Parasitology International 112 (2026) 103228

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Research paper

Beyond the usual suspects: Uncovering less-recognized pathogenic bacteria in Ghanaian blood-feeding *Amblyomma variegatum* ticks using 16S rRNA amplicon sequencing

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ARTICLE INFO

Keywords:
Tick microbiome
Amblyomma variegatum
16S rRNA sequencing
Less-recognized tick-borne pathogens
One health surveillance

ABSTRACT

Ticks are important vectors of bacterial pathogens affecting both human and animal health. In Ghana, *Amblyomma variegatum* is the predominant cattle-infesting tick, yet most studies have focused on a limited range of well-characterized pathogens, potentially overlooking a broader diversity of less-recognized, emerging, or opportunistic bacteria. In this study, we used 16S rRNA amplicon sequencing to characterize the bacteriome of partially blood-fed *Am. variegatum* ticks, with emphasis on underexplored taxa. As ticks were blood-fed at the time of collection, some detected microorganisms may represent transient, host-derived bacteria rather than endogenous tick microbiota; therefore, findings should also be interpreted within the context of xenosurveillance.

Partially-fed ticks were collected from cattle across three ecological zones within Ghana's Greater Accra Region. 11 *Am. variegatum* ticks, confirmed through both morphological and molecular analyses, were subjected to high-throughput sequencing, and bacterial diversity and composition were analysed using established bioinformatics tools. Sequencing generated over 1.75 million high-quality reads and 3172 amplicon sequence variants. Five dominant bacterial phyla were detected, with Actinomycetota and Bacillota being the most abundant. While *Rickettsia* spp. were prevalent in some samples, *Anaplasma* and *Coxiella*, two commonly studied tick-borne bacteria, were not detected. Several less-recognized or opportunistic species, including multidrug-resistant *Corynebacterium resistens* and bovine-associated *Porphyromonas levii*, were identified at high relative abundance.

These findings suggest that *Am. variegatum* may harbor a broader range of bacterial taxa than previously recognized. Incorporating such neglected microorganisms into a One Health tick-surveillance framework may improve disease risk assessment and guide public and animal health interventions in the region.

1. Introduction

Ticks are obligate blood-feeding ectoparasitic arthropods of mammals, birds and reptiles, which transmit various pathogens to their vertebrate host [1]. They are considered second to mosquitoes in their ability to transmit pathogens of veterinary and medical importance [2]. As highlighted by the World Health Organization's One Health approach, many zoonotic diseases, are often spread through these

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https://doi.org/10.1016/j.parint.2025.103228
Received 29 September 2025; Received in revised form 26 December 2025; Accepted 26 December 2025
Available online 29 December 2025

ectoparasites [3,4]. Such diseases include tick-borne bacterial infections such as Lyme disease, anaplasmosis, ehrlichiosis, and rickettsiosis, which pose significant threats to global human and animal health, healthcare systems, as well as the economy [5,6].

Pathogenic bacteria, which are responsible for these diseases, along with non-pathogenic bacteria present in the tick bacteriome [7,8], collectively influence vector competence by shaping the transmission dynamics of these diseases [9,10]. These bacteria also play a role in enhancing host fitness by providing nutrients such as vitamin B and other compounds that are not naturally produced by the host [11,12]. Among these populations are well-known pathogens of tick-borne bacterial infections, including *Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., and others [13]. Other tick-borne bacteria, of low pathogenicity, which could also be transmitted during feeding, include *Spiroplasma* spp., *Candidatus* Midichloria mitochondrii, as well as certain species of *Rickettsia, Coxiella* and *Francisella* [14–16]. Yet still, there are many other tick-borne bacteria, which are linked to significant human and animal infections, but have often been challenging to detect using molecular tools or isolate through culture methods due to their low levels in host ticks or inability to be propagated outside the tick's internal environment, respectively [17,18]. For instance, *Ca.* Neoehrlichia mikurensis is often missed due to its low abundance [19,20], while *Rickettsia africae* is difficult to culture due to its intracellular nature [21]. As a result, these bacteria are less recognized, and often overlooked as important tick pathogens, leading to the exclusion of their associated diseases from the list of recognized tick-borne illnesses.

Until the arrival of next generation sequencing (NGS), the molecular-based detection of tick-borne pathogens has mainly targeted well-known tick-borne pathogens, often to the neglect of these less-recognized, but potentially virulent species [22]. With the emergence of NGS, researchers are now able to explore the bacteriome in ticks through metagenomics [23,24], or metatranscriptomics [25,26], analysing various developmental stages and organs of ticks [8,27,28]. NGS enables the non-specific detection of all bacterial sequences in a single sample by targeting the V3-V4 region of the 16S rRNA gene, found in almost all bacteria [29–32]. This approach is valuable for identifying and assessing the possible transmission of both known and overlooked pathogenic bacteria in blood-feeding ticks [30,32–35].

In Ghana, several tick species have been reported, as well as the pathogens and diseases they transmit [36–43]. Such ticks species include *Amblyomma variegatum,* which has been reported to be the predominant species infesting cattle in Ghana, constituting about 60 % of ticks collected from cattle. [38,41,43–46]. *Am. variegatum* (tropical bont *tick*) is a three-host tick, originally endemic to Africa, but has since spread to several countries including the Caribbeans [47–49]. It is an opportunistic, generalist feeder capable of parasitizing a wide range of vertebrate hosts. Reported blood-feeding hosts include cattle, goats, sheep, horses, donkeys, dogs, several wildlife ungulates such as antelopes and buffalos, and occasionally birds [50]. It is implicated as a vector or potential vector for several diseases, which include Crimean-Congo haemorrhagic fever virus, Dugbe virus, yellow fever virus, *Ri. africae* (African tick bite fever) and Jos virus [51]. In Ghana, various bacterial pathogens have been found in *Am. variegatum* ticks, including *Ri. africae*, *Ri. aeschlimannii*, *Ehrlichia canis*, *Theileria velifera*, *T. mutans*, and a symbiont *Ca.* Midichloria mitochondrii. [52,53]. However, other harmful bacteria, such as *E. ruminantium*, dermatophilosis-causing bacteria, and several others, have not been identified in Ghanaian *Amblyomma* ticks, despite being diagnosed in Ghanaian animals serologically [54]. Employing the 16S rRNA metagenomic approach on blood-feeding *Am. variegatum* ticks may facilitate the detection of such known pathogenic bacteria. In addition, this approach may also help in the identification of less-recognized, but potentially virulent species in these blood-feeding ticks. Furthermore, this approach will possibly provide insights into the microbiome of blood-feeding *Am. variegatum* in Ghana [55]. The efficient detection and identification of these less-recognized, but potentially pathogenic bacteria in blood-feeding ticks can help in the improved assessment of transmission risk, and the development of robust strategies for the control and prevention of tick-borne diseases. In the present study, we analysed the bacteriome of blood-feeding *Am. variegatum* ticks, using the 16S rRNA amplicon sequencing approach for the first time in Ghana, and assessed the possible presence of pathogenic bacteria, with special emphasis on less-recognized or overlooked species.

2. Methods

2.1. Study area

The study was carried out in the Greater Accra Region (GAR) of Ghana, which harbours Ghana's capital city, Accra. It is the smallest of the 16 administrative regions in Ghana, covering approximately 3245 km^2. The region is further subdivided into 29 local government areas (LGAs), the majority of which are urban. For this study, GAR was divided into 3 geographical zones, namely; central (Adenta Municipal, La Dade-Kotopon), eastern (Ada East, Shai Osudoku, Kpone Katamanso) and western (Ga South Municipal, Ga North Municipal) based on similar vegetation features (Fig. 1).

The GAR is generally categorised as tropical savannah with high temperature and humidity levels [56,57], however, increased human activity in various areas have led to slight differences in land cover. While the eastern zone generally assumes a typical grasslike vegetation feature, the vegetation of the central zone has changed mostly to bare lands with sparsely distributed grasses and shrubs, due to high human activity in this zone. The western zone vegetation, however, generally consists of dense clusters of small trees, shrubs and grasses. In the GAR, there is a high rate of cattle rearing for meat and milk production, and for ploughing of land for crop production. There have also been reports of high tick infestation in the kraals where the cattle are housed [41,44,58–60], hence our selection of the GAR as our study site.

2.2. Tick collection and processing

An initial number of 204 partially-engorged adult hard ticks were collected from 5 Sanga (*Bos taurus africanus/Bos taurus* × *Bos indicus*.), 85 Sanga Cross (*Bos taurus* × *Bos indicus*) and 10 N'dama (*Bos taurus taurus*) breed cattle aged at least 6 months from 2 selected farms within each district in the study sites from June to July 2020. Hard ticks attached to the animals' skin were aseptically collected using fine forceps, which were wiped with 70 % ethanol after each tick removal, and transported in specimen collection tubes to the laboratory. Morphological identification of ticks was done by observation of ticks under a dissecting microscope, using standard taxonomic keys [61]. The ticks were stored in 2 mL tubes containing RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4 °C until the extraction of nucleic acids later that week.

2.3. Nucleic acid extraction and purification

Prior to extraction, individual ticks were successively washed in 500 μL of hydrogen peroxide (H_2O_2), 70 % ethanol, and double distilled water (ddH_2O) by vortexing for 15 s in each solution to remove surface contaminants. The ticks were air-dried and each was ground into fine powder using a mortar and pestle, kept in liquid nitrogen. Total nucleic acid was isolated from each individual ground tick powder with the Quick-DNA/RNA Miniprep Kit (Zymo Research, Irvine, CA, US) following the manufacturer's protocol. RNase treatment was performed for each sample to obtain pure genomic DNA (gDNA) using NucleoSpin® Clean-up XS kit (Takara Bio. Inc., Shiga, Japan) according to the manufacturer's protocol. The concentration and purity of each gDNA was measured using the Nanodrop 2000™ (Thermo Scientific, Delaware, ME, USA).

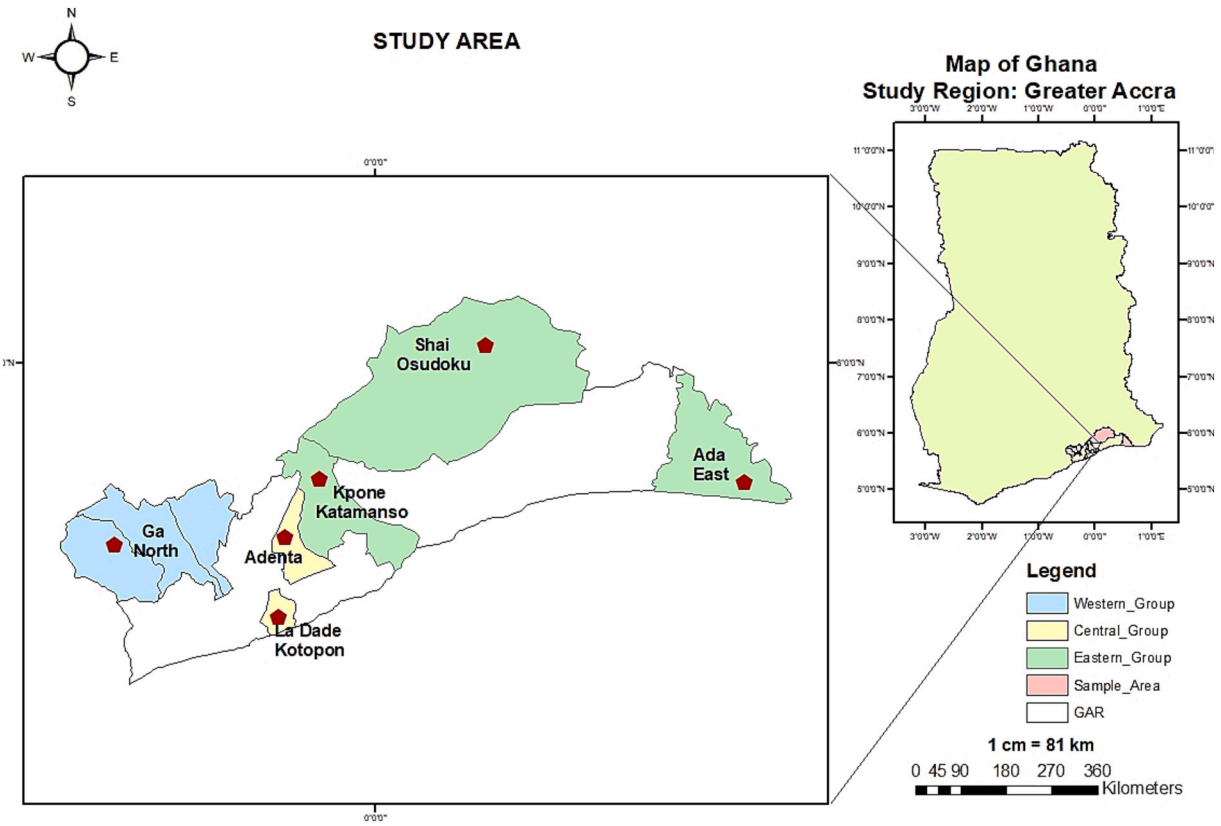

Fig. 1. Sampling locations of cattle ticks from Greater Accra Region (GAR), Ghana. The red pentagons indicate the sampling sites in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Polymerase chain reaction

PCRs to amplify tick Cytochrome oxidase I (COI) and 16S rRNA genes, were performed on individual gDNA samples, with primer sets previously used for the amplification of a 680 bp region of tick COI [62], and a 460 bp region of tick 16S rRNA [63]. Nuclease-free water served as a negative (no template) control. The reaction mixtures for PCR were prepared using the KOD One™ PCR Master Mix (Toyobo, Japan), and amplification was done using a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), with the following cycling conditions; initial denaturation at 98 °C for 1 min, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at either 55 °C (COI) or 57 °C (16S rRNA) for 5 s and extension at 68 °C for 1 s, with a final extension performed at 68 °C for 1 min. The PCR products were separated on a 1.5 % ethidium bromide-stained agarose gel by electrophoresis. PCR products were then purified from the gel with Viogene Gel/PCR DNA isolation system (Viogene Biotek, Taiwan) according to the manufacturer's protocol.

2.5. Sequencing analysis

Sequencing analysis was performed on amplified fragments of the COI and 16S rRNA genes, after the products were cleaned with EXOSAP-IT™ (Themofisher Scientific, USA) according to manufacturer's protocol. The purified amplicons were directly sequenced in both directions using Sanger technology by an independent external company (Eurofins Genomics K.K., Tokyo, Japan).

2.6. Molecular identification of ticks and phylogenetic analyses

The sequences obtained were validated from the chromatograms using BioEdit Sequence Alignment Editor version 7.2.5 (https://bioedit.software.informer. com/7.2/), and confirmed sequences were subjected to a BLAST Search using the National Center for Biotechnology

Information's (NCBI) nucleotide BLAST (BLASTN) program webtool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Primer regions were trimmed off and partial sequences of the COI (~419 bp) and 16S rRNA (~324 bp) genes were used as BLAST queries for *Am. variegatum* species identification. Using these sequences, further multiple sequence alignment was performed with *Am. variegatum* reference sequences retrieved from GenBank by MAFFT web program (https://mafft.cbrc.jp/alignment/server/). Phylogenetic analyses were then run for both COI and 16S rRNA sequences obtained under the maximum likelihood criterion in MEGA v.11 [64]. The analysis was run under the T92 + I model with bootstrap resampling of 1000 replicates for the COI gene. The sequences of the COI genes of *Rhipicephalus microplus* and *Am. parvum* were used as out-groups. A similar analysis was also run under the T92 + G model with bootstrap resampling of 1000 replicates for the 16S rRNA gene. The sequences of the 16S rRNA genes of *Rh. microplus* from Colombia and Mozambique were used as out-groups. The newly generated sequences of the COI and 16S rRNA genes were submitted to the GenBank database under the accession numbers in Table 1.

2.7. High-throughput 16S amplicon sequencing and data processing

Tick gDNA samples (Table 1) were selected for bacteria 16S rRNA amplicon sequencing based on the following criteria: concentration > 5 ng/μL with 260/280 purity ratio ~ 1.8, and the location of sample collection within the GAR. The V3-V4 variable region of the bacteria 16SrRNA were paired-end sequenced on the Illumina platform (250 bp paired-end) using the primers 341F (5'-CCT AYG GGR BGC ASC AG-3′) and 806R (5′-GGA CTA CNN GGG TAT CTA AT-3′) at the Novogene Bioinformatics Technology Co., Ltd. (Japan K.K., Tokyo, Japan). A total

Table 1
Details of study sites and ticks selected for metagenomic sequencing.

Districts	Sample Name	Collecting Sites	Geographical zone within the GAR	Tick sex	Morphological ID	Molecular ID		Gene accession IDs	
						COI	16SrRNA	COI	16SrRNA
Adentan	AD.20.022	Frafraha	Central	Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835925	LC835917
	AD.20.025	Frafraha		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835926	LC835918
	AD.20.026	Frafraha		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835927	LC835919
La Dade Kotopon	ACA.20.003	Burma Camp		Male	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	–	LC835916
Ada East	AE.20.014	Ada Kasseh		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835928	LC835920
Shai Osudoko	SO.20.020	Agomeda	Eastern	Male	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835931	LC835924
Kpone Katamanso	KP.20.006	Appolonia		Male	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835930	LC835923
Ga North	GN.20.003	Ayawaso Amamoley	Western	Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	–	–
	GN.20.004	Ayawaso Amamoley		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	–	LC835921
	GN.20.019	Pokuase		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	–	LC835922
	GN.20.024	Pokuase		Female	*Am. variegatum*	*Am. variegatum*	*Am. variegatum*	LC835929	–

of 1,754,133 demultiplexed paired-end reads were obtained following sequencing and initial quality trimming. Data viewing and further sequence processing were performed with QIIME 2023.7 [65]. A table of amplicon sequence variants (ASVs) and representative sequences were obtained following sequence dereplication, joining of paired reads and chimera filtering with *dada2* plugin [66]. Taxonomic classification was performed using a custom naïve bayes classifier that was extracted from SILVA 138.1 SSU reference [67] based on the primers used. The resulting taxonomic table and representative sequences were filtered to exclude mitochondria, chloroplasts, Archaea and Eukaryota. A rooted phylogenetic tree was constructed with the filtered representative sequences for diversity analyses.

2.8. Statistical analyses

A *phyloseq* object was constructed with the filtered ASV table, rooted tree, taxonomically classified sequences and the sample metadata in R for further analyses. To remove singletons (ASVs that are represented only once), the ASV table was further filtered to retain only taxa that were observed more than once in at least 5 % of the samples. The sequencing depth across samples was also normalised. Alpha and beta diversity analyses were performed on rarefied sequences. Pairwise Wilcoxon tests were used to compare alpha diversity between sample groups. The differences in microbial composition between groups were estimated based on Bray-Curtis distances and statistically tested with the PERmutational Multivariate ANOVA (PERMANOVA) [68]. All visualisation was performed with custom scripts in R [68].

3. Results

3.1. Identification of ticks collected

Individual ticks collected from the cattle were first identified morphologically using standard taxonomic keys. *Am. variegatum* was found to be the predominant tick species, constituting 103 (50.5 %) of the 204 ticks initially collected (Supplementary material 1: Table S1). For the purposes of this study, eleven ticks, individually morphologically identified as *Am. variegatum,* were eventually selected for microbiome analyses, based on the concentration and purity of gDNA extracted, as well as the location of sample collection site within the GAR, as already described. Each tick was analysed separately, allowing assessment of bacterial diversity and composition at the level of individual ticks. Species identification of the 11 tick samples was confirmed by PCR and sequencing analyses of the COI and 16S rRNA genes. Further BLAST analyses of the DNA sequences of all 11 samples revealed high homology (> 90 %) with other *Am. variegatum* sequences stored in GenBank. Detailed information of the 11 tick samples is shown in Table 1.

To obtain further information about *Am. variegatum* ticks in the GAR of Ghana, further phylogenetic analyses were performed (Fig. 2). Samples with ambiguous base readings within their COI and/or 16S rRNA sequences, were however excluded from these analyses. Phylogenetic trees generated by the maximum likelihood method were used in constructing dendrograms for *Am. variegatum,* using the COI and 16S rRNA gene sequences. T92 + I and T92 + G models were used in constructing the COI and 16S rRNA dendrograms, respectively. Both dendrograms revealed a general clustering of all study samples with other *Am. variegatum* isolates previously reported around the world,

Greater Accra region, GAR; mitochondrial cytochrome *c* oxidase subunit 1, COI including Ghana (Fig. 2a and b), suggesting a close relationship and high similarity between our tick samples and other isolates reported elsewhere. The GenBank accession numbers of the COI and 16S rRNA sequences included in the phylogenetic analyses are also listed in Table 1.

3.2. Sequence data statistics

Targeted bacterial 16S rRNA metagenomic sequencing of the11 ticks generated 1,754,133 paired-end reads. Taxonomic classification identified 3435 as bacterial. Following normalization and filtering out ASVs that were present less than once in at least 5 % of the tick samples, 3172 ASVs remained from 674,791 'clean' reads. The average and median of number of reads were 61,345 and 64,503, respectively.

Rarefaction curves (Fig. 3) were drawn to evaluate the coverage of sequences within a sample, by graphing the number of observed sequences relative to sequencing depth [69]. The rarefaction curves showed that the samples approached their true diversity within the first 1000 to 2000 reads (Fig. 3a) and demonstrated that the sequencing depth sufficiently depicted the microbial community diversity. The number of bacterial ASVs generally ranged from 0 to 400 across the

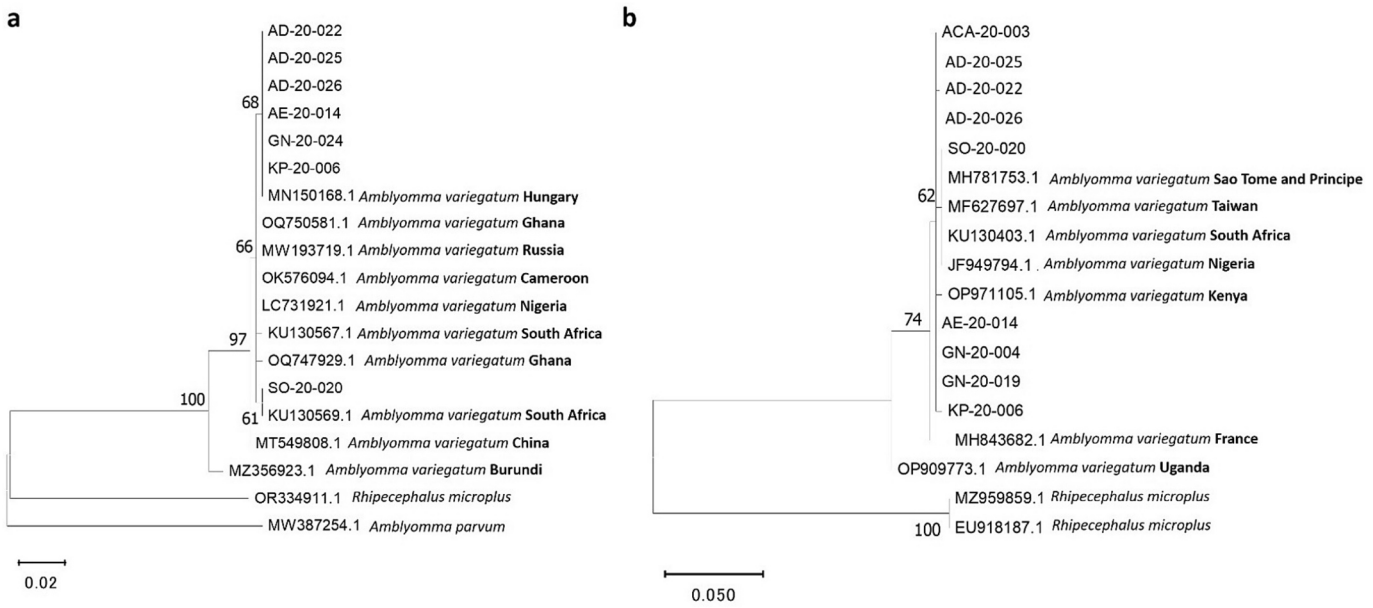

Fig. 2. Molecular phylogenetic analysis based on mtDNA (a) COI and (b) 16S rRNA sequences for exploring the relationships among other *Amblyomma variegatum* strains. Phylogenetic trees were inferred by the maximum likelihood method, based on COI and 16S rRNA obtained from *Am. variegatum* collected samples. GenBank accession numbers of all the reference sequences used to construct the trees are indicated. The numbers at the branches indicate the bootstrap values for 1000 replicates (only bootstrap scores higher than 50 % are shown). The scale bars represent the distance in substitutions per nucleotide.

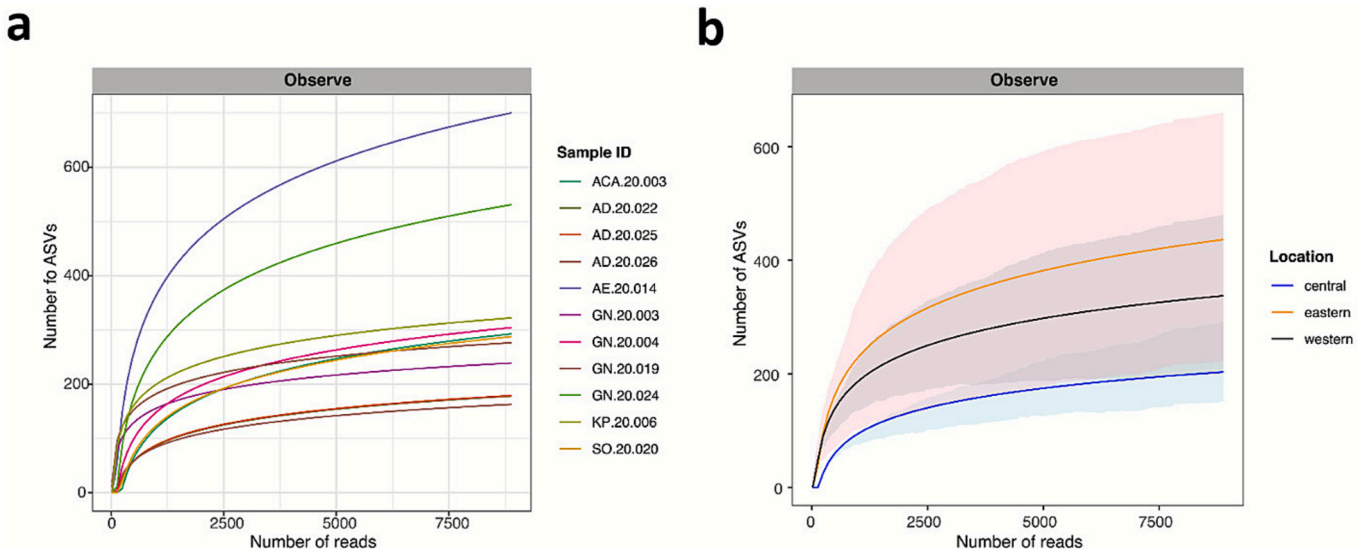

Fig. 3. Rarefaction curves based on the (a) individual ticks and (b) location. (ASVs: Amplicon Sequence Variants).

different locations (Fig. 3b), although one sample from the eastern zone and one from the western zone each exceeded 400 ASVs at the individual sample level (Fig. 3a).

3.3. Alpha diversity

Microbial diversity within each group was estimated using Pielou's evenness, Shannon, and Gini-Simpson indices. Each tick was processed individually to capture diversity at the single-tick level (Supplementary material 2: Table S2). The number of observed taxa did not vary significantly across locations; however, ticks from the central zone exhibited lower diversity and evenness compared to other regions (Fig. 4). Specifically, the Gini-Simpson index in the central zone had a mean of 5.06 (95 % CI: 1.86–8.26), compared to 22.3 (0.0–60.5) in the eastern zone and 21.3 (4.81–37.7) in the western zone. Similarly, the Shannon index was lowest in the central zone (mean 0.760, 95 % CI: 0.529–0.991) compared to 0.934 (0.807–1.06) in the eastern zone and 0.943 (0.898–0.989) in the western zone.

Male and female ticks exhibited similar diversity, with Gini-Simpson means of 13.4 (0.0–29.0) for males and 17.0 (3.95–30.0) for females, and Shannon means of 0.832 (0.521–1.14) for males and 0.898 (0.836–0.960) for females, indicating no significant differences between sexes (Supplementary Fig. S1).

3.4. Beta diversity between ticks from different locations

Ticks from the three locations shared 230 bacterial taxa, representing ~15–20 % of the taxa identified in each region (Fig. 5a). Pairwise comparisons indicated that central and eastern ticks shared 10.6 % of taxa, central and western shared 10.6 %, and eastern and western shared

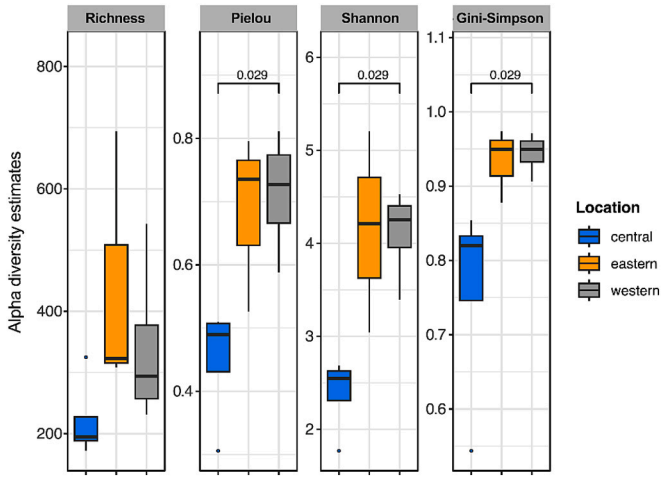

Fig. 4. Alpha diversity (cluster levels observed, Pielou, Shannon, and Simpson indexes) of the tick microbiome based on different locations. $P < 0.05$. Pairwise Wilcoxon tests.

9.7 %. The eastern zone had the highest number of unique taxa, while the number of taxa shared between each pair of locations was relatively similar (Fig. 5a). The reported percentages of shared taxa represent the number of taxa shared between groups (numerator) divided by the total number of unique taxa identified across the indicated regions (denominator), clarifying the basis for these comparisons.

The diversity between groups (β-diversity) was also visualized using multidimensional scaling (MDS) with weighted UniFrac and Bray-Curtis distances to assess the microbial community diversity based on phylogeny and bacteria counts, respectively. The locations shared phylogenetically similar bacteria (Fig. 5b) although the results indicated that there was ~21 % variation between them (PERMANOVA: $R^2 = 0.2097$, $F = 1.062$, $P = 0.37$) (Fig. 5c). They varied more widely and significantly based on bacterial count alone (PERMANOVA: $R^2 = 0.345$, $F = 2.106$, $P = 0.013$) (Fig. 5d).

3.5. Bacterial composition in Am. variegatum

The bacterial community of the tick samples were composed of 5 major phyla: *Bacteroidota*, *Bacillota* (formerly *Firmicutes*), *Pseudomonadota* (formerly *Proteobacteria*), *Actinomycetota* (formerly *Actinobacteriota/Actinobacteria*), and *Fusobacteriota*, which made up ~98 % (Fig. 6a; Supplementary material 3: Table S3). Among these, *Actinomycetota* (39.6 %) was most abundant and the next dominant was Firmicutes (19.0 %) (Fig. 6a). Only 12 bacteria genera had average relative abundance above 1 %, and included *Lachnospiraceae, Anaerococcus, Helcococcus, Peptoniphilus, Bacteroides, Rhodococcus, Trueperella, Porphyromonas, Muribaculaceae, Rickettsia, Fusobacterium,* and *Corynebacterium* (Fig. 6b; Supplementary material 4: Table S4). *Corynebacterium* was a predominant bacterial genus present in all samples and averaged 38.5 % of the bacteria across samples. Notable bacteria species were generally scanty and lowly represented in most samples. For example, the well-known tick-borne bacteria, *Rickettsia*, was identified as high as 72 % in one tick from the central zone, but absent in others from the same zone.

Metastat pairwise analyses were used to identify bacterial species that were differentially abundant between ticks collected. Here we only showed the bacteria species that had at least 1 % relative abundance in any of the two groups being compared. *Porphyromonas* spp. were abundant in ticks from the central zone compared to the eastern (Fig. 7a). *Corynebacterium resistens*, which accounted for ~15 % of the bacterial community of ticks collected in the central location, was also significantly higher than that found in eastern and western samples (Fig. 7a and b). Ticks from the eastern and western locations differed in *Porphyromonas levii* which had relative abundance of 0.2 % and 3 % respectively (Fig. 7c). Given that the sampled ticks were blood-fed at collection, a portion of the bacterial taxa detected here may reflect host-derived or transient organisms, rather than endogenous tick microbiome, limiting the ability to distinguish between superficial and internal bacterial communities.

4. Discussion

In the last 5 to 10 years, there has been a noticeable rise in the occurrence of tick-borne diseases (TBDs) on a global scale [70], particularly those of bacterial aetiology. This surge has been propelled by the expansion of clinically relevant tick-borne bacterial species [5,71]), along with the introduction and broader use of advanced screening/diagnostic technologies, such as NGS [34]. These technologies have improved tick surveillance, resulting in the enhanced detection of less-recognized pathogens and the identification of new pathogens. Ultimately, offering valuable insights into assessing disease risks through the high-throughput analyses of pathogen infection rates in ticks [34,72,73].

In the present study, we investigated the potential occurrence of both well-known and less-recognized tick-borne bacterial pathogens in blood-feeding *Am. variegatum* ticks, across three geographical zones in the GAR of Ghana. This was done by analysing the bacteriome present, using the 16S rRNA amplicon sequencing technology for the first time in Ghanaian ticks. With this, we were able to go beyond the typically targeted pathogens and uncover bacterial signatures that may have otherwise remained undetected by conventional molecular diagnostics.

Consistent with previous studies in Ghana [38,41,44,74], our study also showed that *Am. variegatum* remains the primary tick species infesting cattle, constituting about 50 % of ticks collected. Therefore, our focus on *Am. variegatum* reflects its significance as the major tick species parasitizing cattle in Ghana. Moreover, the phylogenetic clustering of Ghanaian *Am. variegatum* ticks, together with other *Am. variegatum* isolates reported within and outside of Africa, suggests a broader significance of this study, not just limited to Ghana, but to other countries within sub-Saharan Africa and beyond. Furthermore, *Am. variegatum* functions as a major carrier for pathogenic bacteria such as *Ehrlichia* spp. and *Rickettsia* spp. [54,75,76]. Although, only 3–4 ticks were analysed per location, the sequencing depth achieved was sufficient to robustly characterize microbial richness and overall diversity within individual ticks and across locations, consistent with previous small-sample tick microbiome studies [77,78]. As such, the dataset provides a meaningful initial baseline for understanding the bacterial communities of *Am. variegatum* in the study area and offers valuable guidance for future, larger-scale sampling designed to support broader surveillance and ecological assessments. Consistent with patterns commonly observed in arthropod microbiomes, the communities were dominated by a small number of highly abundant taxa, accompanied by a broader array of low-abundance species. [69]. Results of the alpha-diversity analysis indicated variations in tick bacteriome richness across the three geographical locations, with central exhibiting lower diversity than eastern and western. Beta-diversity analysis using weighted UniFrac and Bray-Curtis distances revealed differences in microbial community composition between locations. However, each location also exhibited unique bacterial diversity. The observed variations in diversity within, and across the 3 zones could be attributed potentially to the influence of local environmental factors and/or differences in bacterial flora of these locations, as well as the host blood source, on tick-associated microbiota, as suggested previously [79]. It is thus likely that host community differences may contribute to regional beta-diversity patterns observed in this study, but cannot be conclusively assessed due to a lack of host-specific data. Future research should therefore focus on targeted ecological surveys or molecular blood-meal analysis to better link host identity with tick microbiome variation across regions.

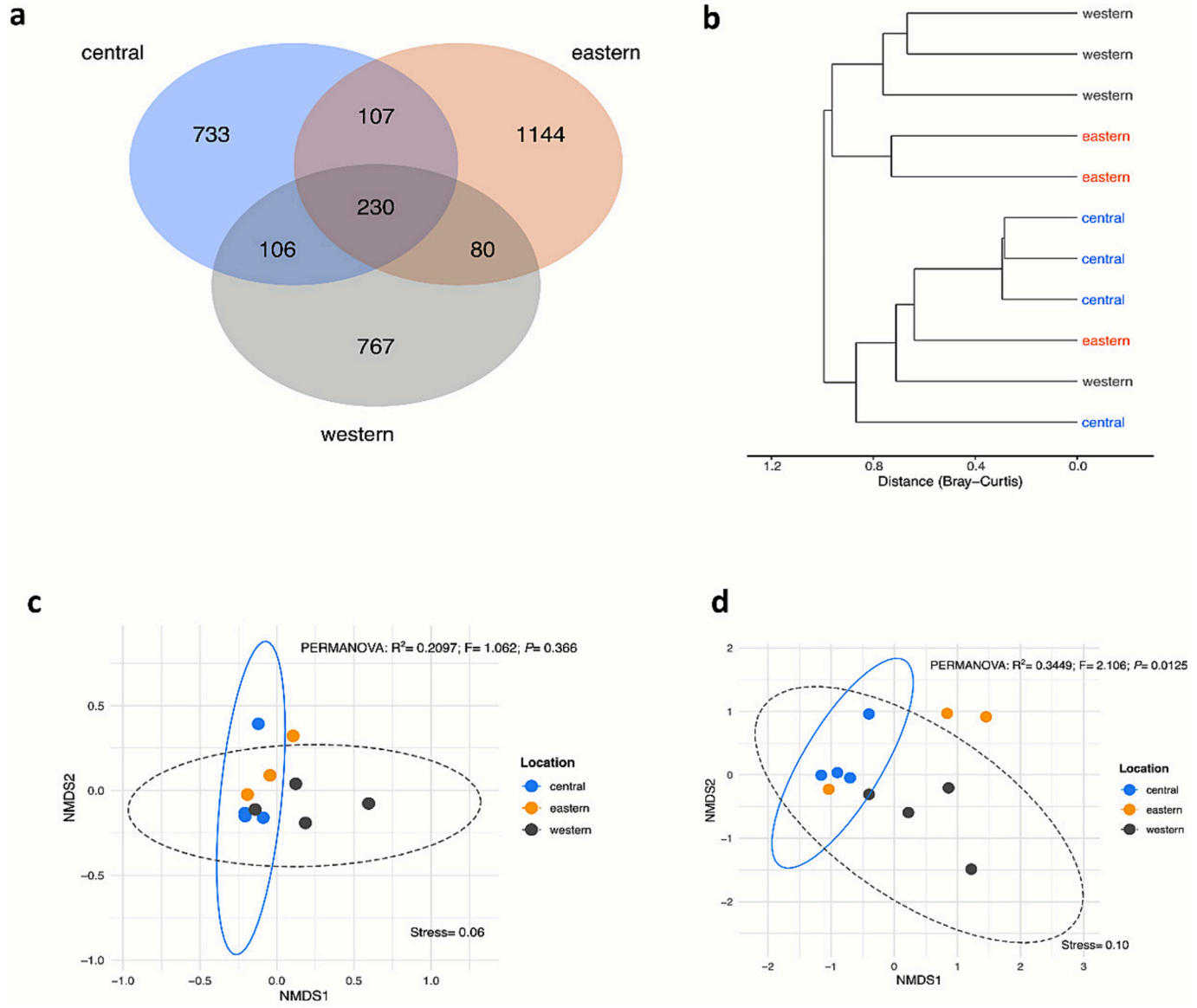

Fig. 5. Beta diversity between ticks from different locations (a) Venn diagram showing number of shared bacterial taxa between the 3 zones. (b) Dendrogram showing the relatedness of locations based on bacterial counts (c) Multidimensional scaling (MDS) clustering analysis using weighted UniFrac and (d) Bray-Curtis distances of samples ($N = 11$). The differences in microbial composition between the 3 geographical locations were statistically tested with the PERmutational Multivariate ANOVA (PERMANOVA), $P > 0.001$.

The identified phyla, *Bacteroidota, Bacillota, Pseudomonadota, Actinomycetota,* and *Fusobacteriota*, dominated the bacterial communities in *Am. variegatum*, accounting for approximately 98 % of the total microbiota. This composition is generally consistent with previous studies of other *Amblyomma* species and tick genera [23,80,81]. Comparable profiles have been reported in *Am. maculatum* and *Am. americanum*, which typically show a strong dominance of *Pseudomonadota* (often >80 %), alongside a good representation of *Bacillota* and *Actinomycetota* [82]. In *Am. variegatum* populations from Ethiopia, targeted molecular studies revealed a high prevalence of *Ri. africae* (Alphaproteobacteria, within *Pseudomonadota*) in up to 38 % of tick pools, further supporting the prominence of this phylum, although comprehensive phylum-level microbiome profiles remain limited [83]. In contrast, other tick genera often display distinct bacterial community structures. For instance, *Hyalomma truncatum* has been reported to harbor microbiomes enriched in *Actinomycetota* and *Bacillota*, with comparatively lower representation of *Pseudomonadota* [84]. Similarly, *Rhipicephalus* species commonly exhibit high abundances of *Bacillota* and *Actinomycetota*, with fewer Proteobacteria-derived symbionts [84]. The genus-specific differences in bacterial symbionts among these ticks are shaped by several biological and ecological factors. These include host preference, as different tick genera feed on distinct animal species with varying microbiota, and environmental exposure, which is influenced by habitat and geography [85,86]. Additionally, life cycle stages affect microbiome composition, particularly in three-host ticks, as larvae, nymphs, and adults often feed on different hosts and encounter diverse microbial communities. *Amblyomma* ticks tend to maintain stable symbionts like *Coxiella*-like bacteria through vertical transmission, whereas *Hyalomma* and *Rhipicephalus* ticks may rely more on environmental acquisition [87]. These suggest that though symbiont composition may be genus-specific, it is also shaped by host interactions, developmental stages, climate, and evolutionary adaptations. Despite these genus-specific differences, our findings affirm the conserved presence and dominance of these five bacterial phyla across *Am. variegatum* and related *Amblyomma* species. Interestingly, across all three geographic zones sampled in this study, *Actinomycetota* emerged as the most abundant phylum, to which

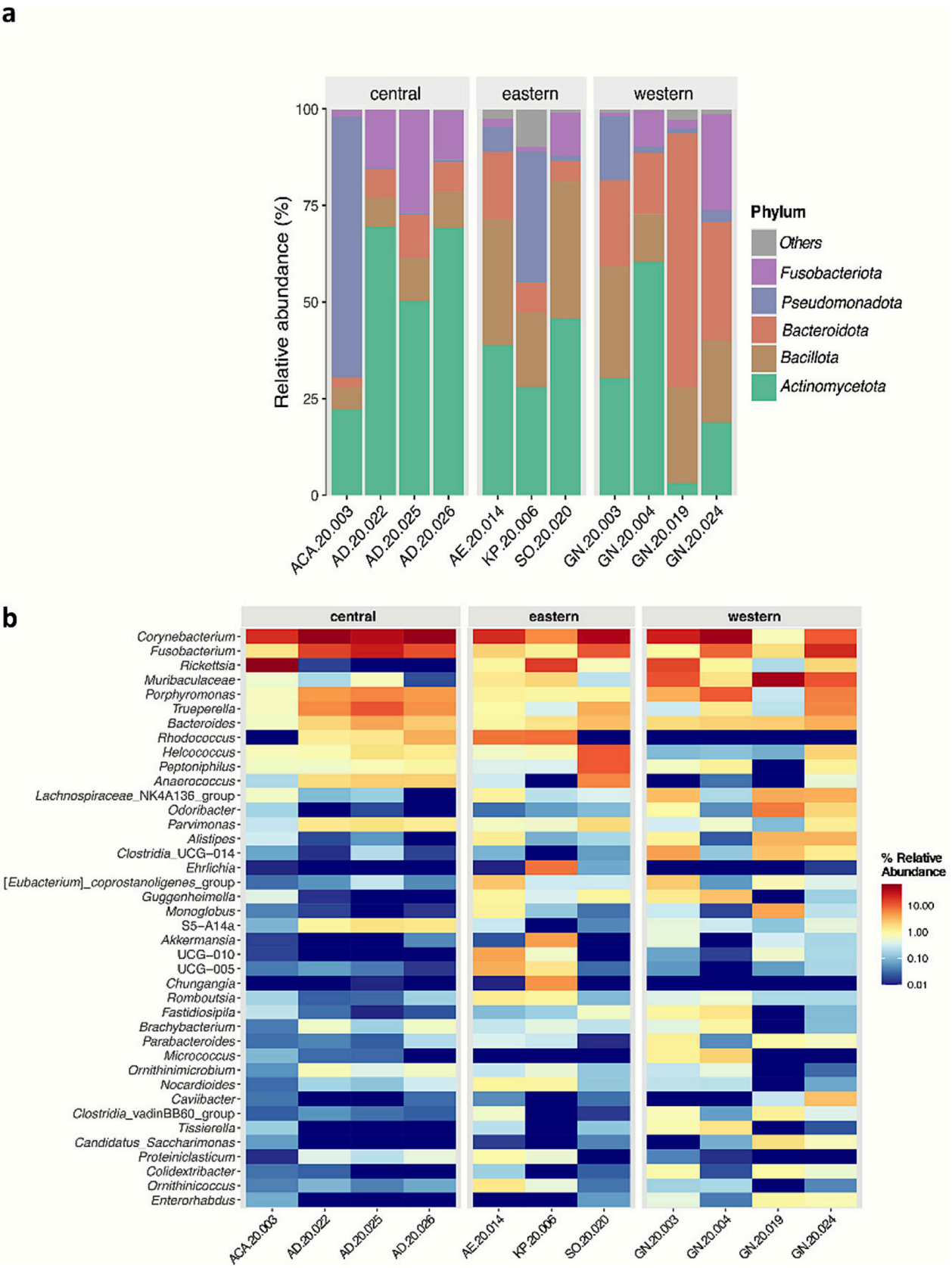

Fig. 6. Relative abundances of potential bacteria in individual tick samples at the (a) phylum and (b) genus level. The relative abundances were based on location. Percentage relative abundance of the top 40 genera.

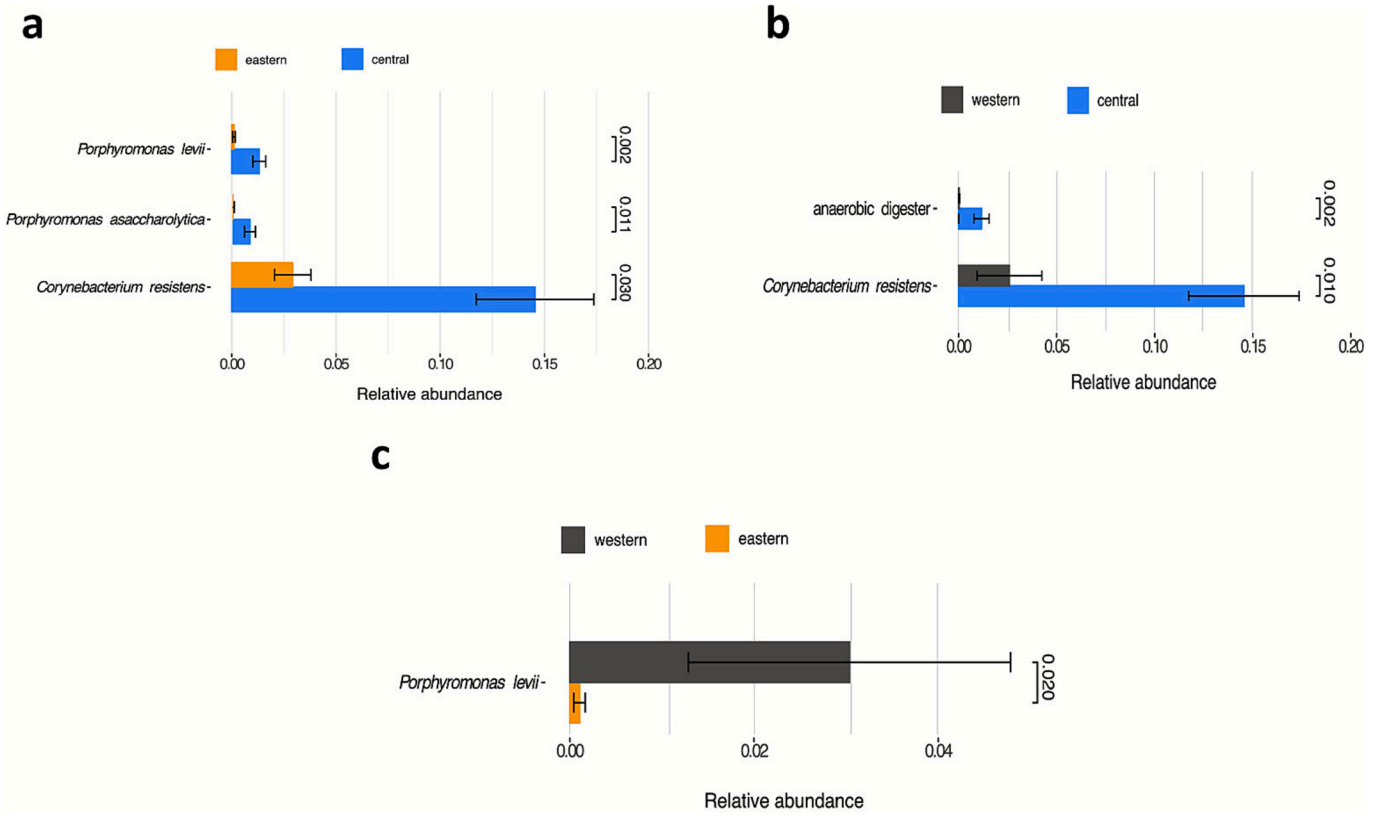

Fig. 7. Comparison of differential abundance between locations at the bacterial species level. Metastats of (a) central and eastern, (b) western and central and (c) western and eastern.

prominent pathogens, such as *Mycobacterium* spp. and *Corynebacterium* spp. belong [88]. This may be due to their wide adaptability to varied environmental conditions [89], which is well represented in the GAR. Additionally, pathogens of this phylum are implicated in causing various human and animal diseases [88,90–96] although are not yet reported to be transmitted by ticks.

Among the genera, *Corynebacterium,* a genus of well-documented symbionts in various tick species [97,98] was the most prevalent, with *Cor. resistens* being significantly enriched in ticks from the central zone. This species, although not typically prioritized in tick-borne disease studies, is an emerging opportunistic pathogen in humans. Originally isolated from human infections, *Cor. resistens* has been associated with skin and soft tissue infections [97,99], and more worryingly, it demonstrates multi-drug resistance, including resistance to macrolides and lincosamides [100]. Its detection in blood-feeding *Am. variegatum* ticks is quite significant, as it raises the possibility that these ectoparasites could act as mechanical vectors or reservoirs for resistant bacteria, potentially transferring them to humans or animals during feeding. However, our data cannot distinguish whether *Cor. resistens* is present as a surface contaminant, a transient environmental or host-derived organism, or a more stable internal symbiont. Future studies incorporating surface sterilization controls, tissue-specific dissection, or metagenomic approaches will be essential to clarify its ecological role within the tick.

Similarly, *Porphyromonas* spp., particularly *Po. levii*, were detected at significant levels, especially in ticks from the western zone. *Po. levii* is typically associated with the oral and gastrointestinal microbiota of animals [101], but has been implicated in bovine foot rot, rumenitis, and reproductive tract infections in livestock [102–104]. *Po. asaccharolytica*, another species from this genus, identified in our study, has also been implicated as a rare causative agent of Lemierre's syndrome with only a few cases reported in humans [105,106]. Although these bacteria have not yet been experimentally confirmed and characterized as tick-borne pathogens, their presence in *Am. variegatum* supports the hypothesis that ticks may play a role in harbouring or mechanically transmitting opportunistic bacteria of medical and veterinary significance [107,108], especially in regions with high livestock-tick interaction, such as Ghana, thus requiring further investigation.

Other genera included *Trueperella*, *Helcococcus*, *Anaerococcus*, and *Peptoniphilus*, many of which are anaerobic or facultatively anaerobic bacteria associated with polymicrobial infections [109,110]. For instance, some *Trueperella* species are well-known agents of pyogenic infections in cattle, including mastitis and abscesses, and has occasionally been reported in human infections [111]. Also, *Anaerococcus* and *Helcococcus* species are implicated in soft tissue and bloodstream infections, particularly in immunocompromised individuals [112,113]. Though often under-recognized, their detection in *Am. variegatum* raises the possibility of co-infection risks and supports the emerging view that ticks may act as complex microbial reservoirs [5,114], and not just vectors for a handful of classic tick-borne pathogens.

Of particular interest was the variable presence of *Rickettsia* spp., which were highly abundant (up to 72 %) in some samples but absent in others from the same location. This sporadic distribution may reflect differences in tick vector competence, host blood sources, microbial competition, bacterial proliferation or environmental exposure [115]. The genus includes important pathogens such as *Ri. africae*, the causative agent of African tick bite fever [21], further highlighting the significance of its detection.

It is important to acknowledge that some of the bacteria detected in this study may represent transient organisms, acquired from host blood or environmental exposure, rather than stable endosymbionts or actively transmitted tick-borne pathogens. A key limitation is that sequencing was conducted without wash controls, despite rigorous surface sterilization prior to DNA extraction. Wash controls are generally recommended to identify and exclude potential contaminating or aberrant DNA and to provide a comparative reference. While we did not sequence the wash solutions, which would have provided an additional

layer of confirmation, we believe the combination of stringent washing and sequencing depth supports the reliability of the microbial profiles presented. Future studies should however incorporate wash controls and sterilization protocols to distinguish stably associated microbes from transient or environmental taxa, and to further evaluate experimentally their potential for biological or mechanical transmission to vertebrate hosts, as is characteristic of established tick-borne pathogens.

Interestingly, although other pathogenic bacteria such as *Anaplasma* species, including those recently detected at low prevalence in ticks from Ghana, and *Coxiella* have both been reported in previous Ghanaian tick studies [38,41], neither genus was detected in this dataset. While the number of ticks analysed in this study may be relatively small, other factors likely to contribute to phenomenon, include heightened tick immune activity during feeding, altered gut conditions in partially-fed ticks, variation in host species, and regional ecological differences. These factors can influence the acquisition and persistence of certain bacterial taxa, especially those not maintained through vertical transmission. Furthermore, the observed regional differences in bacterial profiles in this study, suggest that geographic location, environmental conditions, and possibly cattle management practices may influence the presence and distribution of both well-characterized and less-recognized tick-associated bacterial pathogens. These findings however support the growing need to assess tick-borne pathogens not only from a veterinary or entomological perspective, but also through a broader One Health lens that integrates human, animal, and environmental health.

5. Conclusions

This study represents the first application of 16S rRNA amplicon sequencing to characterize the bacterial communities of *Am. variegatum* ticks in Ghana. By detecting both well-known and less-recognized pathogenic bacteria, the findings provide new insights into the hidden microbial diversity of Ghanaian ticks. In particular, the identification of opportunistic pathogens such as *Cor. resistens* and *Po. levii* expands current understanding of tick-associated microbiota and highlights potential health risks often overlooked in conventional tick-borne disease surveillance.

These results emphasize the need to broaden pathogen surveillance beyond well-established agents such as *Borrelia*, *Anaplasma*, and *Ehrlichia*, and to adopt One Health approaches that consider not only traditional zoonotic threats, but also emerging opportunistic bacteria. Such integrated frameworks are essential for understanding the complex ecology of tick-borne diseases, and their impacts on livestock health and rural livelihoods in Ghana.

Because the study relied on blood-fed ticks, some of the bacterial taxa identified may reflect host-derived microorganisms; therefore, the results should be interpreted as a combination of potential tick-associated bacteria and xenosurveillance signals rather than a definitive characterization of the intrinsic tick microbiome. A key limitation is that sequencing was performed solely on primary tick samples, without wash controls or tissue-specific sampling. Future research incorporating wash samples, tissue-specific or microdissection-based sampling, and expanded collections of both blood-fed and unfed ticks from diverse regions of Ghana will be necessary to accurately identify true internal symbionts and pathogens, and provide a more comprehensive understanding of the tick-associated microbial ecosystem.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2025.103228.

CRediT authorship contribution statement

Jennifer Afua Afrifa Yamoah: Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kofi Dadzie Kwofie:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Jewelna Akorli:** Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Danielle Ladzekpo:** Investigation, Formal analysis, Data curation. **Hayato Kawada:** Investigation. **Kwadwo Yeboah Boateng:** Investigation. **Julius Beyuo:** Investigation. **Antoinette Keleve:** Investigation. **Jonas Bedford Danquah:** Investigation. **Christopher Tawiah-Mensah:** Investigation. **Jane Ansah-Owusu:** Investigation. **Samuel Kweku Dadzie:** Writing – review & editing. **Paul Amponsah Wallace:** Writing – review & editing. **Naotoshi Tsuji:** Writing – review & editing, Resources, Funding acquisition. **Takeshi Hatta:** Writing – review & editing, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

Ethical approval for this study was obtained from the Council for Scientific and Industrial Research (CSIR) Institutional Animal Care and Use Committee (IACUC), Ghana (RPN 001/CSIR.IACUC/2021). The animal owners were verbally informed about the study objectives and sampling procedure. All owners gave their verbal informed consent to collect ticks from their animals.

Funding

This study was supported by a Research Fellowship Program (20G22-2) from the Matsumae International Foundation to JAAY, the JSPS Core-to-Core Program (JSJSCCB20200010) to NT and JSPS KAKENHI Grant Number 17H04641 to TH.

Declaration of competing interest

None.

Acknowledgements

The authors are thankful to Dr. Ebenezer Ansa, the Director of CSIR-Animal Research Institute for his immense support. The authors also thank Associate Prof. Dr. Ryo Nakao from Hokkaido University for his insightful comments on this project, as well as Prof. Kwabena Duedu from Birmingham City University for his continuous mentorship to JAAY. The authors again thank Dr. Bernard Bortei Borteih, Mr. James Myers-Hansen and Ms. Georgina Isabella Djameh for their technical support. We also deeply appreciate farm owners and district officers for their cooperation and assistance in sample collection.

Data availability

The sequence datasets generated and analysed during the current study are available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) under BioProject accession number PRJNA1231535. Additional sample data and analyses results have been included in this article and its supplementary files.

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