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Culture-dependent and metagenomic analysis of lesser horseshoe bats' gut microbiome revealing unique bacterial diversity and signatures of potential human pathogens

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A B S T R A C T

Bats are highly diverse and ecologically important mammals. They harbor various bacteria, viruses, and fungal communities that are either beneficial or potentially pathogenic. Extensive metagenomic studies in bats are limited, particularly for the gut, and to date, there are no reports on the bacterial diversity of *Rhinolophus monoceros* from Meghalaya, India. There are limited studies on the isolation of potential harmful or beneficial bacteria and their interactions with the environment through culture-dependent approaches. Therefore, high-throughput screening was used to understand the population structure, genetic diversity, and ecological role of the microorganisms. High-throughput sequencing of the 16S rRNA marker for gene mapping showed that the gut samples constitute a diverse group of bacteria that is dominated by Proteobacteria, followed by Firmicutes. The bacterial genera *Corynebacterium* and *Mycobacterium* were also observed in the Illumina dataset. Illumina sequencing revealed eight bacterial phyla composed of 112 genera. The metagenomic analysis of the OTUs from the gut revealed diverse bacterial communities as well as zoonotic and human pathogens. There were differences in the bacterial communities between the two methods used in this study, which could be related to host specificity, diet, and habitat. The culture-dependent technique resulted in the isolation of 35 bacterial isolates, of which *Bacillus cereus* and *B. anthracis* are well-known bacterial pathogens that show virulent traits including hemolytic and proteolytic activities. *Pseudomonas stutzeri* is an opportunistic human pathogen that was also isolated and showed similar traits. Antibiotic sensitivity tests were performed on all 35 isolates, and different antibiotics were used for Gram-positive and -negative bacteria. The result showed that some isolates are resistant to antibiotics such as penicillin G and Cefoxitin. This report on gut bacterial communities could attract interest in the possibility of isolating and characterizing bacteria for the production of antibiotics, enzymes, plant growth promoters, and probiotics. However, the presence of potential pathogenic bacteria that may impose health hazards cannot be ignored and needs to be studied further.

1. Introduction

Bats belong to the order Chiroptera and are highly diverse and ecologically important mammals, comprising 20% of known living mammalian species. They feed on nectar, seeds, insects, fruits, fish, frogs, and small mammals [1–4]. Bats have a crucial role in nutrient cycling, and bat guano is rich in nitrogen, carbon, phosphorus, and potassium, making it as an ideal biofertilizer that is marketed in many countries. Bats mostly dwell in caves and are present on every continent in the world except Antarctica [5]. They are highly diverse in their anatomy and lifestyle and probably play a key role in the ecosystem by serving as a natural pest-controlling agent [4,6].

As prey and predators, bats act as a reservoir host of several zoonotic pathogens [7,8]. With more than 1230 bats species throughout

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the world, proper mapping of their microbiome is important to reveal the host-microbe relationship and the possibility of carrying microbial pathogens [9]. There are abundant studies on gut bacterial communities in different animals, but studies on bats' intestinal bacterial communities are inadequate [10,11].

The lesser horseshoe bat (*Rhinolophus monoceros*-) is widely distributed across continents. As bats harbor a diverse community of bacterial species, understanding what constitutes a normal bacterial community and the factors that can influence them could have a profound effect on hosts and ecosystems. Most studies use culture-based approaches to analyze bacterial communities associated with the bat intestinal tract because the bat intestinal environment is nutritionally rich and favors the growth of various microbes [12]. This method has successfully identified bacterial pathogens such as *Salmonella* [13], *Shigella*, *Enterobacter* [14], *Yersinia* [15], and many other intestinal pathogens [16] from bat guts [17]. However, there are limitations in culture-based methods to discover novel and potentially pathogenic bacteria and their interactions with the environment, so the use of culture-independent methods is essential to study the polutation structure, genetic diversity, as well as ecological roles of most microorganisms [18]. Metagenomic studies have used 16S rRNA gene sequencing to characterize gut microbiomes and microbes in the guano of several bat species [19,20] and the feces of other animals to determine their diet and the diversity of metabolic functional genes and enzymes [21,22].

As carriers of pathogenic agents, bats have been found to be associated with more than 200 different types of viruses, including rabies, ebola, and coronavirus [23–26]. Some studies have shown that there may be some novel species associated with and specific to bats as hosts, which may possibly have medicinal importance in humans and other animals [9,27–29]. Analysis of the microbial diversity in bats may help to identify harmful pathogens that can lead to declines in bat populations in caves.

Metagenomic profiling of the bacterial diversity of gut microbiota may provide insights about potential sources of nutrients for plant growth and impacts on human health. So far, there have been no reports on the gut bacterial diversity of *R. monoceros* from the caves of Meghalaya, Northeast India. Thus, we focused our research on metagenomic profiling for bacterial species diversity and explored the role of culturable isolates in terms of their beneficial and detrimental effects on health and the ecosystem.

2. Materials and methods

2.1. Sample site collection

The Arwah Cave (25.2717′N, 91°7308′E) is located at Cherrapunji, Meghalaya (Supplementary Fig.1) is a habitat of many insects and bat species. Geologically, the Khasi Hills is located at the North-eastern extension of the Indian Peninsular Shield [30]. Sampling sites were selected on the basis of available information of the bat roosts and foraging sites. The bats were captured using mist nets and the trapped bats were kept in cages for easy transportation to the laboratory. The DNA was extracted from their gut and were identified using mitochondrial CytB gene amplification and sequenced using primers L14724 (5′ CGCGAAGCTTGATATGAAAAACCATCGTT-3′) and H15149 (5′AAACTGCA GCCCCTCAGAATGATATTTGTCCTCA-3′), that were previously reported for the identification of bat species [31].

2.2. Characterization of bacteria present in the lesser horseshoe bat gut

Three different gut dissection was performed as per [32] with few modifications. Briefly, 1 gm of the sample from each bat was finely ground in a sterile mortar and pestle followed by suspending the aliquot in 1 ml of sterile distilled water. The sample suspension was then mixed thoroughly, and serial dilution was done upto 10^9 and plated onto different bacteriological media like Nutrient Agar, Trypticase Soy Agar, Brain Heart Infusion Agar and Luria Bertani Agar (Himedia). After incubation at 37 °C for 24–48 h the plates were observed and total colony forming units (CFUs) were calculated by plate count technique [33]. Colonies with different morphologies were sub-cultured into pure culture by inoculating in freshly prepared nutrient agar plates [34]. Biochemical tests of the pure cultures were carried out according to Bergey's manual of systematic bacteriology. The bacterial isolates were identified up to genus level according to identification keys available [35–38]. The isolates were tested for Gram staining; motility test [36] followed by catalase and oxidase tests [36]. IMVIC Indole, Methyl red, Voges Praskauer, Citrate utilization and Triple sugar iron tests were performed to identify for genus level identification [39]. Hemolytic activity of the isolates was carried out in triplicates as per [40]. Primary screening of protease enzyme activity was carried out in skim milk agar for testing the ability of the isolates to produce zones of clearance around the colonies which will indicate the production of protease enzyme. The tests were performed in triplicates. All the plates were incubated at 37 °C for 24–48 h and the zones were measured.

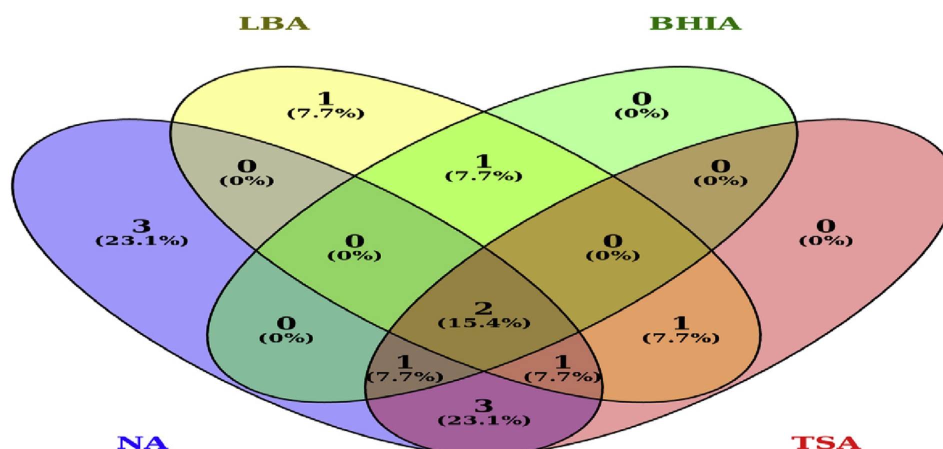

Fig. 1. Venn diagram showing the distribution of bacterial species obtained from different bacteriological media. LA; Luria Bertani Agar, BHIA; Brain Heart Infusion Agar, NA; Nutrient Agar, TSA; Trypticase Soy Agar medium.

2.3. Antibiotic sensitivity tests

Each isolate were individually grown in 10 ml nutrient broth and incubated overnight at 37 °C for 24 h. 0.1 ml of the overnight culture was swabbed onto Mueller-Hinton agar (Sigma, Switzerland) and the antibiotic discs (Sensi Discs, BBL, Becton Dickinson) were placed on the surface of each plate. The antibiotic discs were selected separately for Gram positive and Gram negative bacteria. For gram positive bacteria the antibiotic used include Ciprofloxacin (5 μg), Tetracycline (30 μg), Penicillin G (10U), Chloramphenicol (30 μg), and Vancomycin (30 μg) and Cefoxitin (30 μg). The antibiotics used for gram negative bacteria include Piperacillin (100 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg), Amoxyclav (30 μg), Cephotaxime (30 μg), and Ceftazidime (30 μg). The plates were incubated at 37 °C for 24 h and the diameter of the zones around each disc was measured with reference to interpretive standards [41,42].

2.4. Genomic DNA isolation and 16S rRNA gene sequencing

For bacterial identification, DNA was isolated from pure cultures using a DNA extraction kit (ZymoBIOMICS™ Quick-gDNA™ Miniprep Kit). The 16S rRNA was amplified in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using universal bacterial primer sets 16S-RS-F 5′-CAGGCCTAACACATGCAAGTC-3′ 16S-RS-R 5′-GGGC-GGWGTGTACAAGGC-3’ [43]. Sanger Sequencing was performed using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) as per the manufactures instruction. The sequences obtained from Sanger sequencing were aligned using MEGA 7.0 and manually corrected using the FASTA format. These aligned sequences were then compared in the BLAST (NCBI) database based on the homology sharing percentage for bacterial identification. A similarity rate of 99% was applied for the species level identification while 97% were used for genus level identification. The nearest closest representative of the bacterial isolates were obtained using BLAST search and were used in phylogenetic analysis. A neighbour-joining tree was constructed [44] with Kimura 2-parameter [45] as the model of evolution. Statistical confidence of phylogenetic trees was carried out through bootstrap analysis with 1000 replications. The phylogenetic analyses were generated using MEGA 7.0 version [46,47].

2.5. Metagenomic sequencing and NGS data analysis

2.5.1. DNA extraction

The total DNA extracted from the intestinal region of the bats was stored in 0.5 ml of DNA extraction buffer (100 mM Tris-HCl, 20 mM NaCl and 100 mM EDTA at pH 8). All the samples (in triplicates named as SS, S5 and SL) were placed in sterile containers and immediately stored in liquid nitrogen. DNA extraction was performed with ZymoBIOMICS™ DNA Mini Kit, USA as per manufacturer's instructions. The DNA concentration was quantified using a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm.

2.5.2. Library preparation and PCR amplification of the V1–V4 region of bacterial 16S rRNA gene

The library preparation involves steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual index barcodes to the amplicon target. Hypervariable regions V3–V4 of 16S rRNA gene were amplified using the forward primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW-GCAG; and reverse primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAA-GAGACAGGACTACHVGGGTATCTAATCC) [48]. The PCR condition involved is 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and final elongation at 72 °C for 5 min. High-throughput Illumina Miseq sequence data was generated by Bionivid, Bangalore, India.

2.5.3. Data analysis

Raw fastq sequences were processed and analyzed using QIIME software [49,50]. USEARCH was performed in order to remove chimeric sequences. Preprocessed consensus sequences were clustered into operational taxonomic units (OTUs) using the denovo approach (similarity cutoff = 0.97). Representative sequence for each OTU was classified using the Greengene OTU database. Diversity measurements were performed using QIIME.

2.5.4. Comparative study with gut and guano data

For a comparative study we retrieved data from SRA (Sequence Read Archives) NCBI in fastq format of the bat guano which had previously been published from the same geographical region of the guano microbiome [21] and compared it with the present bat gut metagenome. All the files were quality filtered and sequence of good quality (quality score > 25) was used for the comparison. All the sequences were clustered into OTU based on 97% sequence similarity using pick_closed_reference_otus.py command with the Greengenes database (version 13_5). A closed reference approach using Greengenes as reference was used to pick OTUs. Further, the alpha and beta diversity were carried out after rarefying the OTUs in QIIME pipeline [49,50].

3. Results

3.1. Bat identification

Bats were captured using mist nets at various locations, and species identification was performed using the sequenced amplified Cytochrome B (CytB) gene. Phylogenetic analysis was performed as shown in Supplementary Fig.2 The results confirmed that the collected samples (n = 3) were *R. monoceros* (lesser horseshoe bat), which is a predominant bat species in the Arwah Cave, East Khasi Hills, Meghalaya, India.

3.2. Cultured bacterial community associated with bat gut microbiome

The plate-count-based bacterial enumeration indicated an aerobic bacterial load of 1.86×10^5–1.43×10^8 CFUs/ml in the bat gut microbiome. Bacterial isolates were identified by sequencing the 16S rRNA gene followed by BLAST analysis. The 16S rRNA gene sequencing data of the isolates were further analyzed for media-wise genus level distribution. The 19 isolates retrieved on Luria Bertani Agar (LBA) belonged to *Staphylococcus, Hafnia*, *Serratia,* and *Pseudomonas*. The 11 isolates obtained on Nutrient Agar (NA) belonged to the genera *Escherichia* and *Bacillus,* and 5 isolates retrieved on Brain Heart infusion Agar (BHIA) belonged to *Rhodococcus*, *Enterobacter,* and *Brevibacteria*. The LBA media retrieved maximum cultured bacterial diversity (four different bacterial genera) (Fig. 1).

A total of 35 bacterial species belonging to different genera were isolated from the bat gut by the conventional plating method, of which the most dominant bacterial genera obtained were *Staphylococcus*, *Bacillus,* and *Hafnia* (6 isolates each), followed by *Escherichia* (5 isolates), *Serratia* (4 isolates each), *Pseudomonas* (3 isolates), *Enterobacter, Brevibacteria* (2 isolates each), and *Rhodococcus* (1 isolate). Gram staining revealed 7 Gram-positive cocci, 6 Gram-positive rods, and 22 Gram-negative bacilli isolates. The biochemical results of 35 intestinal isolates are summarized in (**Appendix1)**. The biochemical characteristics confirmed that there were a total of 9 genera in the gut microbiome: *Staphylococcus*, *Hafnia*, *Bacillus*, *Escherichia*, *Brevibacillus*, *Serratia*, *Pseudomonas*, *Enterobacter,* and *Rhodococcus*. Species identification was performed using phylogenetic analysis, and the results are shown in (Fig. 2). The sequences were deposited at NCBI, and the obtained accession numbers are given in (**Appendix2**).

The 35 bacterial isolates identified were *Staphylococcus succinus* (1), *Staphylococcus xylosus* (1), *Staphylococcus equorum* (1), *Staphylococcus lentus* (1), *Staphylococcus scuiri* (2), *Bacillus cereus* (1), *Bacillus aerius* (1),

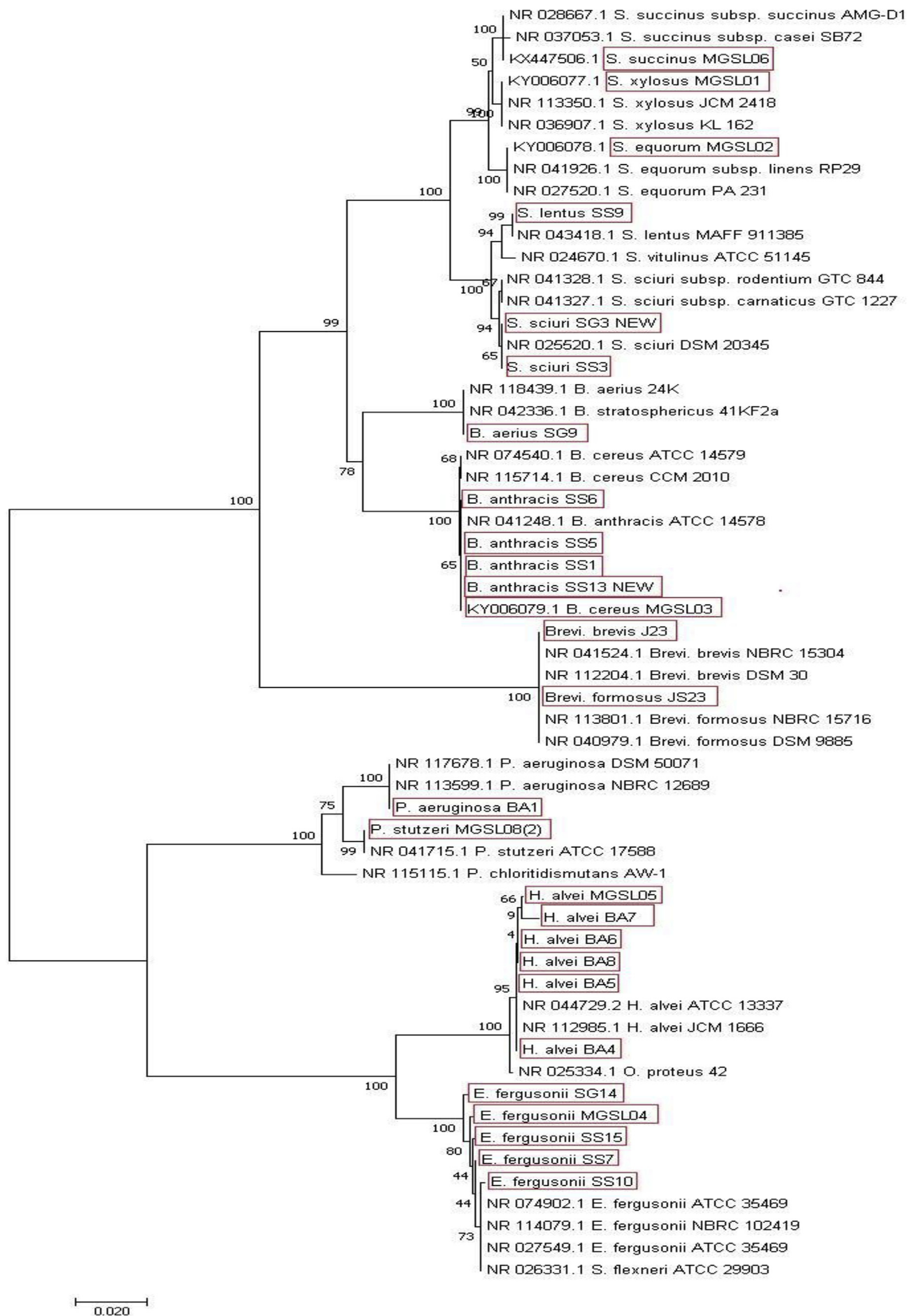

Fig. 2. Neighbour-joining tree of bacterial isolates in this study reconstructed using Kimura 2-parameter model with 1000 bootstrap replications.

Table 1
Antibiotic sensitivity tests performed on bat intestinal isolates. A: Gram negative bacteria; B: Gram-positive bacteria.

	Strain Id	PI	GNM	AMC	CTX	CAZ	CIP
A.	MGSL04	29 ± 1	20 ± 2	16.33 ± 0.57	20 ± 14.73	26.66 ± 1.52	35 ± 2
	MGSL05	20 ± 1	17 ± 1	0	22.33 ± 1.52	16.66 ± 1.15	31.66 ± 2.08
	MGSL08	18.33 ± 1.52	19.33 ± 1.15	20 ± 1	30.33 ± 1.52	16.33 ± 1.15	34.66 ± 1.52
	BA7	26.66 ± 0.57	16.66 ± 0.57	0	24.66 ± 0.57	15.66 ± 0.57	45.66 ± 0.57
	JS23	28.33 ± 0.57	18.33 ± 0.57	36 ± 1	28.33 ± 0.57	9 ± 1	34.66 ± 0.57
	BA8	19 ± 1	15 ± 1	8.33 ± 1.52	23 ± 1	19.33 ± 1.15	27 ± 1
	BA1	24.66 ± 0.57	18.66 ± 0.57	0	22.66 ± 0.57	21.66 ± 0.57	36.66 ± 0.57
	BA6	24 ± 1	17.66 ± 2.08	14.66 ± 1.52	26 ± 2	21 ± 1	29.66 ± 1.52
	BA4	25.33 ± 1.52	18.33 ± 0.57	19 ± 1	30.66 ± 1.52	21 ± 1	28.66 ± 2.08
	SG14	23.66 ± 2.51	20.66 ± 1.15	14.66 ± 2.51	23.66 ± 2.08	27.33 ± 1.52	30 ± 2
	SS10	20 ± 1	18.66 ± 1.15	22 ± 1	41 ± 1	25.66 ± 0.57	29.66 ± 0.57
	BA5	24.33 ± 1.15	17.33 ± 1.15	16 ± 1	25.66 ± 1.15	20 ± 1	37.66 ± 2.51
	SS15	24.66 ± 0.57	16.66 ± 1.15	16 ± 1	28.66 ± 0.57	21 ± 1	35.66 ± 0.57
	SS7	30.66 ± 1.15	18.33 ± 2.08	28.66 ± 1.15	30.33 ± 1.52	R	30 ± 1
	UK2	20.66 ± 0.94	16.66 ± 1.24	R	20.66 ± 0.94	20.66 ± 0.94	31.66 ± 2.62
	SG02	22 ± 0.81	13.5 ± 1.08	R	21.16 ± 0.84	20.66 ± 0.47	29.83 ± 0.23
	SG11	22.5 ± 1.2	16.3 ± 1.2	R	21 ± 0.81	20.6 ± 0.94	23.5 ± 1.08
	SL	22.83 ± 2.01	14 ± 0.81	R	23.83 ± 1.02	21 ± 0.81	27.6 ± 2.05
	MGSL11	21.16 ± 1.02	20 ± 0	20 ± 0	26.83 ± 1.31	16.16 ± 0.84	20.66 ± 0.47
	CSL	31.3 ± 1.24	14 ± 0.81	25.5 ± 1.08	30.66 ± 0.94	11.5 ± 1.08	21.16 ± 1.54
	OR	28.3 ± 1.24	18 ± 0	17.16 ± 0.62	28 ± 1.63	18.33 ± 0.94	40.66 ± 0.94
	M5	30 ± 1	12 ± 2	25 ± 1	30	13 ± 2.64	31.33 ± 1.52
B.	Strain Id	**CIP**	**TE**	**PENI G**	**C**	**VA**	**FOX**
	MGSL01	29.66 ± 0.57	33.66 ± 2.51	22.66 ± 3.78	27.66 ± 2.51	21.33 ± 3.51	27 ± 2.64
	MGSL02	36.66 ± 5.77	21.66 ± 2.88	26.66 ± 1.52	29.66 ± 2.08	20 ± 2	28.66 ± 1.15
	MGSL03	31 ± 1	29 ± 1.73	R	28 ± 2	19.33 ± 1.15	R
	MGSL06	23 ± 2	29 ± 1	17 ± 2	27.33 ± 1.52	17.66 ± 2.51	18 ± 2
	SG09	46.66 ± 4.16	38 ± 2	30.33 ± 1.52	30.33 ± 2.88	24.33 ± 2.51	0
	SG3	27 ± 1	32.33 ± 1.52	19.33 ± 1.52	29 ± 1	17.33 ± 1.15	19 ± 1
	SS3	12.33 ± 16.16	29.33 ± 1.52	20 ± 2	30.66 ± 3.05	15.33 ± 0.57	39.66 ± 1.52
	SS5	R	R	9 ± 1	29.33 ± 1.15	10 ± 1	26.33 ± 1.52
	SS6	19 ± 1	21.66 ± 1.15	17 ± 1	29.66 ± 0.57	41.66 ± 1.52	R
	SS9	28 ± 1	23.66 ± 0.57	30	32 ± 2	26.66 ± 0.57	R
	SS13	34.33 ± 0.57	24.33 ± 0.57	33.66 ± 0.57	33.33 ± 1.15	39 ± 1	R
	SG06	27.66 ± 2.05	29.33 ± 0.47	28.6 ± 0.94	28.3 ± 1.24	31.3 ± 1.24	R
	SS1	20.5 ± 1.2	19.33 ± 1.52	28 ± 1.63	25.66 ± 0.57	17.33 ± 1.15	R

Values are the mean of duplicate experiments and represented as mean ± SD. PI- piperacillin, GNM – gentamycin, AMC – amoxyclav, CTX – cefotaxime, CAZ – ceftazidime, CIP – ciprofloxacin, TE – tetracycline, PENI G – penicillin G, C – chloramphenicol, VA – vancomycin, FOX- orfloxacin. R – Resistance.

Bacillus anthracis (4), *Hafnia alvei* (6), *Escherichia furgusonii* (5), *Brevibacillus formosus* (1), *Brevibacillus brevis* (1), *Pseudomonas stutzeri* (1), *Pseudomonas aeruginosa* (2), *Rhodococcus jialingiae* (1), *Enterobacter xiangfangensis* (1) *Enterobacter cloacaea* (1), *Serratia marcescens* (2), *Serratia liquefaciens* (1), and *Serratia quinivorans* (1). Phylogenetic analysis revealed 2 major and minor clades. Based on the analysis, we observed that the most dominant species was *H. alvei*.

The cultured bacteria were screened for virulence tests in vitro, such as tests for hemolytic activity, protease enzyme production, and screening of the antibiotic resistance profile. Among the 35 isolates, *B. cereus* (MGSL03) isolates SS1, SS5, SS6, and SS13 were identified as *B. anthracis,* and MGSL08 was identified as *P. stutzeri.* These isolates showed zones of beta hemolysis around the colony on blood agar plates and also the secretion of extra-cellular protease enzyme on skim milk agar. The values of hemolytic and protease activity of the isolates are presented in (Supplementary Table 1 **and** Supplementary Figs. 3A–C **and** Supplementary Figs. 4A–C**.**).

The antibiogram profile of the isolates summarized in (Table 1) was measured in millimeters and compared to a standard interpretation chart to categorize the isolates as susceptible, intermediately susceptible, or resistant. *B. cereus* (MGSL 03) showed resistance to penicillin G and Cefoxitin. *B. anthracis* (SS1, SS6, and SS13) showed resistance to only Cefoxitin, and *B. anthracis* (SS5) showed resistance to ciprofloxacin and tetracycline. *P. stutzeri* (MGSL08) did not show resistance to any of the antibiotics tested.

3.3. Metagenomic profile of bat gut microbiome

High-throughput Illumina sequencing of the V3–V4 hypervariable region of the 16S rRNA gene was performed to analyze the bacterial community associated with the gut of *R. monoceros*. The number of reads in the metagenomic sample ranged from 168,114 in sample SS to 209,454 in sample S5. The data from Illumina sequencing have been deposited at GenBank in the Sequence Reads Archive under accession numbers SL (SRR7100944), sample S5 (SRR710095), and sample SS (SRR7100942).

3.4. Metagenomic bacterial community composition

Analysis of the Illumina sequence data revealed the presence of 19 bacterial phyla (Fig. 3): Thermi,Tenericutes,Spirochaetes,Planctomycetes,OD1,Firmicutes, Cyanobacteria, Chlamydiae, Armatimonadetes, Acidobacteria, Verrucomicrobia, TM7, Proteobacteria, OP11, Fusobacteria, FBP, Chloroflexi, Bacteroidetes, and Actinobacteria. Samples SS and S5 showed an abundance of the phylum Proteobacteria (70.4% and 93.2%), whereas sample SL showed an abundance of Firmicutes (50.6%). At the family level, more than half of the identified reads fell under the Enterobacteriaceae family (62.2%). Other dominant families included Streptococcaceae, Staphylococcaceae (2.9%), Peptostreptococcaceae (1.8%), Hyphomicrobiaceae (1.7%), and Moraxellaceae (1.09%). The most abundant genera was *Citrobacter* (9.8%), *Lactococcus* (8.9%), *Staphylococcus* (2.9%), *Devosia* (1.6%), *Acinetobacter* (0.9%), *Arthobacter* (0.9%), *Streptomyces* (0.7%), and *Bacteriodetes* (0.5%). Other dominant genera included *Alkanindiges*,

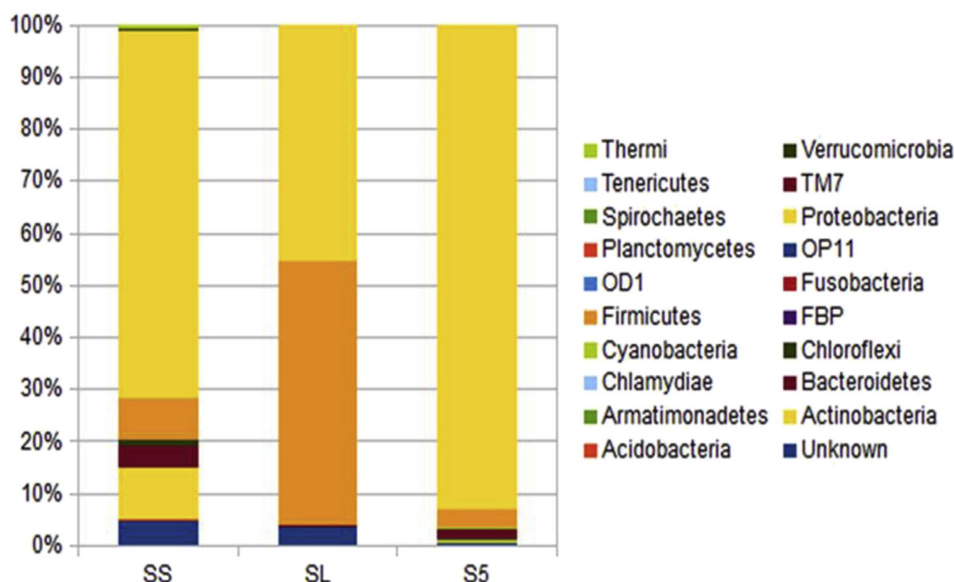

Fig. 3. Phylum level distribution of Illumina sequences of different intestinal bat samples SS, SL and S5.

Hyphomicrobium, *Exiguobacterium*, *Erwinia,* and *Citrobacter*.

A number of identified genera are known to be potential human pathogens, including *Bartonella, Rickettsia*, *Burkholderia*, *Flexispira*, *Arcobacter*, *Clostridium*, *Corynebacterium*, *Mycobacterium*, and *Staphylococcus*. There were also phylotypes belonging to different families (Pasteurellaceae, Neisseriaceae, Helicobacteraceae, and Leptospirae) that harbor zoonotic pathogens. The alpha and beta diversity analysis results are presented in **Appendix 3**. Sample SS showed the highest bacterial diversity (Chao1 value 884.25, Shannon value 5.94, Simpson index 0.94), followed by sample SL (Chao1 780.65, Shannon 3.24, and Simpson 0.78). Sample S5 showed the least diversity (Chao1 320, Shannon 3.05, Simpson 0.78).

3.5. Comparative analysis of gut bacterial diversity in the bats of Arwah Cave and guano

The bat gut microbiome was compared with a previously published report on bat guano samples, which revealed that the distributions of bacterial phylotypes were different between habitats. The beta diversity analysis using the unweighted UniFrac distance revealed that the gut samples SL, SS, and S5 and the guano sample (CPN) contained different bacterial communities (Fig. 4). The guano sample CPN had a different bacterial complex from the gut samples, which had similar bacterial communities, indicating that the three gut samples belonged to the same bat species (*R. monoceros*).

On average, the bat gut microbial communities were dominated by Proteobacteria (70.9%) and Firmicutes (24.9%), whereas the bat guano was dominated by Actinobacteria (32.5%), Proteobacteria (38.4%), and AD3 (18.9%), as indicated in (Fig. 5). A higher alpha diversity (Choa1 index) was also found in the guano sample than the gut microbiomes of the Arwah cave samples (Fig. 6). The results indicate that Proteobacteria is the most dominant bacterial phylum in both types of bat samples. Bacterial phyla such as Actinobacteria, Acidobacteria, and AD3 were abundant in the guano samples but were not found or had very low numbers in the gut samples.

4. Discussion

Bats are ecologically very important and play vital roles like controlling arthropod populations, pollination, biofertilizer production, and recycling processes [51]. Evidence suggests that bats harbor several viruses and fungal pathogens. However, scarce information is available about bacterial pathogens, which constitute a major part of the bat microbiome [52]. Data on the bacterial counts from the intestinal content of *R. monoceros* have not been reported previously.

We used culture-dependent and metagenomic approaches to determine unique bacterial diversity and bacterial pathogens. The results obtained from the culture-dependent method showed a relatively low count of intestinal aerobic bacteria, which ranged from 1.86×10^5 to 1.43×10^8 CFUs/ml in plate count agar. In contrast, other studies on bat intestinal bacteria showed bacterial counts of 1.92×10^{10}–6.10×10^{15} CFU/ml [53]. These differences in bacterial counts suggested by previous studies [54] can be attributed to factors like host specificity, size, diet, and geographical location. The bacterial identification was based on a biochemical test and 16S rRNA gene sequence analysis, which provided more concrete data on the cultured bacteriome of *R. monoceros*.

Of the 35 bacterial isolates found in this study, *B. cereus* (MSGL 03) and *B. anthracis* (SS1, SS5, SS6, SS13) are known bacterial pathogens in humans. Another bacterium isolated was *P. stutzeri* (MGSL08), which is also reportedly an opportunistic pathogen in humans. The virulence cascade and pathogenicity of the isolates remain to be investigated to establish the transmission of these human pathogens from bats. The isolates of *B. cereus*, *B. Anthracis,* and *P. stutzeri* showed common virulence traits such as the production of hemolysin and protease enzyme and resistance to a few antibiotics. Evidence of the presence of *B. cereus* has been previously reported in studies conducted on insectivorous bats [15,55,56]. *B. cereus* has also been previously been reported as an opportunistic human pathogen [57].

This study also reported the isolation of the genus *Enterobacter,* which may have a function of breaking down most sugars, including xylose, which is a principal component of plants [58]. Four species of *Serratia* were also reported, which are known to exhibit cellulolytic properties [59]. We also found that *R. monoceros* harbors *H. alvei* in its intestinal microbiome, which has previously been reported in two other bat species: *Myotis lucifugus* and *M. septentrionalis.* Whitaker et al. [56] reported that it could be a putative chitinase-producing bacterium that helps in the degradation of chitin in insectivorous bats. The presence of the genera *Enterobacter*, *Serratia,* and *Hafnia* in the gut microbiome could contribute to the degradation of food in such bats.

The metagenomic approach using Illumina sequencing of the 16S rRNA gene is routinely used to study the bacterial communities in different species [60]. The paired end Illumina sequencing of the V3–V4 region of the 16S rRNA gene was used for in-depth understanding of the

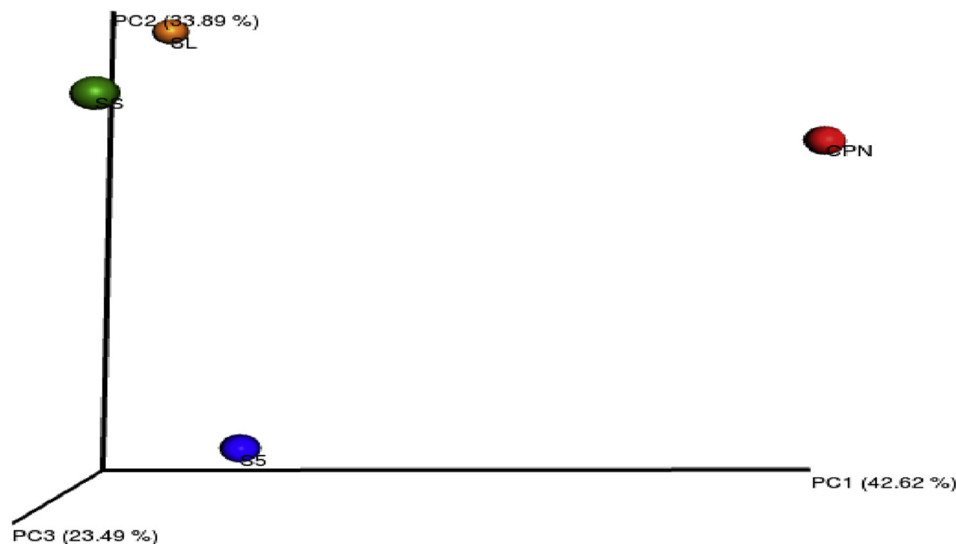

Fig. 4. Comparison of composite guano (CPN) and gut samples (SS,SL and S5) used in this study. Both representatives of guano (Red) are far apart from the rest of fresh bat gut samples (Blue, green and yellow) in PCoA plot generated using the unweighted UniFrac distance.

gut bacterial community of *R. monoceros*. The data generated from the culture-independent approach showed that Proteobacteria was the most dominant bacterial phylum in the bat gut microbiome [61–63]. The second most dominant phylum was Firmicutes, which comprises commensal inhabitants of the intestinal tract [61,64]. According to Phillips et al. [65], herbivorous individuals harbor more diverse microbiota than carnivorous individuals. Interestingly, there was a difference in the distribution of the bacterial families between the three individuals belonging to *R. monoceros* (SS, SL, and S5). This could be due to the differences in individual diets and the specificity of the hosts [66].

The metagenomic data of *R. monoceros* were compared with previously published data on bat guano samples collected from the same geographical region [21] but from different caves and from an unidentified bat species. The results showed that bacterial diversity was

Legend	Taxonomy	Total %	guano %	gut %
	k__Bacteria;p__AD3	9.4%	18.9%	0.0%
	k__Bacteria;p__Acidobacteria	2.8%	5.6%	0.0%
	k__Bacteria;p__Actinobacteria	17.4%	32.5%	2.3%
	k__Bacteria;p__Bacteroidetes	0.8%	0.0%	1.5%
	k__Bacteria;p__Chlorobi	0.0%	0.0%	0.0%
	k__Bacteria;p__Chloroflexi	0.1%	0.1%	0.2%
	k__Bacteria;p__Cyanobacteria	0.1%	0.0%	0.1%
	k__Bacteria;p__Elusimicrobia	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes	12.6%	0.3%	24.9%
	k__Bacteria;p__Fusobacteria	0.0%	0.0%	0.0%
	k__Bacteria;p__Gemmatimonadetes	0.0%	0.0%	0.0%
	k__Bacteria;p__Nitrospirae	0.1%	0.1%	0.0%
	k__Bacteria;p__OP11	0.0%	0.0%	0.0%
	k__Bacteria;p__Planctomycetes	0.1%	0.1%	0.0%
	k__Bacteria;p__Proteobacteria	54.7%	38.4%	70.9%
	k__Bacteria;p__SBR1093	0.0%	0.0%	0.0%
	k__Bacteria;p__Spirochaetes	0.0%	0.0%	0.0%
	k__Bacteria;p__TM7	1.8%	3.5%	0.0%
	k__Bacteria;p__Tenericutes	0.0%	0.0%	0.0%
	k__Bacteria;p__Verrucomicrobia	0.0%	0.0%	0.0%
	k__Bacteria;p__WPS-2	0.2%	0.4%	0.0%
	k__Bacteria;p__[Thermi]	0.0%	0.0%	0.1%

guano

gut

Fig. 5. B Phylum level distribution of Illumina sequences of different intestinal bat samples (SS, SL and S5) and guano (CPN) samples.

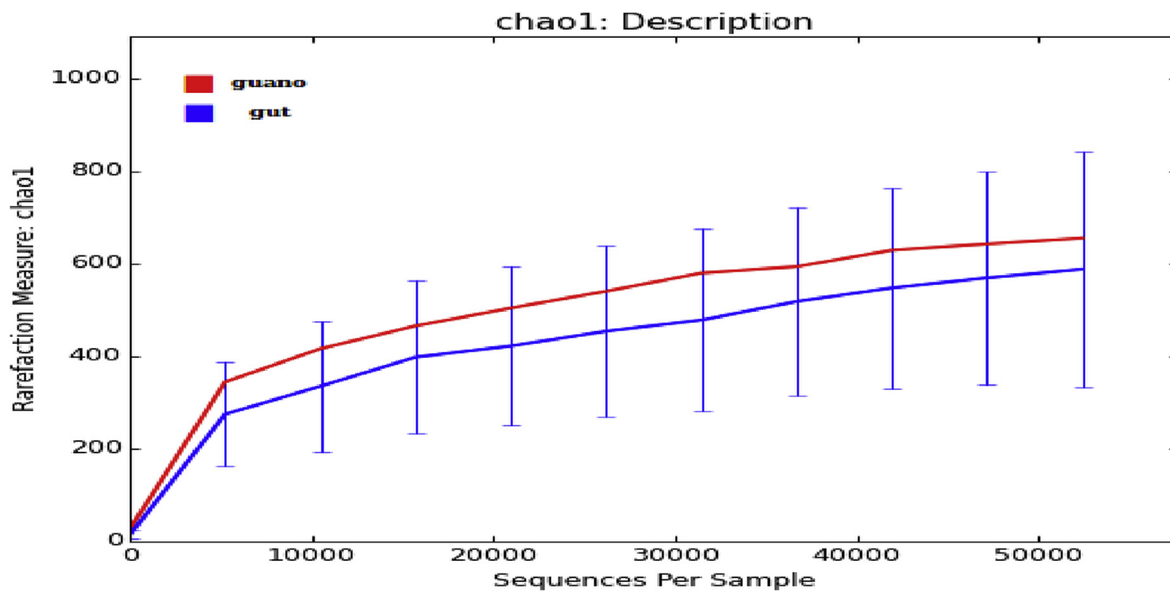

Fig. 6. Comparison of alpha diversity in guano and gut samples.

higher in the guano sample than the gut samples, which might be due to the influence of the cave environment. The results confirm that Proteobacteria is the most dominant bacterial phylum in both bat species. Bacterial phyla such as Actinobacteria, Acidobacteria, and AD3 were abundant in the guano samples but were not found and or had very low numbers in the gut samples. The bacterial abundance in the gut microbiome depends on the niches of the bat. However, differences in the sampling process can influence the inferences drawn from the metagenome because fecal and intestinal samples differ substantially. Fecal samples are best for analyzing the microbiome in the context of host diet, whereas intestinal samples are best for analyzing the evolution of the host [67].

We also identified several genera representing potential pathogens, as reported in other bat microbiome studies [19,68]. The identified phenotypes included opportunistic pathogens such as *Bartonella*, which can infect healthy people, usually through vectors such as ticks, fleas, sand flies, and mosquitoes [69]. Dietrich et al. [61] found that *Bartonella* is commensal in many bat species, which suggested that it may also be transmitted within bat populations through behavioral transmission. Another identified genus was *Rickettsia*, which are obligate intracellular parasites associated with both human and plant diseases that are transmitted through arthropods like chiggers, ticks, fleas, and lice [70].

The genera *Corynebacterium* and *Mycobacterium* were also observed in our Illumina dataset. *Corynebacterium diphtheria* can cause diphtheria, skin infections, and septicemia, while *Corynebacterium ulcerans* is recognized as a causative agent in the re-emergence of diphtheria. They are mainly transmitted through droplets, secretions, or direct contact [71]. The genus *Mycobacterium* is a causative agent for tuberculosis. A few reports also describe a potential etiological relationship between *M. avium paratuberculosis* and Crohn's disease in humans, but the issue is still under debate [72]. Similar findings were also observed by Banskar et al. [19]. Two bacterial genera, *Staphylococcus* and *Streptococcus,* may cause pneumonia, meningitis, bacteremia, infectious lesions, neonatal infections, and septicemia [73,74]. We observed that the *R. monceros* gut microbiome also harbors known pathogenic microbes, but their pathogenicity has not been determined.

Metagenomic analysis of the OTUs from the gut revealed diverse bacterial communities as well as zoonotic and human pathogens. The Illumina sequencing also revealed eight bacterial phyla comprising 112 genera, which are largely dominated by *Citrobacter*, *Lactococcus*, *Staphylococcus*, *Devosia*, *Arthrobacter*, *Acinetobacter*, *Streptomyces*, *Ochrobactrum*, *Dietzia*, *Bacteroides*, *Enterococcus*, *Brevundimonas*, and *Lentzea*. Hence, using both culture-dependent and metagenomic approaches, abundant bacterial species were isolated. However, when using the culture-dependent approach, the identified bacterial species were limited in comparison to the metagenomic approach. This could be due to the inability to cultivate some of the bacterial species, which could only be extracted by the metagenomic approach, where the total DNA is extracted from the bat gut.

Using the culture-dependent approach, a total of 35 isolates were identified in the gut samples (n = 3 individuals), and the total viable count was between 2.86×10^5 and 1.43×10^8. The most predominant family was Enterobacteriaceae. The cultured bacteria revealed a unique diversity and included *H. alvei*, *E. furgusonii*, *S. marcescens*, *S. liquefaciens*, *S. quinivorans*, *P. stutzeri*, *P. aeruginosa*, *E. xiangfangensis*, *E. cloacaea*, *S. succinus*, *S. xylosus*, *S. equorum*, *S. lentus*, *S. scuiri*, *B. cereus*, *B. aerius*, *B. anthracis*, *B. formosus*, *B. Brevis,* and *R. jialingiae*.

In conclusion, the high-throughput sequencing of the 16S rRNA marker gene mapping showed that the gut samples harbor a diverse group of bacteria that are dominated by Proteobacteria, followed by Firmicutes. The genera *Corynebacterium* and *Mycobacterium* were also observed in the Illumina dataset. The Illumina sequencing revealed eight bacterial phyla comprising of 112 genera. The metagenomic analysis of the OTUs from the gut revealed diverse bacterial communities as well as zoonotic and human pathogens. There were differences between the two methods used, which could be related to host specificity, diet, and habitat. This first report on the bat gut microbiome collected from Arwah Cave could stir scientific interest in the possibilities of isolating and characterizing novel bacteria with both beneficial and harmful effects. These bacteria could contribute to gut metabolism and the development of anti-infectives and probiotics. Some may also act as plant growth-promoting bacteria and degrade enzymes like cellulase and chitinase. Human pathogenic bacteria were also identified, but their virulence traits in humans have yet to be determined.

Conflicts of interest

The authors declare no conflicts of interest.

Ethics statement

Due to ethical considerations and permit limitations, three individuals *Rhinolophus monoceros* were collected. Ethical clearance was

obtained from the Chief Conservator of Forest of the state of Meghalaya States, (No: 23(4)/(MEMO.NO.FWC/G/173/PT-III/2270/74). In total, 3 intestinal biopsy samples were used for this study and it was conducted under proper biological safety conditions.

Consent for publication

The guano data which was retrieved from SRA (Sequence Read Archives) NCBI in fastq format of the bat guano which had previously been published by De Mandal has been performed with the authors consent and relevant information has been given separately.

Authors' contributions

SL designed the objectives of the paper, performed all experimental work and analysis. SL and SDM written the manuscript; SDM performed the NGS analysis; DS and HS provided ideas and infrastructure to conduct the research and corrected the final manuscript; NSK coordinated the NGS study, and corrected the final manuscript; GS helped in the designing the work and outlining the paper and JS planned, coordinated the whole study and corrected the final manuscript. All authors revised and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2019.103675.

Appendix 1. Biochemical tests performed on intestine isolates according to gram type (+) - positive, (−) - negative, GPC – Gram positive cocci, GNB – Gram negative bacilli, GPB – gram positive bacilli, K/K – base by base, A/A – acid by acid, M- motile, NM – non motile

Strain.no	GRAM STAIN	CATALASE	OXIDASE	INDOLE	MR	VP	CITRATE	TSI	HEMOLYTIC ACTIVITY	MOTILITY	IDENTIFICATION
MGSL01	GPC	+	-	-	+	-	-	K/K	-	NM	*Staphylococcus xylosus*
MGSL02	GPC	+	-	-	-	-	-	K/K	-	NM	*Staphylococcus equorum*
MGSL03	GPB	+	+	-	+	-	-	K/K	β-Hemo	NM	*Bacillus cereus*
MGSL04	GNB	-	+	+	+	-	+	K/A	-	NM	*Escherichia furgusonii*
MGSL05	GNB	-	+	-	+	-	+	K/A	-	NM	*Hafnia alvei*
MGSL06	GPC	-	-	-	+	-	+	K/A	-	NM	*Staphylococcus succinus*
MGSL08	GNB	+	+	-	+	-	+	K/A	-	NM	*Pseudomonas pstutzeri*
JS23	GNB	-	-	-	-	-	-	K/K	-	NM	*Brevibacillus formosus*
M5	GNB	-	-	-	-	-	-	K/K	-	NM	*Brevibacillus brevis*
BA1	GNB	+	+	-	-	-	+	K/K	β-Hemo	M	*Pseudomonas aeruginosa*
BA4	GNB	-	+	-	-	+	+	A/A	-	NM	*Hafnia alvei*
BA5	GNB	+	+	-	-	-	+	A/A	-	NM	*Hafnia alvei*
BA6	GNB	-	-	-	+	+	-	A/A	-	M	*Hafnia alvei*
BA7	GNB	-	+	-	+	+	-	K/A	-	M	*Hafnia alvei*
BA8	GNB	-	+	-	+	-	+	K/K	-	NM	*Hafnia alvei*
SS1	GPB	+	-	-	+	-	-	K/A	-	NM	*Bacillus anthracis*
SS3	GPC	-	-	-	+	-	+		-	NM	*Staphylococcus scuiri*
SS5	GPB	-	-	-	-	-	-	A/A	-	NM	*Bacillus anthracis*
SS6	GPB	-	+	-	-	-	+	K/K	-	NM	*Bacillus anthracis*
SS7	GNB	-	+	+	+	-	+	A/A gas	-	M	*Escherichia furgusonii*
SS9	GNB	-	-	-	+	-	+	K/A	-	NM	*Staphylococcus lentus*
SS10	GPC	-	-	+	+	-	+	A/A gas	-	NM	*Escherichia furgusonii*
SS13	GNB	+	+	-	-	-	+	A/A	-	NM	*Bacillus anthracis*
SS15	GPB	-	+	+	+	-	+	A/A	-	NM	*Escherichia furgusonii*
SG3	GPC	-	-	+	+	-	-	K/K	-	NM	*Staphylococcus scuiri*
SG9	GPB	-	-	+	+	-	-	K/K	-	NM	*Bacillus aerius*
SG14	GNB	-	+	+	+	-	-	A/A	-	NM	*Escherichia furgusonii*
CSL	GNB	-	+	+	+	+	-	A/A	-	M	*Pseudomonas*
MGSL11	GNB	-	+	+	+	+	+	A/A	-	M	*Serratia*
SG02	GNB	-	+	+	+	+	+	K/K	-	M	*Serratia*
OR	GNB	-	+	+	+	+	+	K/K	-	M	*Serratia marcescens*
SG11	GNB	-	+	+	+	+	+	A/A, gas	-	M	*Enterobacter hormaechei*
SG06	GPR	+	_	+	-	-	+	K/K	-	NM	*Rhodococcus* spp
UK2	GNB	-	+	+	+	+	+	K/K	-	M	*Serratia quinivorans*
SL	GNB	-	+	+	+	+	+	K/K	-	M	*Enterobacter* spp

Appendix 2. Accession no of bacterial 16S rRNA gene sequences used in the phylogenetic analysis

Bacteria species	Strain no	GeneBank accession no.
Staphylococcus xylosus	MGSL01	KY006077
Staphylococcus equorum	MGSL02	KY006078

Bacillus cereus	MGSL03	KY006079
Staphylococcus succinus	MGSL06	KX447506
Pseudomonas pstutzeri	MGSL08	KY006080
Brevibacillus formmosus	JS23	MF509664
Brevibacillus brevis	M5	MG593959
Pseudomonas aeruginosa	BA1	MG593960
Bacillus anthracis	SS6	MG593961
Hafnia alvei	BA4	MG593962
Hafnia alvei	BA5	MG593963
Hafnia alvei	MGSL05	MG593964
Staphylococcus scuiri	SG3	MG593965
Bacillus anthracis	SS5	MG593966
Staphylococcus lentus	SS9	MG593967
Escherichia furgusonii	SG14	MG593968
Hafnia alvei	BA7	MG593969
Hafnia alvei	BA8	MG593970
Escherichia furgusonii	MGSL04	MG593971
Bacillus anthracis	SS1	MG593972
Staphylococcus scuiri	SS3	MG593973
Escherichia furgusonii	SS7	MG593974
Escherichia furgusonii	SS10	MG593975
Escherichia furgusonii	SS15	MG593976
Hafnia alvei	BA6	MG593977
Bacillus anthracis	SS13	MG593978
Bacillus aerius	SG9	MG583979
Pseudomonas aeruginosa	CSL	MH285867
Serratia liquefaciens	MGSL 11	MH285868
Serratia marcescens	SG02	MH285869
Serratia marcescens	OR	MH285870
Enterobacter xiangfangensis	SG11	MH285871
Rhodococcus jialingiae	SG06	MH285872
Serratia quinivorans	UK2	MH285873
Enterobacter cloacaea	SL	MH285874
Rhinolophus monoceros 1	L3	MH356774
Rhinolophus monoceros 2	L2	MH356775
Rhinolophus monoceros 3	H3	MH356776

Appendix 3. Analysis of alpha diversity by Chao, Shannon and Simpson index

Sample ID	Chao	Shannon	Simpson
CPN	783	4.854564	0.896401
SS	884.25	5.949008	0.944343
SL	780.6517	3.249033	0.785309
S5	320	3.054009	0.787326

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