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Fecal Microbiota of Free-range Pigs (*Sus scrofa domesticus*) Scavenging on a Municipal Dumpsite is a Potential Reservoir of Pathogens

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Abstract Free-range food animals scavenging in urban and peri-urban dumpsites are exposed to diverse microbes of public health importance, yet little is known of their fecal microbiota and public health implication. We characterized the fecal microbiota of pigs scavenging at a municipal dumpsite (FecD, *n* =19) by MiSeq sequencing for 16S rRNA and compared with conventionally indoor reared pigs (FecI, *n* = 21). A total of 4,364,507 sequences with an average of 114,852 reads per sample passed quality control. The predicted mean of species per sample was 5,979. There was no difference in alpha diversity between free-range and indoor pigs (InvSimpson 23.68 vs 38.06, p = 0.1091 and Shannon 5.60 vs 6.41, p = 0.053). The community membership and population structure were significantly different (Yue and Clayton p = 0.001 and Jaccard p = 0.014). Bacterial genera significantly associated with free-range pigs were *Bifidobacterium*, *Enterococcus*, *Turicibacter and Cellulosilyticus*; while in indoor pigs were *Prevotella*, *Fibrobacter*, *Megasphaera*, *Allisonella*, *fibrobacteres* and *Phascolarctobacterium*. Metagenome prediction revealed that Tetracycline biosynthesis, *Staphylococcus* infection, sporulation and *Vibrio cholerae* pathogenic pathways are significantly ($p < 0.05$) associated with scavenging pigs. The organism-level phenotype prediction revealed that free-range pigs were also dominated with *Proteobacteria* rich in mobile elements and pathogenic potential. Free-range pigs scavenging in urban and peri-urban areas are potential reservoirs of pathogens of public health importance. These findings suggest indoor management of animals in urban and peri-urban areas to mitigate possible health risks from free-range animals which might get into food chain. Further study of the gut microflora of free-range pigs at dumpsites and their clinical significance to humans and other animals is warranted.

Keywords: *pigs, fecal microbiota, 16S rRNA, municipal dumpsites, free-range pigs, scavenging*

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1. Introduction

Studies of fecal microbiota of animals have received increasing attention due to the complexity of gut microflora as well as its importance for public health and disease. Despite the known importance of fecal microbiota [1,2,3], there is limited information on its composition in free-range animals raised in urban and peri-urban areas and its impact on the environment. The current rising trend of urban and peri-urban agriculture in most countries in Africa has resulted in pig production under free-range management becoming a viable livestock farming system playing a major role in meat production either for home consumption or income generation [4-6]. One advantage of a free-range farming system, especially for pigs, include low capital investments, which allow small holder farmers the opportunity to enter into livestock keeping. However, such a farming system increases the risk of pigs acquiring diseases, either production-limiting or zoonotic in nature [7,8,9].

In urban and peri-urban farming settings with high human-animal interaction; free-ranging pigs may be a cause of transmission of zoonotic diseases [6,10,11]. For example, some reported diseases in Tanzania include African Swine Fever [12,13], Leptospirosis [14] and Campylobacterosis [15,16] which have serious health implications for humans and other animals. Of interest to this study, was the management environment of free-range pigs, which scavenged on dumpsites in a peri-urban area. Dumpsite composition included solid waste, typically organic waste from households, markets and abattoirs, waste from agriculture and industries as well as chemical/ pharmaceutical/ biomedical waste, on which the pigs scavenged. Since free-range management of pigs would

presumably have a profound effect on the composition of gut flora, detailed study of fecal microbiota of pigs scavenging on dumpsite would help in surveillance of pathogens of potential public health importance. Despite the importance of fecal microbiota in different animal species [2,3,17], there are no reports on microflora of pigs free-ranging on dumpsites in Africa.

In the current study, we compared fecal microbiota of free-range pigs, which continuously scavenged on the dumpsite with that of pigs reared indoors using the V4 region of 16S rRNA gene and high throughput Illumina MiSeq sequencing technology. To our knowledge, this is the first study in East Africa reporting the influence of free-range pig management on the composition of enteric microbiota.

2. Materials and Methods

2.1. Study Site and Fecal Samples

The site for this study was the Arusha municipal dumpsite in Tanzania which is near the household of smallholder farmers, who allow their livestock, mostly pigs, to scavenge on the dump. It is the site where solid waste from different urban sources is thrown. This is comprised of a variety of solid waste from households and markets (foods remnants, rotten fruits and vegetables, diapers, clothes, etc.), chemical and biomedical waste (drug containers, used syringes, swabs), various plastics and used glassware, waste from abattoirs and brewers, as well as fecal matter from animals scavenging on the dump itself. The dumpsite is close to the river into which solid wastes drains over during rainy season. Animals like cattle, goats, dogs, birds and people are all interacting at the dump (Figure 1). Animals for this study were adult pigs, aged 8 -10 months. Samples were the fresh fecal material of free range pigs scavenging on the dump (FecD, n = 19) where solid waste was the only source of food, no supplemental feeding was given, and the control samples were indoor reared pigs (FecI, *n* = 21) from the Livestock Training farm (LITA, Tengeru, Arusha) 30 km away from the dumpsite. Indoor pigs were raised under good management practices, and their feed comprised of plant-based diets such as cereal grains, corn bran, vegetables, fruits, potatoes and bananas. Samples were the core of fresh fecal matter of pigs, which was collected into sterile plastic containers, and within 1 hour transported on ice to the laboratory, where total genomic DNA was extracted.

2.2. Extraction of Total Genomic DNA

About 250 mg of fecal sample was used to extract total DNA using PowerSoil™ DNA extraction kit (MOBIO Laboratories, Carlsbad, CA) as per manufacturer's protocol. Quality and quantity of total DNA was verified with Nano Drop ND-2000c spectrophotometer (Thermo Scientific) and gel electrophoresis run in 0.8 % agarose. The DNA was stored at -20℃ until further processing.

2.3. 16S rRNA Amplification, Library Construction and Sequencing

The 16S rRNA amplification, library preparation and sequencing was done as described in Mwaikono et al,. [18]. Briefly, PCR primers and protocol were adapted from Caporaso [19]. PCR reaction was done in 20 µl AccuPower® Taq PCR PreMix composed of 0.5 µl of 10pmol/ µl each for the forward and reverse primers, 17 µl molecular grade water and 2 µl DNA template. The PCR program was run on GeneAMP™ PCR system 9700 set at 95℃ for 3 min, 35 cycles of 94 ^{o}C for 45 s, 50℃ for 60 s and 72℃ for 90 s and a final extension at 72℃ for 10 min. Amplicon quality was visualized using gel electrophoresis, then pooled and purified using QIAquick® PCR purification kit (Qiagen, German) following manufacturer's protocol. Purified PCR products were normalized to 120 ng of DNA and then pooled to form three replicates libraries. Quantification of DNA was done using Qubit® dsDNA assay kit in Qubit fluorometer 2.0 (Invitrogen, Life Technologies) and quality checked using Agilent DNA 1000 Chip in Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) following manufacturer's protocol.

Figure 1. Animal and human interaction at the municipal dumpsite: (a) a truck offloading garbage and people scrambling for recyclable materials, (b) pigs scavenge on dumped solid waste

Library denaturing, dilution and PhiX control preparation were done as described in the 16S metagenomic sequencing library preparation guide [20]. Three primers (Read 1, Read 2 and index sequencing primers) described in Caporaso [19] were used. Sequencing of the library was done using Illumina MiSeq platform (San Diego, USA) and 2×250 PE chemistry at the BecA –ILRI Hub genomic platform, Nairobi, Kenya

2.4. Quality Control of Sequence Data and Statistical Analysis

The Mothur package algorithms (v1.39.5) were used for quality control and some statistical data analysis [21]. After paired end reads were assembled, sequences were aligned with the Silva 16S rRNA reference database (www.arb-silva.de) [22]. Sequences that were < 239 bp and > 260 bp in length or contained > 2 ambiguous base calls or long runs (> 8 bp) of homopolymers or did not align with the correct region were removed. Chimeras were identified using VSEARCH v2.3.4 [23] (chimera.vsearch) and eliminated. Catchall analysis was used to assess species richness [24]. Taxonomy was assigned using the RDP taxonomy database (http://rdp.cme.msu.edu/index.jsp) [25]. Sequences were binned into operational taxonomic units (OTUs) at 97% sequence similarity level.

Subsampling of 9,115 sequence reads was done to normalize sequences for further comparison [26], and consisted of random selection of a number of sequences from each sample consistent to the lowest abundance of all samples. Population diversity, richness estimation and coverage were established by generating collector's curves of the Chao1 richness estimator [27], the inverse Simpson diversity index [28] and Shannon weaver index for bacterial population evenness. Community membership was compared using the traditional Jaccard index, while community structure was assessed using the Yue & Clayton measure of dissimilarity.

Dendrograms were created using Mothur to compare the fecal microbiota among all samples using both Jaccard index and Yue & Clayton measure which account for the relative abundances in each sample. Parsimony (unifrac unweighted and unifrac weighted) tests were applied to the Jaccard and Yue & Clayton OTU based trees to determine significance of clustering between the pig management systems. The statistical significance of the separation was also assessed using Analysis of Molecular Variance and Homogeneity of Molecular Variance.

Further, samples of free-range pigs, which clustered separately, were re-assigned OTUs using the Greengenes database, and then, features that significantly explain the difference in the fecal microbiota between free-range and indoor pigs were determined using the linear discriminatory analysis (LDA) effect size (LefSe) in Galaxy [29,30]. The metagenome prediction and functional gene profiling was done using the PICRUSt [31] and its statistical analysis in STAMP [32]. The BugBase tool (https://bugbase.cs.umn.edu/index.html) [33] was used to predict the organism-level phenotype composition of the fecal microbiota. A p-value of ≤ 0.05 was considered significant for all comparisons.

3. Results

A total of 4,364,507 V4 region of 16S rRNA gene sequences of fecal microbiota passed the quality control. The number of sequences per sample ranged from 551 to 291,467 (mean 114,852, SD 56,720). Only 37 samples with at least 9,155 sequences per sample were used in the downstream analyses. A total of 40,803 OTUs were identified, and Catchall analysis of richness predicted a mean of 5,979 species per sample (range 1271 – 18,422, SD 4,011). Coverage ranged from 0.9876 – 0.9962 (mean 0.9876, SD 0.00617). Population diversity was high with an average inverse Simpson index of 37.75 (SD 27.93, range 2.9514 – 99.8023), Shannon's evenness values were on average 5.8138 (SD 1.05, range 3.35 – 7.80) and Chao1 richness estimator was on average 3,193.9 (SD 2,076.4, range 823 - 8,318). A total of 830 bacteria genera were found in all management systems. Seventy-four genera were significantly different between the free-range and indoor management system this includes some bacteria genera associated with known pathogens (Figure 2).

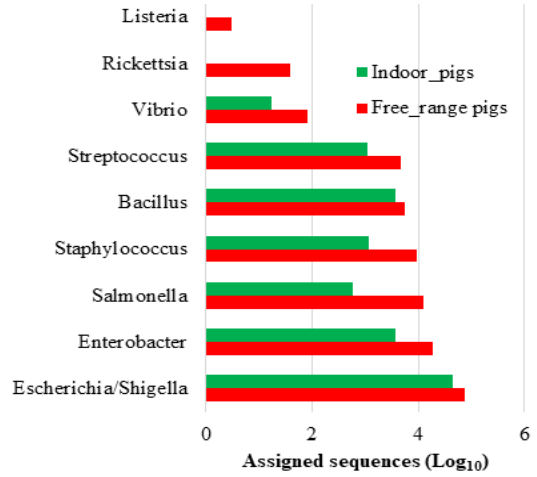

Figure 2. Profile of potential pathogenic genera in free-range and indoor pigs (log transform of assigned sequence reads in each management system).

When each management system was individually analysed, the most abundant phyla in free-range pigs were *Firmicutes* (56%), *Proteobacteria* (23%), *Bacteroidetes* (8%), *Actinobacteria* (3%), *Spirochaetes* (2%), *Chloroflex* and *Acidobacteria* had 1% while indoor reared pigs were dominated with *Firmicutes* (42%), *Proteobacteria* (25%), *Bacteroidetes* (20%), *Spirochaetes* (4%) and *Acidobacteria* (2%).

Comparison of the alpha diversity of the fecal microbiota revealed no difference between free-range and indoor pigs (InvSimpson 23.68 vs 38.06, p = 0.1091 and Shannon 5.60 vs 6.41, p = 0.053) while community membership and population structure were significantly different (Yue and Clayton P = 0.001and Jaccard P = 0.014). Similarly, the difference was also found with AMOVA (P < 0.001) and HOMOVA (P = 0.03). The difference was also revealed using principal component analysis; where both PC1 at 69.5% and PC2 at 20.3% clearly show a cluster (circled) of some samples from free-range pigs separated from the rest (Figure 3).

Linear discriminant analysis (LDA) effect size (LefSe) revealed bacterial genera that explained the greatest difference between the fecal microbiota of free-range and indoor reared pigs. *Bifidobacterium*, *Enterococcus*, *Turicibacter and Cellulosilyticus* genera were significantly associated with free-range pigs' while *Prevotella*, *Fibrobacter*, *Megasphaera*, *Allisonella*, *fibrobacteres* and *Phascolarctobacterium* accounted for the pigs raised under indoor management (Figure 4)

Imputed metagenome prediction revealed significant differences in functional profiles between the fecal microbiota of free-range and indoor reared pigs ($P < 0.05$). Free-range pigs had higher proportions of functional genes involved in biological pathways of public health importance, such as Tetracycline biosynthesis, *Staphylococcus* infections, sporulation and *Vibrio cholerae* pathogenic cycle (Figure 5).

The organism-level phenotype prediction revealed a significant difference in the fecal microbiota of free-range and indoor reared pigs. Indoor pigs were found to have higher abundance of gram negative bacteria contributed by *Proteobacteria*, *Bacteroidetes* and *Spirochaetes*; while scavenging pigs weres rich in gram positive bacteria dominated with *Firmicutes.* The predominance of the mobile elements and potential for pathogenic composition, in each case contributed highly by *Proteobacteria*, were abundant in free-range pigs pigs (Figure 6).

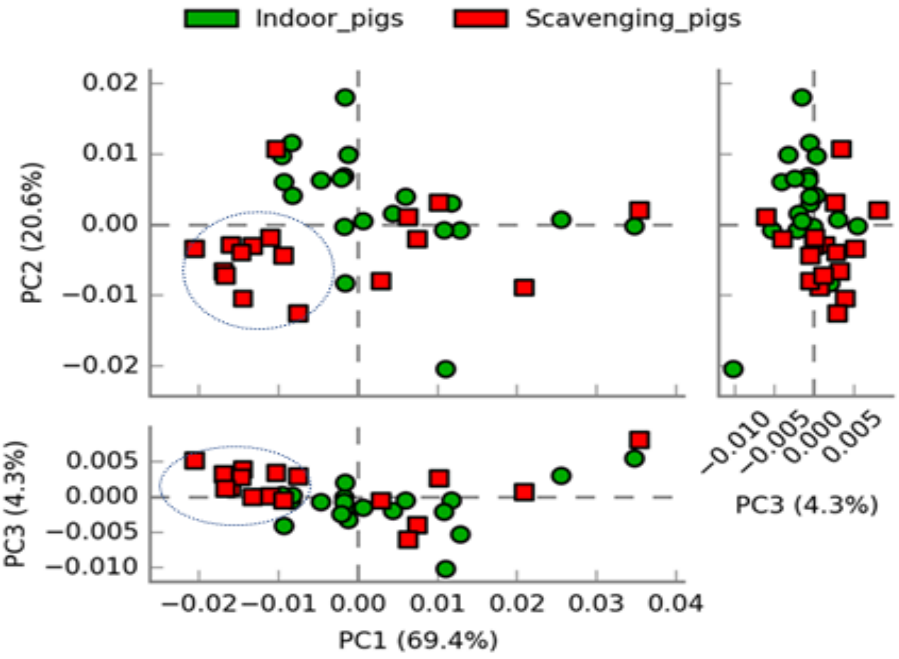

Figure 3. Principal component analysis of the fecal microbiota from free-range and indoor pigs: Analysis was performed using Statistical Analysis of Microbial Profile (STAMP) tool, using genus-level organism abundance profiles derived from free-range and indoor pig's management systems. A cluster of free-range pigs separated from others is clearly seen. Percentage of variation explained by principal component1 (PC1) was 69.4% and principal component 2 (PC1) was 20.6%. Normalized sequence reads used was 9,155

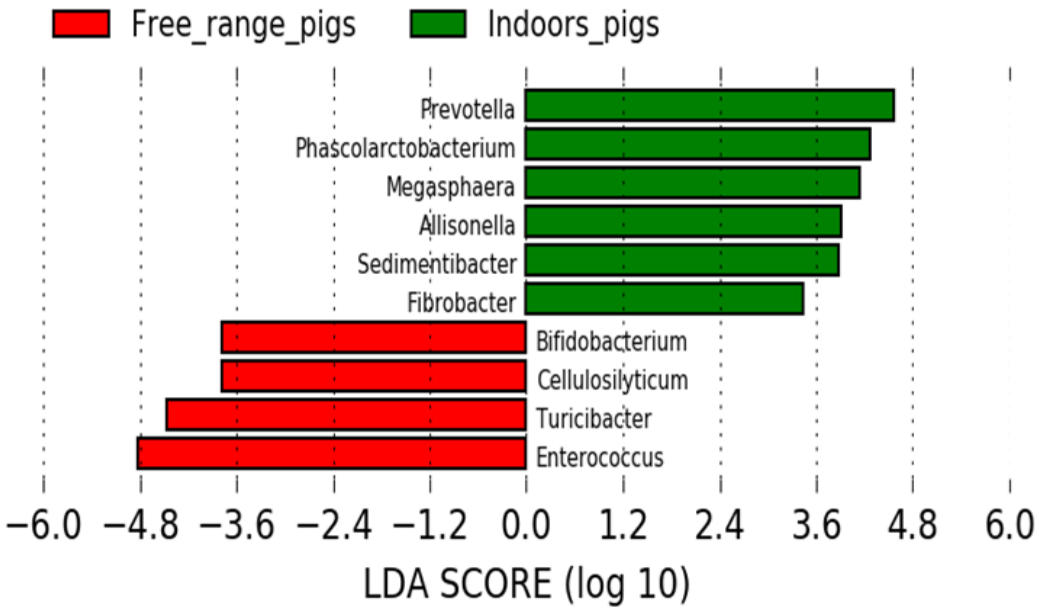

Figure 4. Differentially abundant bacterial from the fecal microbiota of free-range and indoor pigs: Result displays the linear discriminant analysis scores effect size and illustrates which bacterial groups are significantly associated with either free-range pigs (red) or indoor reared pigs (green)

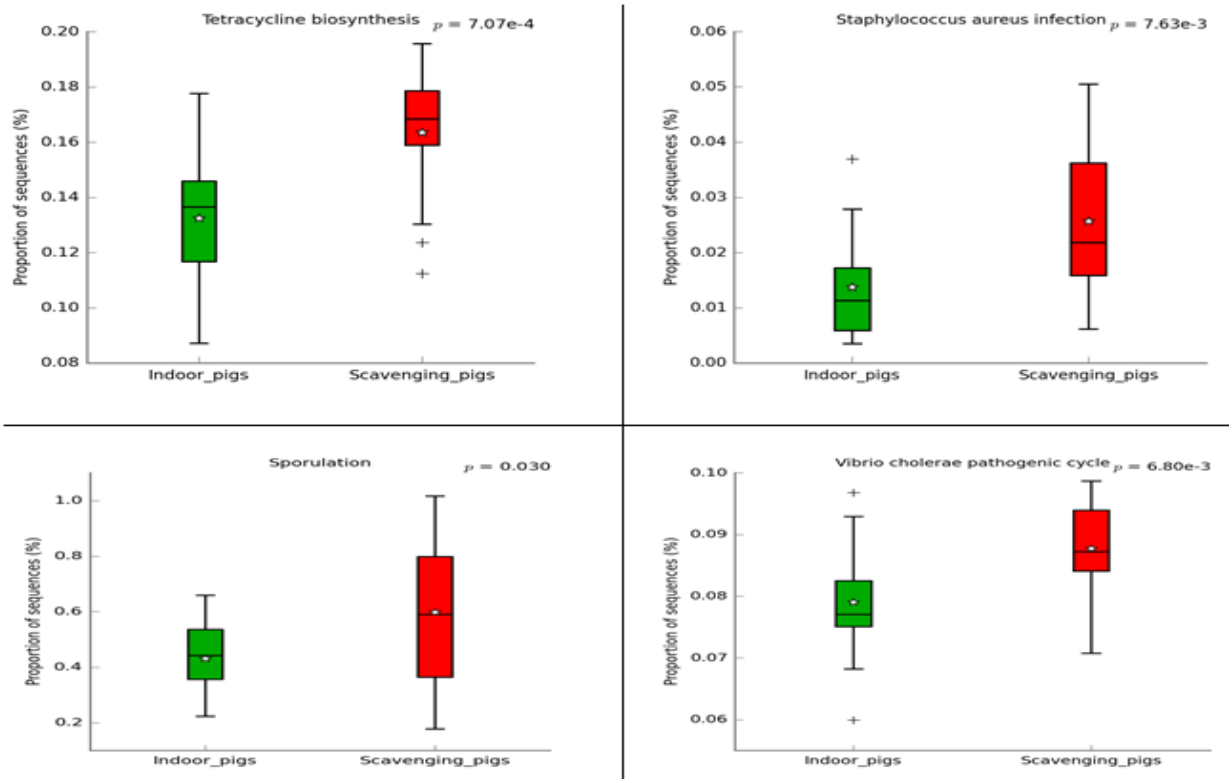

Figure 5. Predicted metagenome functional composition based on 16S rRNA: PICRUSt results revealed functional pathways significantly different between the fecal microbiota of scavenging and indoor pigs. (a) Tetracycline biosynthesis, $p = 7.05e^{-4}$ (b) *Staphylococcus aureus* infection, $p = 7.63e^{-3}$ (c) Sporulation, $p = 0.030$ and (d) *Vibrio cholerae* pathogenic cycle, $p = 6.80e^{-3.}$ Normalized sequence reads used was 9115

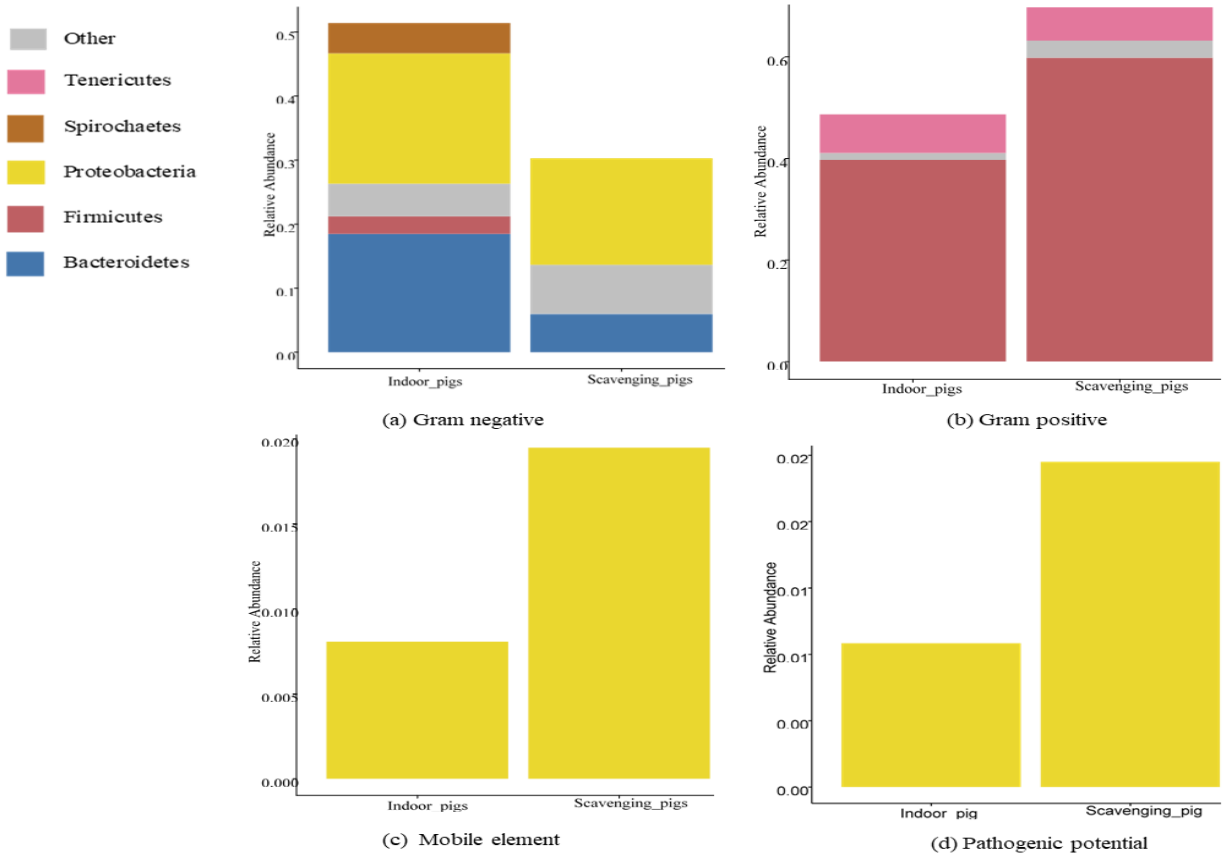

Figure 6. Organism-level metagenome prediction of the fecal microbiota of pigs: The Bugbase plots shows the organism-level proportion of the fecal microbiota of pigs. (a) there is higher proportion of gram negative bacteria in indoor pigs than in free-range pigs, and they are dominated with phyla Proteobacteria and Bacteroides, (b) there is higher proportion of gram positive bacteria in free-range than in indoor pigs, and is dominated with phylum Firmicutes, (c) there is high proportions of mobile element genes from Proteobacteria in free-range pigs than in indoor pigs (d) there is high proportion of genes from Proteobacteria with pathogenic potential in free-range than indoor pigs

4. Discussion

In the current study, the fecal microbiota of pigs scavenging on the municipal dump was compared to indoor reared pigs using high throughput Illumina MiSeq sequencing technology. While most studies report *Firmicutes* and *Bacteroidetes* as the major components of the fecal microbiota of pigs [34,35], in this study *Proteobacteria*, *Spirochetes* and *Actinobacteria* were inclusive, each contributing over 2% of all sequences. Since there was no sorting of solid waste prior to disposal, which significantly contributes to the diversity of bacteria at the dump, it is apparent that such an environment has an impact on the abundance and diversity of the fecal microbiota of pigs scavenging therein.

Our results show a significant difference in the community membership and structure between the fecal microbiota of free-range and indoor reared pigs. A cluster unique to free-range pigs, and a group of bacteria which has higher discriminatory effect size, is evidence of their unique microbial composition. The predominance of *Bifidobacterium* in GIT is considered as an indicator of good health in humans and other animals [1,36], and indeed some studies have shown that presence of *Bifidobacterium* promotes immune response in pigs [37,38]. The significant association of *Bifidobacterium* with pigs scavenging in the dump rich in microbes from a variety of unsorted solid waste suggest that, probably *Bifidobacteria* play role to inhibit proliferation of pathogenic bacteria in free-range pigs, hence their adaptation to the dumpsite environment. It has also been found that *Bifidobacteria* utilize a diverse range of plant derived oligosaccharides and polysaccharides that escape degradation in the upper parts of the intestine [39, 40]. This unique feature is likely to give a competitive advantage at the dumpsite where a variety of solid waste from domestic, market, food and beverage industries and medical waste are the only source of food in free-range pigs.

Along with *Bifidobacteria*; *Turicibacter* was also associated with scavenging pigs. A previous study has shown that, microbiome dominated with *Turicibacter* are associated with a healthy colon mucus layer which cannot be penetrated with pathogenic bacteria [41,42]. The observed association with pigs that are always exposed to a variety of polluted solid waste suggest that, they could be having a mutualism relationship with free-range pigs which enables to resist attack of pathogenic bacteria by establishing a healthy colon mucous layer.

Even though *Enterococci* belong to the lactic acid bacteria and are cautiously used as probiotics in human and slaughter animals [43,44], its predominance in scavenging pigs may be beneficial to these animals through protection against gut pathogens and modulation of the immune system. However, several studies have reported public health risks associated with *Enterococcus* such as spread of multidrug-resistance from animals to humans [45,46], human and pigs sharing vancomycin resistant enterococci [47] and potential agents of nosocomial infections [48]. The fact the dumpsite was not fenced, animals move freely from the dumpsite to nearby residences, people working in the same dumpsite; and the fact that these animals end-up in the food chain, it implies that there is a potential risk of spreading pathogenic *Enterococci* to humans and other animals.

As opposed to scavenging pigs, the fecal microbiota of indoor reared pigs was significantly associated with *Allisonella*, *Prevotella*, *Megasphaera*, *Sedimentibacter* and *Fibrobacter* genera. Though these bacteria are common in the GIT of pigs [49,50,51,52], it appears that diet was the driving feature for the fecal microbiota composition. For example, *Prevotella* and *Fibrobacter* are associated with the digestion of complex carbohydrate and plant rich diets [53,54], and indeed indoor pigs were fed mainly a plant based diet such as cereal grains, corn bran, vegetables, fruits, potatoes and bananas, which constitutes the energy source for *Prevotella* and *Fibrobacter*. The predominance of *Allisonella* spp in indoor reared pigs is also supported by the fact that, their diet was rich in grains which is a good source of histidine, which is well known as *Allisonella's* sole source of energy [55, 56]. Therefore, it is likely that the unique bacteria that discriminate indoor pigs are derived from the feed scavenged at the dumpsite.

Despite the fact that the PICRUSt metagenome functional prediction using the16S rRNA gene can only reach up to 80 - 85% [31] of the whole genome sequencing, it was interesting to find significantly higher predominance of the functional pathway for Tetracycline biosynthesis, Staphylococcus infection, Sporulation and Vibrio cholerae pathogenic cycle in scavenging pigs. Though it is natural for some bacteria like *Streptomyces* to synthesize tetracycline [57,58], it is obvious that these bacteria have developed immunity or mutations which protect them against their metabolites action. The question here is "what happens to other bacteria when exposed to this antibiotic?". These synthesized antibiotics are likely to create selection pressure on other bacteria, hence rendering them resistant. This finding is in agreement to our previous study on the same dumpsite [59], where we found that a majority enteric bacteria isolates were multi drug resistant. The fecal microbiota of free-range pigs is likely to be spreading resistant genes or bacteria to the environment.

The prediction of *Staphylococcus aureus* infection pathway in pigs is consistent with previous research [60,61] where *S. aureus* was found in pigs and also, livestock associated methicillin-resistant *S. aureus* in field workers after exposure to pigs. In this study, 9304 sequence reads were assigned to free-range pigs, versus 1141 in indoor pigs suggesting that *Staphylococcus aureus* are predominant in scavenging pigs. Though there are limited cases of *Vibrio cholerae* linked to fecal microbiota of pigs, its high proportion in scavenging pigs could be attributed to the variety of unsorted domestic and medical waste, which included diapers and swabs, probably originating from infected people.

Even though organism-level microbiome prediction shows a high proportion of gram-positive bacteria dominated with *Firmicutes* in scavenging pigs and *Proteobacteria* in indoor pigs, it was interesting that the organism-level microbiome prediction found that scavenging pigs were rich in both mobile elements and bacteria with pathogenic potential from *Proteobacteria*. Although this is the first report on organism-level fecal microbiota prediction in pigs, the predominance of mobile

elements from *Proteobacteria* which constitute major clinical pathogens, is a serious alert. For example, some studies have shown that the majority of antibiotic resistance genes from human GIT are from *Proteobacteria* [62]. It is also well known that mobile elements carries resistance, virulence, as well as fitness genes associated with entry and survival in the host [63,64]. These genes, through horizontal gene transfer, can lead to the exchange of genetic material between taxonomically diverse bacteria [65,66] leading to the evolution of multidrug resistant bugs. The fact that scavenging pigs are roaming between the dump and nearby residential areas, interacting with other animals at the dumpsite, means it is likely that the fecal microbiota and associated risks contaminate environments, other animals and people working in the dump. Certainly, in our previous study [59] in the same dumpsite we found high prevalence of multidrug resistant bacteria from solid waste and fecal samples of animals scavenging therein. The role of animals and a polluted environment in the evolution and transfer of antibiotic resistant genes is reported elsewhere [63,65,67,68]. The same phenomenon is likely to be happening to pigs scavenging in urban dumpsites.

5. Conclusion

This study has shown a difference in the fecal microbiota of pigs scavenging in dumps and indoor reared pigs. Though free-range pigs were found with some bacteria likely to confer adaptation to the polluted environment; the risk of predicted functional pathways like Tetracycline biosynthesis, *Vibrio cholerae* pathogenic cycle, *Staphylococcus aureus* infection and the predominance of mobile element to the environment, animals, people and food derived from these animals is worthy of further investigation.

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Competing Interests

The authors declare that they have no competing interests.

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