculated agar plates were incubated between 28^{o}C to 37^{o}C for 24 - 48 hours. The colonies observed were enumerated and expressed as colony forming units per gram (CFU/g). Distinct colonies were sub-cultured on fresh agar plates, kept under incubation at 37°C for 24 h to get pure colonies and subsequently transferred to agar slants for further tests. Isolates were labeled in line with their sample codes as displayed in Table 1.

2.4.2 Biochemical characterization and identification of bacterial isolates

To obtain pure isolates, a portion of an isolated colony was streaked on NA and incubated under aerobic conditions at 37°C for 24 h and Gram staining was performed on the isolates. Preliminary biochemical tests like coagulase, catalase, triple sugar iron (TSI) test and oxidase test were done on the isolates following the method of Cheesbrough [17].

Further characterization and identification were performed with the aid of Analytical Profile Index (API 20E) (Biomerieux, France) test strips [18]. The test was conducted, adopting the manufacturer's instructions at the Department of Biotechnology, Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria. Reading and interpretation of results were done accordingly via the API catalog or apiweb: https://apiweb.biomerieux.com

The various tests are represented thus; Orthro-Nitrophenyl-beta-DGalactoPyranosidase (ONPG), Arginine DiHydrolase (ADH), Lysine DeCarboxylase (LDC), Ornithine DeCarboxylase (ODC), Citrate (CIT), Hydrogen sulphide Production (H2S), Urease (URE), Tryptophan DeAminase (TDA), Indole production (IND), Voges Proskauer (VP), Gelatinase (GEL), D-glucose (GLU), D-mannitol (MAN), Inositol (INO), DSorbitol (SOR), L-Rhamnose (RHA), Saccharose (D-Sucrose) (SAC), D-melibiose (MEL), Amygdalin (AMY), L-Arabinose (ARA), CytochromeOxidase (OX), Motility (MOB), MacConkey medium (McC), Fermentation – under mineral oil (OF-F), Oxidation – exposed to the air (OF-O).

2.4.3 Antibiotic sensitivity test and multi-antibiotic resistance (MAR) indices

Antibiotic sensitivity of the bacteria was ascertained by adopting the Kirby Bauer disc diffusion technique described by Cheesbrough [17] and interpretations were made in line with breakpoints of Clinical and Laboratory Standard Institute [19,20]. Mueller-Hinton agar (MHA) was prepared in line with the manufacturer's instructions. Young broth cultures (18-24 h-old) of the isolates were standardized by diluting to 0.5 Mcfarland's standard. A sterile cotton swab stick was introduced into each standardized inoculum (1×10^{8} CFU/ml), drained and spread uniformly onto prepared MHA plates. The inoculated MHA plates (with the lid closed) were subsequently left to stand at room temperature for a few minutes; thereafter the antibiotic-impregnated discs were aseptically placed on the MHA plates, with the aid of sterile forceps. Plates were subsequently kept under incubation at 37°C for 18-24 h. At the end of incubation time, the diameters of the zones of inhibition were measured with a metre rule and recorded in millimetres. Maxi disc antibiotic sensitivity disc (Maxicare Laboratory) was used. Antibiotic-impregnated discs (and concentrations) included; **Gram-negative**: Chloramphenicol (CH) (30µg), Septrin (SXT) (30µg), Ciprofloxacin (CPX) (30µg), Sparfloxacin (SP) (10µg), Streptomycin (S) (30µg) Amoxicillin (AM) (30µg), Augmentin (AU) (10µg), Gentamycin (CN) (30µg), Pefloxacin (PEF) (30µg), Tarivid (OFX) (10µg).

Gram-positive: Amoxicillin (AM) (30µg), Pefloxacin (PEF) (10µg), Rocephin (R) (25µg), Gentamycin (CN) (10µg), Zinnaclef (Z) (20µg), Ciprofloxacin (CPX) (10µg), Streptomycin (S) (30µg), Septrin (SXT) (30µg), Ampiclox (APX) (30µg), Erythromycin (E) (10µg).

It is noteworthy that following the standard by CLSI [19], Ampicillin is representative of Amoxicillin. Results for Ampicillin can be used to predict results for Amoxicillin.

3. RESULTS

3.1 Physico-chemical Characteristics of the Fertilized Soil Samples and Control Sample

Physico-chemical characteristics of the diverse bat guano fertilized soil samples analysed in this study are displayed in Table 2. Across the soil samples, the control soil had the least organic carbon content (0.14%) compared to other soils fertilized with bat guano. Sample code A had the highest Nitrate content (1.74%) compared to the other soil samples; B (1.57%), C_1 (1.32%), C_2 (1.42%), and Control (1.39%). The highest organic matter content was recorded in sample code A (1.09%), while the control had the least organic matter content of 0.42 percent. The particle size analysis as presented in Table 3, suggested that all the soil samples used in this study were sandy [20].

3.2 Enumeration of Bacterial Isolates on Various Microbiological Media

Bacterial counts obtained on the various microbiological media used are displayed in Table 4. Sample A had the highest Total culturable heterotrophic bacterial (TCHB) counts (8.0×10^5 CFU/g), relative to other bat guano fertilized soil samples and control soil. The highest bacterial counts recorded on eosin methylene blue agar (EMB) was from sample C_1 (2.56×10^6 CFU/g), closely followed by sample B (1.52×10^6 CFU/g) and no colony was observed in control soil cultured on EMB. The highest count recorded on salmonella-shigella agar was (1.81×10^6 CFU/g) from sample C_2 and on blood agar (BA) the highest bacterial count recorded was (5.1×10^5 CFU/g).

3.3 Biochemical Identities of Bacteria

The results for the preliminary biochemical characterization and biochemical characterization using the analytical profile index (API 20E) test strip are presented in Table 5 and Table 6 respectively.

Table 2. Physicochemical analysis of the bat guano fertilized soil samples and control

Sample code	pH	Electrical Conductivity (µS/cm)	Moisture content (%)	Organic carbon (%)
A	7.8	336	7.49	0.37
B	8.4	342	7.81	0.26
C_1	8.9	1382	6.09	0.24
C_2	8.7	949	5.51	0.31
Control	8.6	101.5	6.09	0.14

Sample code	Organic matter (%)	Phosphate (%)	Nitrate (%)
A	1.09	0.35	1.74
B	0.77	0.34	1.57
C_1	0.71	0.40	1.32
C_2	0.92	0.49	1.42
Control	0.42	0.36	1.39

Table 3. Particle size analysis of the soil samples

Sample	Sand (%)	Silt (%)	Clay (%)	Textural class
A	97.16	2.84	0.00	Sandy soil
B	98.47	1.53	0.00	Sandy soil
C_1	98.28	1.72	0.00	Sandy soil
C_2	98.41	1.59	0.00	Sandy soil
Control	96.59	3.41	0.23	Sandy soil

Table 4. Mean values of bacterial counts on various microbiological media

Isolates from Sample	NA (CFU/g) ±SD	EMB (CFU/g) ±SD	SSA (CFU/g) ±SD	BA (CFU/g) ±SD
A	8.0 ×10^5±2.8	Negligible	Negligible	4.8 ×10^5±2.8
B	2.7× 10^5±2.8	1.52 ×10^6±2.8	2.15× 10^5±4.2	5.1× 10^5±11.3
C_1	Negligible	2.56 × 10^6±1.4	7.2× 10^4±7.0	Negligible
C_2	1.2× 10^5±1.4	1.41× 10^6±5.6	1.81× 10^6±2.8	3.1× 10^5±5.6
Control	1.09× 10^5±5.6	NIL	NIL	4.2 ×10^5±2.8

**CFU/g= colony forming unit per gram; negligible= < 30 colonies; nil= no colonies observed; sd= standard deviation*

3.4 Distribution and Frequency of Occurrence of Culturable Bacteria across the Soils

The control soil had the least distribution of bacterial isolates, while the bat guano fertilized soils had the highest distribution of bacterial species. The frequency of occurrence of the bacteria across the bat guano fertilized soil samples is presented in Fig. 1. They included *Pseudomonas* (2.60%), *Escherichia coli* (5.25%). *Enterobacter* spp. (18.40 %), *Enterobacter cloacae* (5.20 %), *Citrobacter* spp. (2.60%), *Klebsiella* spp. (15.70%), *Klebsiella pneumoniae ssp. Ozaenae* (2.60%), *Pleisiomonas shigelloides* (2.60%), *Hafnia alvei* (2.60%), *Salmonella spp.* (7.80%), *Salmonella typhimurium* (2.60%), *Salmonella typhi* (5.20%), *Aeromonas hydrophilia* (5.20%), *Staphylococcus aureus* (2.60%) and Coagulase Negative *Staphylococcus* (18.40%) had the highest frequency across the bat guano fertilized soil samples.

3.5 Antibiogram of Bacterial Species

The antibiogram (antibiotic resistance profile) of the bacterial species is shown in Table 7. *Hafnia alvei*, *Salmonella typhimurium*, *Pleisiomonas shigelloides,* and *Klebsiella* spp. displayed multi-antibiotic resistance to some of the antibiotics they were exposed to, while other bacteria were susceptible to the antibiotics used.

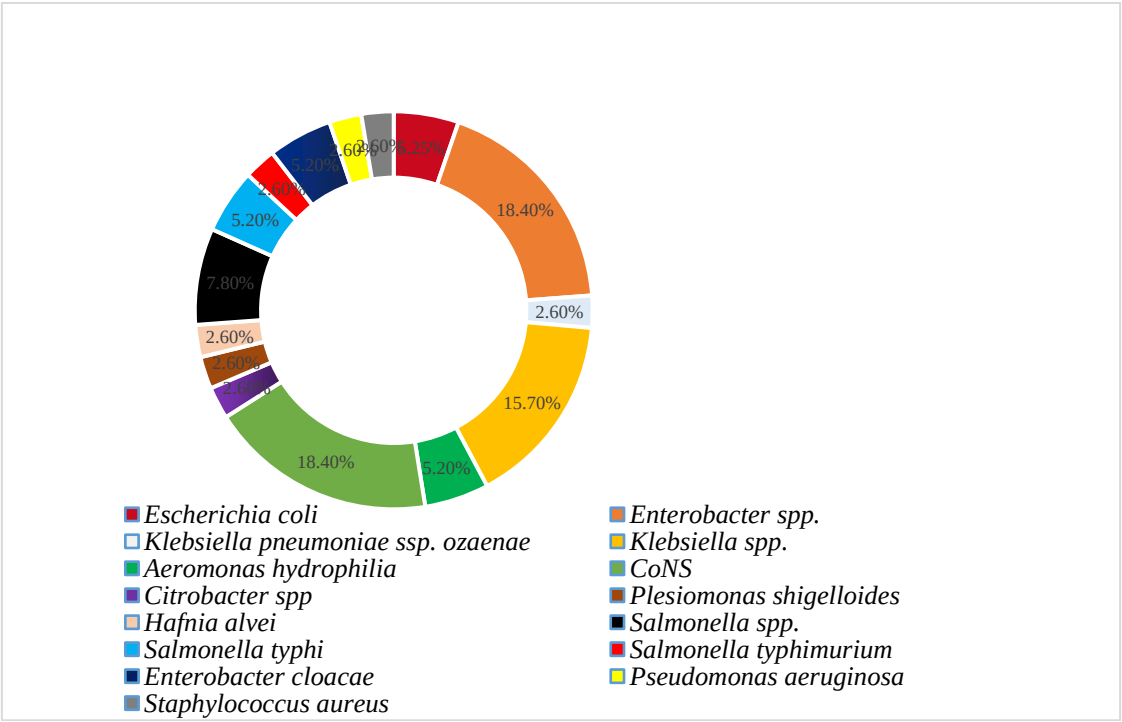

Fig.1. Frequency of occurrence of bacterial species across the bat guano fertilized soil samples

Table 5. Biochemical characteristics & tentative identities of some of the bacteria

Isolate ID	Gram reaction	CAT	COA	TSI	H_2S	GAS production	Tentative ID of organism
B2	-ve rods	-	-	-	-	-	*Hafnia alvei*
B3	-ve rods	+	-	-	-	+	*Salmonella* spp.
B7	+ve cocci in clusters	+	+	NT	NT	NT	*Staphylococcus aureus*
B5, $C_2$9, Control 2, B13, $C_2$3	+ve cocci in clusters	+	-	NT	NT	NT	Coagulase negative *Staphylococcus* (CoNS)
B6	-ve rods	+	-	+	+	+	*Citrobacter* spp.
B15	-ve rods	+	-	-	+	-	*Salmonella typhimurium*

**ID = Identity; NT = Not Tested; CAT= catalase; COA= coagulase; TSI= triple sugar iron; H2S= Hydrogen sulphide*

Table 6. Identification of some bacteria via Analytical Profile Index (API 20E) test

Isolate ID	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO2	N_2	MOB	MoC	OF-O	OF-F	API % SIMILARITYIDENTITY	Tentative Identity of Organisms
Control 1	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	+	-	+	-	+	-	-	+	+	+	+	99.0%	*Aeromonas hydrophilia*
B4, $C_2$7	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	95.0%	*Enterobacter cloacae*
A4	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+	99.9%	*Escherichia coli*
A1, B8	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	+	-	+	+	+	+	99.9%	*Salmonella typhi*
$C_2$1	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+	-	-	+	+	+	99.1%	*Klebsiella pneumoniae* ssp *ozaenae*
B16	+	+	+	+	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	+	-	+	+	+	+	99.9%	*Plesiomonas shigelloides*
A2, B3, B6, $C_1$2	-	-	+	+	-	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+								90.5%	*Salmonella* spp.
B12	+	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	99.5%	*Enterobacter* spp.
A3, A6, $C_2$8' B11, C13	+	-	+	-	+	-	+	+	+	+		+	+	+	+	+	+	+		+	-	+		-				82.5%	*Klebsiella* spp.
A14	+	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	89.5%	*Enterobacter* spp.
$C_1$1	+	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	90.5%	*Escherichia coli*
B14													+															97.8%	*Pseudomonas aeruginosa*

Table 7. Antibiogram of the bacterial species

Antimicrobial class	Antibiotics	CoNS (mm)	*Aeromonas hydrophilia* (mm)	*Salmonella typhi* (mm)	*Salmonella typhimurium* (mm)	*Plesiomonas shigelloides* (mm)	*Hafnia alvei* (mm)
Sulfonamides	SXT	31 (S)	28(S)	24(S)	0(R)	14(I)	25 (S)
Phenicols	CH	NT	NT	20(S)	0(R)	18(S)	NT
Fluoroquinolones	CPX	29 (S)	29(S)	28(S)	28(S)	29(S)	21(S)
	SP	NT	NT	29(S)	28(S)	29(S)	NT
	OFX	NT	NT	22(S)	21(S)	28(S)	NT
	PEF	24 (S)	24(S)	24(S)	23(S)	24(S)	23 (S)
Macrolides	E	32 (S)	NT	NT	NT	NT	NT
Cephalosporins	R (ceftriaxone)	26 (S)	29(S)	NT	NT	NT	20 (I)
	Z (cefuroxime)	17 (I)	19(S)	NT	NT	NT	0 (R)
Aminoglycosides	CN	24 (S)	24(S)	23(S)	0(R)	19(S)	0 (R)
	S	27 (S)	28(S)	24(S)	23(S)	0(R)	27 (S)
Amoxicillin-clavulanate	AU	NT	NT	24(S)	0(R)	0(R)	
Penicillinase-labile penicillins	AM	24 (R)	25(S)	0(R)	0(R)	20(S)	0 (R)
	APX	0 (R)	27(S)	NT	NT	NT	0 (R)

Table 8. Antibiogram of the bacterial species cont'd

Antimicrobial class	Antibiotics	*Klebsiella* spp. (mm)	*Enterobacter* spp. (mm)	*Klebsiella pneumonia* ssp. *Ozaenea* (mm)
Sulfonamides	SXT	0(R)	26(S)	27(S)
Phenicols	CH	0(R)	27(S)	29(S)
Fluoroquinolones	CPX	25(S)	28(S)	29(S)
	SP	25(S)	29(S)	NT
	OFX	0(R)	24(S)	22(S)
	PEF	0(R)	23(S)	23(S)
Cephalosporins	R (ceftriaxone)	NT	NT	NT
	Z (cefuroxime)	NT	NT	NT
Aminoglycosides	CN	0(R)	25(S)	28(S)
	S	21(S)	24(S)	26(S)
Amoxicillin-clavulanate	AU	0(R)	23(S)	23(S)
Penicillinase-labile penicillins	AM	0(R)	26(S)	22(S)

**S=Sensitive, I=Intermediate, R= Resistant, mm= zone of inhibition measured in millimeters, SXT=Septrin, CH=Chloramphenicol, CPX=Ciprofloxacin, SP=spafloxacin, OFX=Ofloxacin(tarivid), E=Erythromycin,R=Rocephin, Z=Zinnat, CN=Gentamycin, S=Streptomycin, AU=Augmentin, AM=Amoxacillin, APX=Ampiclox, CoNS = Coagulase negative Staphylococcus, NT= Not tested*

Table 9. Culturable bacterial diversity and community structure of bat guano fertilized soils

Bacteria community structure	A	B	C_1	C_2	Control
Bacteria	*Klebsiella* spp., *Escherichia coli, Salmonella* spp., *Enterobacter* spp., *Salmonella typhi*, CoNS	*Enterobacter cloacae, Pseudomonas aeruginosa, Citrobacter* spp*., Klebsiella* spp*., Pleisiomonas shigelloides. Hafnia alvei, Salmonella* spp*., Salmonella typhimurium, Aeromonas hydrophilia,* CoNS, *Staphylococcus aureus*,	*Klebsiella* spp., *Escherichia coli, Salmonella* spp.	,*Enterobacter cloacae, Enterobacter* spp., *Klebsiella* spp., *K. pneumoniae* ssp. *ozaenae*, CoNS	*Aeromonas hydrophilia*, CoNS
Coliforms	*Enterobacter* spp., *Klebsiella* spp., *Escherichia coli*	*Enterobacter cloacae, Citrobacter* spp*., Klebsiella* spp*., Hafnia alvei*	*Klebsiella* spp., *Escherichia coli*	,*Enterobacter cloacae, Klebsiella* spp., *Enterobacter* spp.	Nil
Enteric food-borne pathogens	*Escherichia coli, Enterobacter* spp., CoNS,	*Enterobacter* spp., *Pleisiomonas shigelloides, Hafnia alvei*, *Staphylococcus* spp., *Pseudomonas aeruginosa*	*Escherichia coli*	*Enterobacter* spp., CoNS	CoNS
Bacterial pathogens common in human/animal diseases	*Salmonella* spp., *Escherichia coli*, *Klebsiella* spp., CoNS	*Klebsiella* spp., *Staphylococcus* spp., *Hafnia alvei*, *Enterobacter* spp., *Salmonella* spp.	*Salmonella* spp., *Klebsiella* spp., *Escherichia coli*	*Enterobacter* spp., *Klebsiella* spp., CoNS	CoNS
Zoonotic pathogens	Not cultured	Not cultured	Not cultured	Not cultured	Not cultured
Drug-resistant bacterial pathogens	Nil	*Hafnia alvei* (resistant to CN, APX, Z, AM); *Salmonella typhimurium* (resistant to SXT, CH, AM, AU, CN); *Pleisiomonas shigelloides* (resistant to AU, S); *Klebsiella* spp. (resistant to PEF, SXT, CH, AM, CN, OFX, AU)	Nil	Nil	Nil

**SXT=Septrin, CH=Chloramphenicol, CPX=Ciprofloxacin, SP=Spafloxacin, OFX=ofloxacin(tarivid), E=Erythromycin, R=Rocephin, Z=Zinnat, CN=Gentamycin, S=Streptomycin, AU=Augmentin, AM=Amoxacillin, APX=Ampiclox, CoNS = Coagulase negative Staphylococcus*

3.6 Culturable Bacteria Diversity and Community Structure of Bat Guano Fertilized Soils and Control

Bacterial diversity and community structure of the bat guano fertilized soils and control are displayed in Table 9. The bat guano introduced majorly potential enteric pathogens, multi-drug resistant pathogens, and pathogenic bacteria involved in animal and human diseases, to the bat guano fertilized farmland soil samples compared to the control.

4. DISCUSSION

This research focused on evaluating the effect of bat guano fertilizer on soil bacterial communities (diversity and abundance) and the antimicrobial resistance pattern of the bacterial species recovered from the soils. This was necessary following earlier reports about bats and their guano being natural reserves for a diversity of mammalian pathogenic microbes, and the ease of transfer of these pathogenic microbes into the environs following association with other animals, consumption of uncooked food or infected water and likely human infection, especially as it is used as an organic fertilizer to enhance biological and physicochemical characteristics of soil for better crop yield [4].

4.1 Impact of Bat Guano on Soil Physicochemical Characteristics

The freshly fertilized soils (C_1 and C_2) had higher pH compared to the control, suggesting that the addition of bat guano initially slightly increased soil pH (Tables 2 and 3), however, as the bat guano aged and continued to decay, soil pH decreased as seen in soil sample A and B. Corresponding results were documented by Mulec et al. [21,22, 23], who stated that fresh bat guano is slightly alkaline, however, the pH of bat guano changes according to its age, as the pH decreases with the age of the guano. The reason is that, in aged guano, water percolates to form acidic solutions thus reducing the soil pH [23].

Also, the presence of the bat guano increased soil electrical conductivity (which is a measure of soil salinity) compared to the control. However, the electrical conductivity of the freshly fertilized soils C_1 (1382µS/cm) and C_2 (949 µS/cm) were higher than that of soil samples A (336 µS/cm), B (342 µS/cm) and Control (101.5 µS/cm). These results further buttress the impact of aging and continued decay of the bat guano fertilizers on the pH and electrical conductivity of the soils. Also, these results confirm the report of Elango et al. [24,25], that high sodium content (which reflects electrical conductivity) gives rise to high soil pH. It can be observed from the results in Table 2 that soil samples C_1 and C_2 with higher soil electrical conductivity, had higher soil pH relative to soil samples A and B.

Bat guano fertilization across the soils also increased organic carbon content, organic matter content, nitrate, and phosphate compared to the control. The increased percentage of carbon and nitrate elements in this research aligns with the findings of Wurster et al. [26] which showed that bat guano contains high amounts of organic matter, carbon, nitrate, and phosphate. They reported that these elements were higher in aged guano than in fresh guano because of the chemical reactions that occur in aged bat guano to form other minerals, like phosphates [27]. However, in this research the SOM content was recorded in the order thus; A (1.09%) > C_2 (0.92%) > B (0.77%) > C_1 (0.71%) > Control (0.42%). Sample B (aged bat guano fertilized soil) had lower organic matter content relative to fresh bat guano fertilized soil (C_2). This can be attributed to the composition of the bat guano, as it has been reported that bat guano composition varies owing bat species, diets, and geographical location of the site of production [26]. Karimou et al. [23] documented that guano from insectivores bats (i.e. feed on insects) has higher organic matter content relative to frugivores (i.e. bats that feed on fruits).

The particle analysis of both the fertilized soil samples and control showed that they all contained significantly greater proportions of sand which places the samples in a sandy soil textural class. Despite this, the moisture content of the fertilized soil samples was fairly higher than the control's (Table 2), which suggests that bat guano improves soil texture by holding together loose soils and prevent leaching of soil nutrients. This supports the finding of Adam et al. [28].

4.2 Enumeration and Culture-dependent Identification of Bacterial Isolates

Test soil samples including the control had appreciable numbers of bacteria (Table 4). Higher numbers of total culturable heterotrophic bacterial (TCHB) recorded in soil sample A could be attributed to its increased organic matter

content which makes it an excellent source of nutrients for microbial growth and activity [25]. Sample A had negligible (< 30 colonies) population of coliforms, while there were none detected in the control soil. However, in other bat guano fertilized soils (Sample B (1.52×10^6 CFU/g), C_1 (2.56×10^6 CFU/g) and C_2 (1.41×10^6 CFU/g), a high population of coliforms was recorded. Grantina-Ievina and Ievinsh [29] observed similar results. Coliforms were not detected in the soil sample used in their study, but populations of coliforms (4.24×10^4 CFU/g) were detected in bat guano used as organic fertilizer in their study. In this study, *Salmonella* species were observed in high numbers all across the bat guano fertilized soils but not in the control soil. This finding contrasts reports from similar studies conducted by research frontiers [4,16,29,30,31,32] who used both culture-dependent and molecular (High-throughput 16S rRNA sequencing) approach to analyse bat guano samples from different countries in the world (such as India, Serbia, United States of America, Central Slovakia, Netherlands, and others). Several bacterial species most especially from the phyla *Firmicutes* and *Proteobacteria* were reported by these authors, however, *Salmonella* species were not reported by any of these authors as dominant bacterial genera. This contrast could be attributed to influences by environmental factors, variations in bat species, and bat diet [33]. Bat digestive tract (gut) is responsible for the predominant bacterial genera detected in studies.

Culture-dependent method (Analytical Profile Index (API 20E) test kit) used in this study identified 10 bacterial genera (*Enterobacter, Citrobacter, Klebsiella, Pleisiomonas, Hafnia, Salmonella, Aeromonas, Staphylococcus*, *Pseudomonas,* and *Escherichia*) across the bat guano fertilized soils and control (Table 5 and 6). Similar findings were reported by [4,16,30, 31].

4.3 Frequency of Occurrence and Distribution of Bacterial Species Across the Soil Samples

Above thirty-six percent (36.80%) of the culturable bacterial population across the soil samples were equally represented by *Enterobacter* spp. and Coagulase Negative *Staphylococcus* species (Figure 1). [16], however, cultured similar bacterial species but they did not match genera that were identified in lower populations from their molecular analysis. Members of the family *Enterobacteriaceae* (Genus: *Pleisomonas*) and *Pasteurallaceae*, had a greater relative population in bat guano samples used in their study. Also, 15.70% of the cultured bacterial population across the bat guano fertilized soil samples were represented by *Klebsiella* species. A similar finding was reported by Li et al. [34], who studied randomly obtained fresh bat guano from bats with varying dietary sources in Guangdong, Guangxi, and Yunnan, China.

Low population and diversity of cultured bacteria were recorded particularly in the control soil and across the bat guano fertilized soils. This could be attributed to different culture media types used for isolating these bacterial species, competition amongst the species, and growth conditions [16,33]. Bacterial species not recovered via cultivation methods may have more specific nutritional and environmental needs not provided by the selected media used in this study. Isolation or recovery of more bacterial species from bat guano fertilized soils and the control may be done by using molecular techniques to comprehensively analyse bacterial diversity across the soil samples and culturing the soil samples under anaerobic conditions [4,16]. Another reason for the low population of bacteria recorded across the soil samples and control may be a result of the fact that microbial species could be controlled by the richness (abundance) of each specie present in the soil sample, thus causing the richest species or the fastest growing population to create a detectable limit which invariably out-competes or shields the growth of less-rich or abundant population or slow-growing species present in the soil sample [16].

4.4 Antibiogram of the Bacterial Species and Multi-Antibiotic Resistance Indices

The antibiogram of the bacterial species is shown in Table 7, revealed that members of the *Enterobacteriaceae* family; *Hafnia alvei*, *Salmonella typhimurium*, *Pleisiomonas shigelloides,* and *Klebsiella* spp. displayed multi-antibiotic resistance to some of the antibiotics they were exposed to, while other bacteria were susceptible to the antibiotics used. *Hafnia alvei* was resistant to Gentamycin, Ampiclox, Amoxicillin, and Zinnat, which represents the antimicrobial class of antibiotics; Aminoglycosides, Penicillinase-labile penicillins, and Cephalosporins (cefuroxime), respectively.

Salmonella typhimurium expressed resistance to Septrin, Chloramphenicol, Amoxicillin, Augmentin, and Gentamycin, which represents the antimicrobial class of antibiotics; Sulfonamides, Phenicols, Penicillinase-labile penicillins, Amoxicillin-clavulanate, and Aminoglycosides, respectively. *Pleisiomonas shigelloides* was resistant to Augmentin, and Streptomycin, representing the antimicrobial class; Amoxicillin-clavulanate and Aminoglycosides, respectively. While *Klebsiella* spp. expressed resistance to Septrin, Chloramphenicol, Amoxicillin, Augmentin, Gentamycin, Pefloxacin, and Ofloxacin (Tarivid). These are representatives of the antimicrobial class; Sulfonamides, Phenicols, Penicillinase-labile penicillins, Amoxicillin-clavulanate, Aminoglycosides, and Fluoroquinolones, respectively. Dimkić et al. [33] reported similar findings in their review.

The recovery of bacterial isolates with multi-antibiotic resistance from bat guano in Nigeria have also been reported by Ajayi et al. [7,35]. Most of the reported Gram-negative species exhibited resistance to penicillins and cephalosporins. Also, [36] reported for the first time the presence of multi-resistant Extended Spectrum Beta-lactamase (ESBL)-producing *Enterobacteriaceae* in frugivorous bats in Makokou. The species displayed greater resistance to ofloxacin, ciprofloxacin, and tetracycline. The major *Enterobacteriaceae* species that displayed resistance to ESBLs are in the order; *Escherichia coli* > *Klebsiella pneumoniae* > *Enterobacter cloacae*.

The multi-antibiotic resistance (MAR) index of these bacteria was thus; *Hafnia alvei* = 0.44, *Salmonella typhimurium* =0.5, and *Klebsiella* spp. = 0.7. These MAR indices are greater than the 0.2 threshold, suggesting that these bacterial species originated from potentially dangerous sources (i.e. bat guano) and were likely introduced into the soils via faecal contamination (i.e. guano fertilization of farmland soils) of animal (bat) origin.

These challenges of antibiotic resistance reported from this study and supported by several others suggest that the antibiotic resistance exhibited by the bat guano microbial community could differ for location/region and may also be affected or influenced by exposure to medicinal wastes (antibiotic medications) of humans and animals [37].

4.5 Culturable Microbiological Diversity of Bat Guano Fertilized Soils

Microbiological diversity of the bat guano fertilized soils and control as displayed in Table 8, revealed that the bat guano introduced majorly potential enteric food-borne pathogens (*Escherichia coli, Enterobacter* spp, *Pleisiomonas shigelloides, Staphylococcus* spp., *Pseudomonas aeruginosa,* and *Hafnia alvei*), coliforms (*Enterobacter cloacae, Citrobacter* spp*., Klebsiella* spp*., Hafnia alvei*, *Enterobacter* spp. and *Escherichia coli*), multi-drug resistant pathogens (*Hafnia alvei*, *Salmonella typhimurium*, and *Klebsiella* spp.) and pathogenic bacteria involved in human and animal diseases (*Salmonella* spp. *Escherichia coli*, *Klebsiella* spp., *Klebsiella pneumoniae* ssp. *ozaenae*), to the bat guano fertilized farmland soil samples compared to the control. This corroborates the findings of [3, 4,30,33]. In addition, [16] in their study involving functional characterization of the bat guano bacterial community, reported that 0.85–0.87% of protein-encoding genes implicated in human diseases (metabolic diseases, cancers, neurodegenerative diseases, immune system diseases, and infectious diseases) were identified in all the bat guano samples analysed in their study.

5. CONCLUSION

Despite of the limitations of culture-dependent approach, this study has been able to provide relevant data concerning the impact of bat guano fertilization on soil biological (bacterial) diversity and richness (abundance) as well as the antimicrobial resistance pattern of the microbial isolates. The use of bat guano as organic fertilizer in agricultural lands is advantageous owing to its high organic matter and elemental nutrient content, however, it poses more risk to farmers and consumers of foods (especially those eaten raw or slightly cooked) cultivated with them. This was depicted by the greater than the 0.2 threshold multi-antibiotic resistance indices documented in this research, suggesting that the bacterial species originated from a potentially dangerous source (i.e. bat guano) and were likely introduced into the soils via faecal contamination (i.e. guano fertilization of farmland soils) of animal (bat) origin. Also, the bat guano introduced majorly potential enteric food-borne pathogens, coliforms, and pathogenic bacteria implicated in animal, and human diseases to the bat guano fertilized farmland soil samples compared to the control soil.

Howbeit, other bacterial pathogens were sensitive to some of the easily accessible over-the-counter antibiotics like Chloramphenicol, Ampiclox, Amoxicillin, and others. Bat guano fertilization should be done with caution or alternative form of biological fertilizers should be used, such as plant growth promoting microorganisms (PGPM). These PGPM advances plant development, yield and quality in the most sustainable way. Findings from this study, thus, alert Universities of agriculture, Research institutes, Agriculture business firms and Farmers cooperatives, who will consequently inform local farmers via radio, television and private extension agents, on the insecurity of food and human health posed by bat guano fertilizer.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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