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Research Article

Characterization of oral microbiome from black rat (*Rattus rattus*) and assessment for pathogenicity

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Abstract

The present study, conducted from August to November 2022 in the district of Okara, Pakistan, focused on assessing the bacterial characterization in oral saliva swabs of black rats (Rattus rattus). DNA extraction was performed using the QIAamp DNA Microbiome kit, and the 16S rRNA gene was amplified using universal primers to amplify variable regions (V) V1 to V8 of 1380 bp. The identified bacterial phyla were as follows: Proteobacteria 98%, Firmicutes 1%, Actinobacteria 0.4%, and Bacteroidetes 0.05%. The bacterial classes included Gammaproteobacteria 95%, Alpha Enterobacterales 3%, and Bacilli 1%. The relative abundance of different bacterial orders was Pseudomonadales 40%, Enterobacterales 30%, Xanthomonadales 25%, Sphingomonadales 3%, Lactobacillales 1%, Micrococcales 0.4%, and Bacteroidales 0.05%. The identified families followed the order of Pseudomonadaceae 40%, Enterobacteriaceae 30%, Xanthomonadaceae 25%, and Sphingomonadaceae 3%. The percentage distribution of Pseudomonas was 40%, Stenotrophomonas 25%, Sphingomonas 3%, Pantoea 2%, and Porphyromonas 0.05%. This knowledge enhances our understanding of bacterial infections in rodents, serving as crucial baseline data for bacterial species. The high prevalence of potentially pathogenic bacteria like Pseudomonas suggests a significant risk of zoonotic diseases that could affect both local wildlife and human populations. It is crucial that future studies should focus on identified bacterial communities in other rodent species and small mammals to compare their roles in disease ecology. This ongoing research could identify specific species that are particularly significant in zoonotic transmission, thereby guiding future studies and public health measures.

Keywords: NGS, Okara, Pathogens, zoonosis, Proteobacteria, Pseudomonas

Introduction

The oral microbiome harbors around 200 predominant bacterial species in the oral cavity and hosts approximately 50 to 100 billion bacteria. There are around 700 predominant taxa; out of these, only one-third still have not been cultured in labs (Krishnan et al., 2017). Microbial communities that inhibit the oral cavity are important to understand to know their role in maintaining health and causing diseases (Sedghi et al., 2021). Different types of oral microorganisms (bacteria, viruses, fungi, protozoa, and archaea) form oral microbiomes that live in human hosts in a symbiotic relationship with each other. These microorganisms interact with each other and the host, influencing oral and systemic health (Radaic & Kapila, 2021). Microbial communities are essential for preserving oral health as they actively participate in crucial physiological processes, including digestion, regulation of the cardiovascular system, antioxidant activity, supporting the host defense function, having anti-inflammatory properties, and the maintenance of a stable oral microenvironment (Kilian et al., 2016).

The oral bacteria are also associated with or act as bioindicators for specific general diseases like cancer of the pancreas (Sun et al., 2020). The oral cavity is an entry point to the respiratory and digestive systems, which are highly vascularized. This allows for the potential dissemination of oral microorganisms throughout the body, leading to systemic infections and various diseases. Studies highlight the association between the oral microbiome and various oral and systemic diseases, including periodontal diseases, oral cancer, diabetes, and cardiovascular diseases (Sampaio-Maia et al., 2016).

According to recent research, understanding the composition and dynamics of the oral microbiome is crucial for identifying potential disease-causing microorganisms and developing targeted preventive and therapeutic strategies (Arweiler et al., 2020). Rodents are commonly employed in in vivo research, with rats being the predominant species utilized to investigate peri-implantitis disease and its progression (Schramm et al., 2023). The Black Rat (*Rattus rattus*) is a Muridae family member known for its flexible nature and ability to live in different environmental conditions (Chellappan, 2021). Examining the oral microbiome of Black Rats can provide valuable insights into both rat and human health. With the increasing understanding of the oral microbiome and its role in overall health, it becomes important to explore and characterize the oral microbiome of different animal species, including the Black Rat (Radaic & Kapila, 2021).

Oral microbiome communities were evaluated in mice soon after birth to adulthood, up to 1 year of life, in a controlled manner by using sequential oral samples from the same mice over time to examine the establishment and stability of oral mucosal microbiome in mice. Transmissibility of oral microbes from parents and during cohousing experiments was evaluated along with the evaluation of vulnerability to oral inflammatory disease in mice hosting diverse microbiomes. This research reveals vital main concepts related to the formation and durability of a healthy oral microbiome post-birth and offers valuable understanding for microbiome-host studies in animal models (Abusleme et al., 2020).

The oral microbiome of wild mammals plays an essential role in both health and diseases, covering a complex community of microorganisms that inhabit the mouth cavity. Several studies have been conducted on the oral microbiome of humans and domestic animals, but not much information is available on the microbial communities of wild rodents. Humans and other animals are exposed to many emerging infectious diseases. Rats are typically nocturnal and live near humans. In the wild, they often construct burrows into the ground or use already available hollow spaces for shelter. Rats can be reservoirs of pathogens, and characterization of oral microbiomes is essential because they can cause severe health concerns to humans and animals. Considering the abovementioned factors, the present study was planned to characterize the oral microbiome of Black rats (*Rattus rattus*).

Material and methods

Study area and specimen collection

A total of 5 specimens (n=5) of *Rattus rattus* from each tehsil (Renal Khurd, Deepal Pur, and Okara) of district Okara were captured using Sherman traps. The district Okara is situated in the Punjab, Pakistan, with an area of 4419 km^2. The geographical position of the study area is 30.80138^{o}N, 73.448334^{o}E. The average annual rainfall is almost 200 mm (DDMP-Okara, 2022). The hottest months considered are May and June, with high temperatures around 44^{o}C and the lowest temperature in December at 20^c (Khalil & Zahid, 2018). Figure 1 shows the map of the study area.

Sample collection

The captured specimens were brought to the Post Graduate lab, Department of Wildlife and Ecology, UVAS, Lahore, to collect and identify saliva samples. Saliva samples were collected using sterile cotton swabs. After sample collection, all specimens were released into their natural

habitat. Collected swab samples were stored at -80ºC for further processing and analysis (Shriner et al., 2012; Hallmaier-Wacker et al., 2018; Walther et al., 2021).

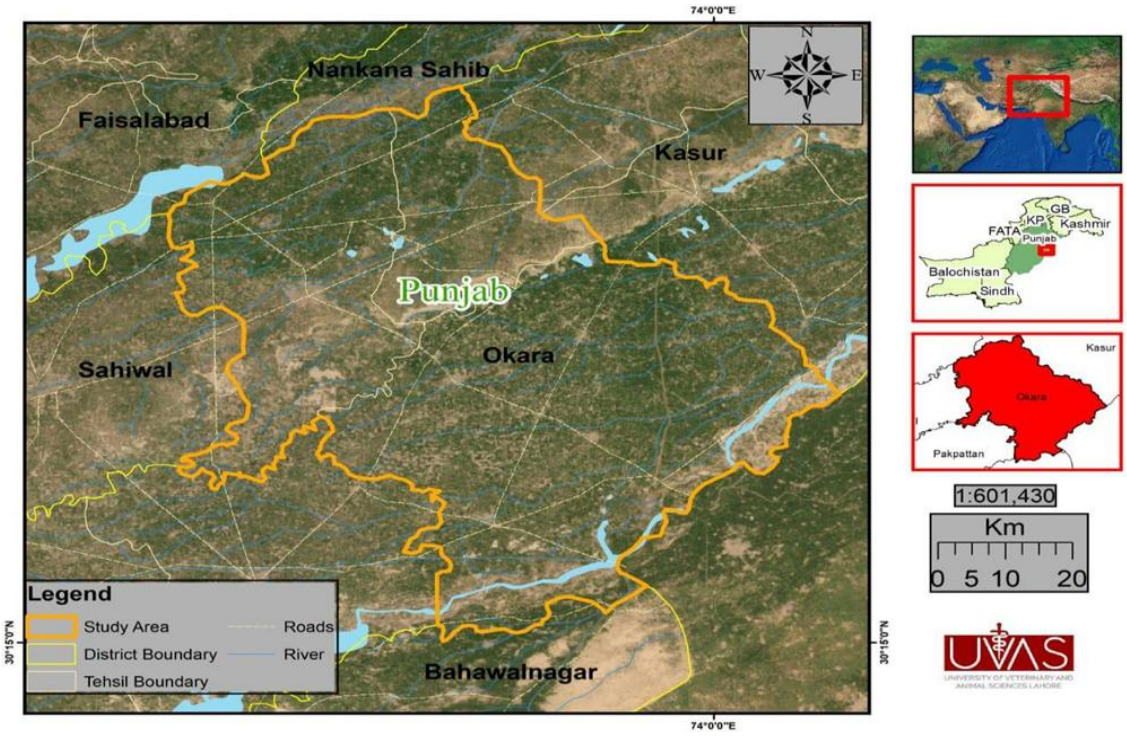

Figure 1. Map of the study area

DNA extraction, quantification, and sequencing

According to the manufacturer's instructions, a QIAamp DNA Microbiome kit was used to extract DNA from the oral swab of the rat. The quality of DNA was checked through gel electrophoresis, and quantity was measured using Nano-Drop One at Alpha Genomics lab, Islamabad, Pakistan. The 16S rRNA gene was amplified using the universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and BS-R1407 (5-GACGGGCGGTGWGTRC-3) to amplify variable regions (V) V1 to V8 of 1380 bp (Klindworth et al., 2013). The PCR was done according to Quast et al., 2013. PCR reaction was performed in 25 µL reaction mix using 12 µL PCR Master Mix, 1µL forward primer, 1 µL reverse primer, and 10 µL of double distilled water and 1µL DNA. Thermocycler parameters were initially denaturing at 95 °C for 3 minutes, followed by 30 cycles of 95°C for 45 seconds, 50°C for I min, 72°C for 90 seconds, and a final extension of 72 °C for 10 minutes. The NGS (next-generation sequencing) of PCR products was done using an overseas commercially available facility at Macrogen Korea.

Data analysis

The PKSSU4.0 catalog database of prokaryotic 16S rRNA gene structures was used for pre-process analysis. The effective DNA reads were used for Operational Taxonomic Unit (OTU) picking. Using OTU data, the relative abundance of bacterial species was estimated (Chao & Chiu, 2016; Magurran, 2021). The VSEARCH application was used to organize OTUs based on closed references. The genomes for reference were stored in a FASTA file with the QIIME 2 data type Feature Data. The reference databases for 16S rRNA were SILVA (https://www.arb-silva.de/download/archive/qiime). The q2-feature classifier plugin and the Nave Bayes classifier were used to assign plausible categories to each read. This classifier was trained using OTU segments with a 97 percent consistency from a database table. The microbial communities at the genus and species level were grouped. The sub-populations were compared using both ordination analysis and hierarchical clustering. Raw paired-end reads (FASTQ) from the original DNA fragments were imported in QIIME 2 v2021.4 software, and a Krona plot was constructed.

Spatial Distribution of Bacteria Taxa:

We used IDW-Interpolation techniques from spatial analyst tools of ArcGIS 10.1 to show bacterial taxa's spatial distribution and variation between selected sampling stations.

Results

The distribution bar plot of the comparative abundance of the top 10 bacterial groups in saliva samples of black rats (*Rattus rattus*) is shown in Figure 2. An interactive bar plot has been created to observe the taxonomic composition of each sample across seven classification levels. This visualization utilizes bar plots to represent the relative abundance of Operational Taxonomic Units (OTUs) at all classification levels. The elimination of non-bacterial OTU sequences was achieved through the feature-table-

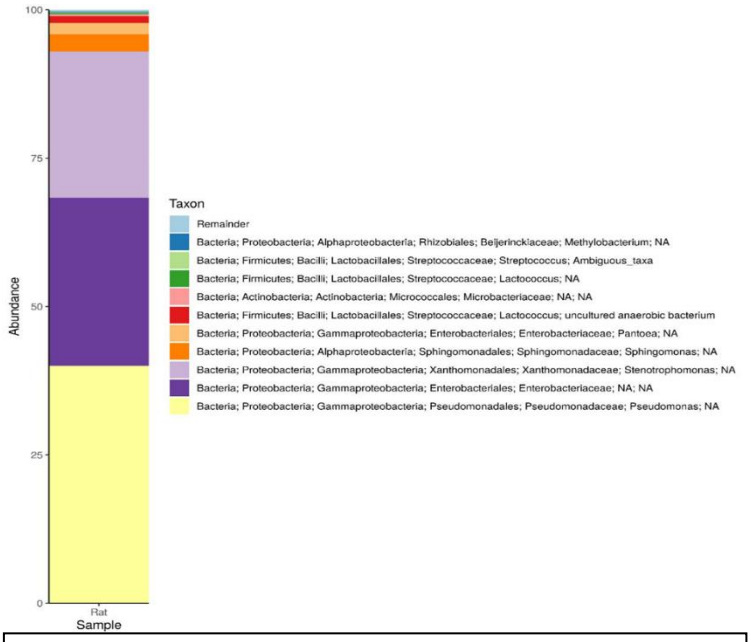

Figure 2. Taxa bar plot showing the abundance of top 10 bacterial groups in saliva samples of black rat (*Rattus rattus*)

filtering method in QIIME 2. The plot also provides information on the absolute abundance of species or OTUs across the samples.

Taxa annotation and visualization

The identified bacterial Taxa's annotation is visually shown in the KRONA plot (Figure 3). In the result display, circles from inside to outside stand for different classification levels, and the area of the sector means the respective proportion of different OTU explanation results.

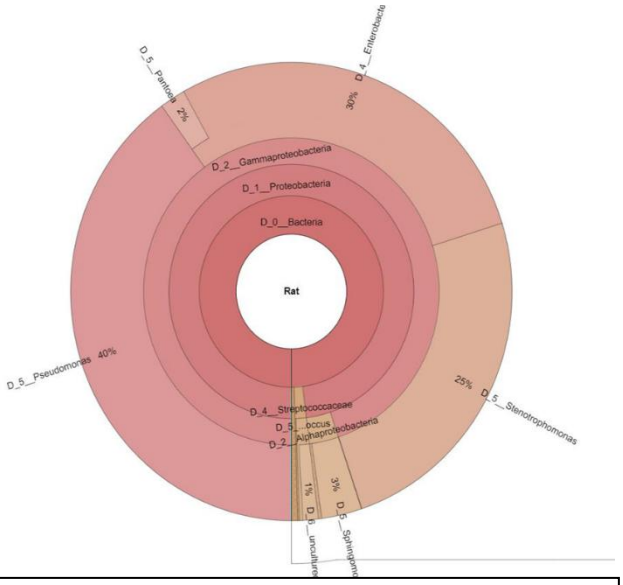

Figure 3. Krona plot showing identified bacterial taxa and their relative abundance

The following Phyla were identified, and their average percentages are as follows: Proteobacteria 98% > Firmicutes 1% > Actinobacteria 0.4% and Bacteroidetes 0.05%. The mean order of abundance of Bacterial classes includes Gammaproteobacteria (95%), Alphaproteobacteria (3%), and Bacilli (1%) (Figure 4). In this way, Pseudomonadales, Enterobacterales, Xanthomonadales, Sphingomonadales, Lactobacillales, Micrococcales, and Bacteroides were different bacterial order taxas' identified in the saliva of the black rat, the average order of abundance was as follows 40% > 30% > 25% > 3% > 1% > 0.4 % > 0.05% respectively. The mean order of identified families were Pseudomonadaceae 40% > Enterobacteriaceae 30% > Xanthomonadaceae 25% > Sphingomonadaceae 3% (Figure 5) while the average percentage of identified genera including *Pseudomonas* was 40% > *Stenotrophomonas* 25% > *Sphingomonas* 3% > *Pantoea* 2% > *Porphyromonas* 0.05% (Figure 6).

Pseudomonas is a pathogen that can cause multiple diseases in healthy persons but is most likely to attack hospitalized persons or those with weak immune systems. It can result in low blood pressure (hemodynamic shock), which can lead to organ failure in the heart, kidneys, and liver. Fever, chills, weariness, muscular and joint discomfort are all possible symptoms. It's also implicated in causing skin, ear, and eye disorders. Stenotrophomonas is a genus with at least ten species and a gram-negative bacteria.

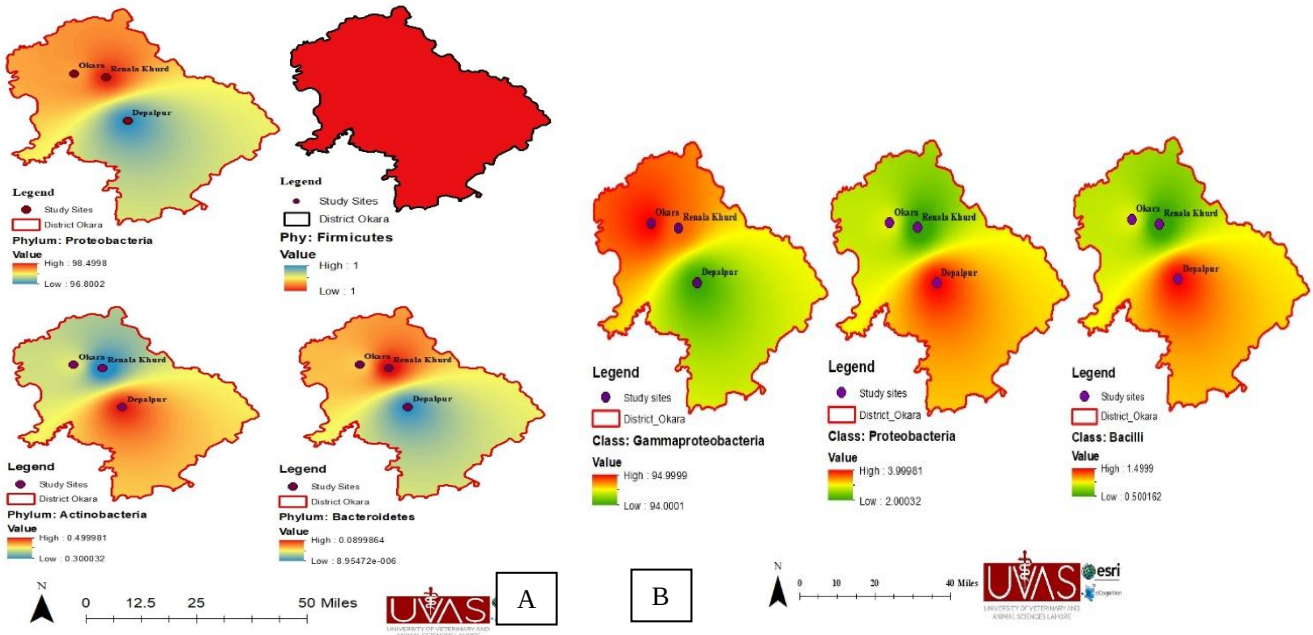

Figure 4. A) Abundance of bacterial phylums. B) Abundance of bacterial classes in district Okara on different sampling stations from saliva samples of black rat (*Rattus rattus*).

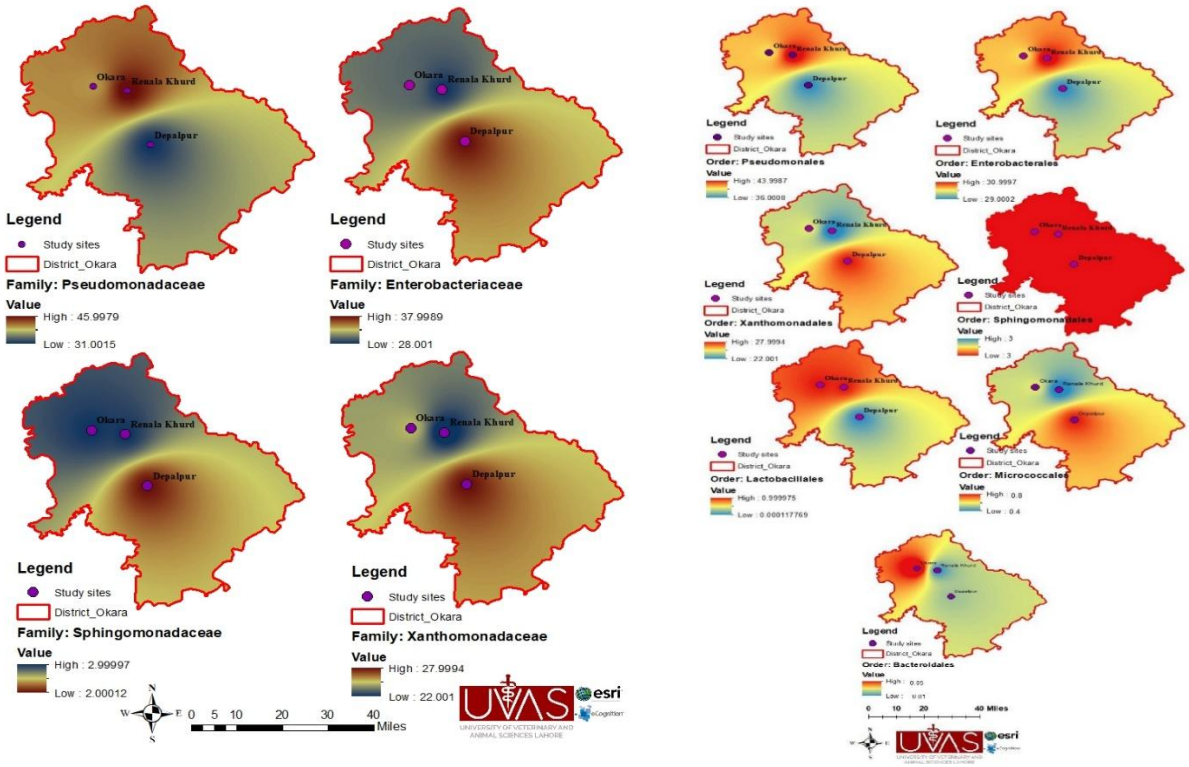

Figure 5. A) Abundance of bacterial orders B) Abundance of bacterial families in district Okara on different sampling stations from saliva samples of black rat (*Rattus rattus*)

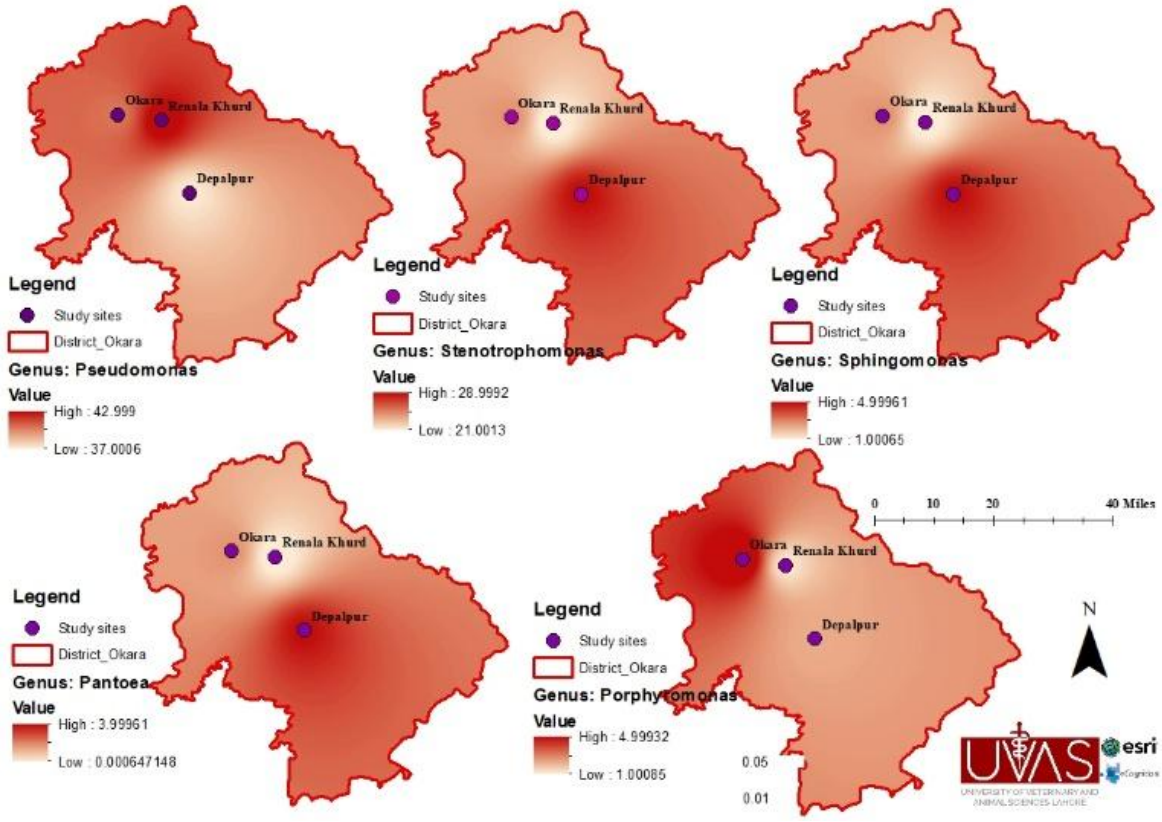

Figure 6. Abundance of bacterial genus in district Okara on different sampling stations from saliva samples of black rat (*Rattus rattus*).

Discussion

We collected saliva samples from captured black rats (*R. rattus*) from different sampling sites in district Okara. The Rattus genus encompasses at least 56 species (retrieved from the Integrated Taxonomic Information System online database on January 28, 2014, http://www.itis.gov). The Norway rat (*Rattus norvegicus*) and the black rat (*Rattus rattus*) are the two species most frequently linked with the genus among these species (Otto et al, 2015). In the present study, the oral microbiota of black rats (R. rattus) exhibits a distinct bacterial composition, as revealed by the phylum-level analysis; Proteobacteria dominated the oral saliva, constituting an overwhelming 98%, followed by Firmicutes at 1%, Actinobacteria at 0.4% and Bacteroidetes at 0.05%, which varies from other mammals, like in humans. The percentage of Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes is 4.5%, 24.5%, 0.0%, and 65.4%, respectively (Shin et al., 2015). Sturgeon et al. (2014) characterized the oral microbiota of 11 healthy cats through next-generation sequencing; eight bacterial phyla comprised 97.6% of the sequences, with the

predominant ones being Proteobacteria (75.2%), Bacteroidetes (9.3%), Firmicutes (6.7%), SR1 (2.7%), Spirochaetes (1.8%), Fusobacteria (1.3%), and Actinobacteria (0.6%). Proteobacteria are very common in mammals with very diverse metabolisms, and they can break down and ferment complex carbohydrates and create vitamins (Colston & Jackson, 2016). The abundance of various bacterial taxa in rodents varies between domestic and wild animals and depends on dietary compounds (Camps-Bossacoma et al., 2017; Bensch et al., 2023). Vasques-Monteiro et al.'s (2021) study reveals that prolonged high-fructose diet intake in adult mice leads to a notable increase in Proteobacteria. This disrupts the gut-liver axis, causing heightened hepatic steatosis, oral glucose intolerance, elevated blood pressure, and endotoxemia. In our study, at the class level, Gammaproteobacteria takes precedence, accounting for 95% of the identified classes, followed by Alphaproteobacteria at 3% and Bacilli at 1%.

Similarly, Actinobacteria, Alpha- and Gammaproteobacteria, Bacilli, and Clostridia are the predominant five classes identified by Garcia et al. (2024). In present results, the order-level analysis sheds light on the diversity within the identified bacterial classes; Pseudomonadales takes the lead with an average order abundance of 40%, followed by Enterobacterales at 30% and Xanthomonadales at 25%. Shah et al. (2023) annotated the bacterial composition through OTUs into 104 families, 168 genera, and 17 phyla in the intestine of *Rattus norvegicus*. Moving down to the family level in current studies, the prevalence of Pseudomonadaceae (40%), Enterobacteriaceae (30%), and Xanthomonadaceae (25%). Bacterial taxa may differ across various sections of the gut and respiratory system. In the findings of Shah et al. (2023), the stomach exhibits a prevalence of Lactobacillaceae and Bifidobacteriaceae at the microbial family level. Conversely, the gut displays a higher abundance of Lactobacillaceae and Erysipelotrichaceae, while the lungs show a notable presence of Alcaligenaceae. Zooming in further to the genus level in present study, *Pseudomonas* emerges as the most abundant genus, constituting 40% of the identified genera. *Stenotrophomonas* follows at 25%, *Sphingomonas* at 3%, *Pantoea* at 2% and *Porphyromonas* at 0.05%. Several species within the *Pseudomonas* genera are known zoonotic pathogens. Alike *Pseudomonas aeruginosa* poses a significant threat to commercial poultry, particularly affecting young birds and causing substantial losses. Infection during embryonic stages results in death within the shell, while infection in chicks leads to septicemia, respiratory and enteric infections, and increased mortality rates. *P. aeruginosa* is also highly infectious to humans, causing severe lung damage, especially in immunocompromised individuals. The transmission of *P. aeruginosa* to humans is facilitated by chicken carcasses and related poultry

retail products, particularly following processing in abattoirs (Abd El-Ghany, 2021). *Stenotrophomonas maltophilia* complex (Smc) from *Stenotrophomonas* genre comprises a group of emerging pathogens affecting individuals with compromised immune systems and those with cystic fibrosis (Mercier-Darty et al., 2020). The *Sphingomonas* bacterial genera was detected in spleen samples from six rodent species (*Liomys adspersus*, *Melanomys caliginosus*, *Mus musculus*, *Proechimys semispinosus*, *Rattus rattus*, *Zygodontomys brevicauda*) but was present in lower abundance. This genus holds clinical importance (García et al., 2024). *Pantoea agglomerans*, a bacterium within the *Pantoea* genus, stood out as the most frequently isolated bacterium from parrots in pet shops, constituting 23.5% of the isolates (Marques et al., 2021).

Notably, *P. agglomerans* is a Gram-negative facultative anaerobic bacillus known to cause various opportunistic infections in humans, such as septicemia, pneumonia, septic arthritis, wound infections and meningitis (Guevarra et al., 2021).

Porphyromonas has been identified in various pristine and human-altered environments, indicating a broader host range than previously understood. This includes detection in aquatic animals, arthropods, and birds, although humans, pets, and farm animals remain their predominant hosts. *Porphyromonas* is primarily linked to mammals and is implicated in chronic oral infections, along with secondary pathologies like cancers and neurodegenerative diseases. De-Cock et al (2022) sequencing data revealed 14 potentially zoonotic bacterial genera, confirming the presence of zoonotic *Leptospira spp*. and *Bartonella tribocorum* through (q)PCR or Sanger sequencing in wild rats. Additionally, one of three dominant bacterial taxa *Streptococcus*, *Mycoplasma* and *Leptospira*, accounted for more than 50% of the reads. Bacterial communities vary among rodent species. Naked mole rats show an overabundance of Bacteroidetes and a reduction in Firmicutes. At the family level, Lactobacillaceae (Firmicutes) constitutes around 25% of the mouse gut microbiome but is scarce in naked mole rats. In contrast, Prevotellaceae (Bacteroidetes) and Erysipelotrichaceae (Firmicutes) together contribute over 40% to the naked mole-rat gut microbiome, whereas they are minimally present in mice (Fitzpatrick et al., 2022). The studies indicate that there may be inherent differences in the salivary microbiota between male and female adolescents and changes during growth (Chandel et al., 2018; De-Andrade et al., 2020). The study demonstrated that *R. rattus* exhibited a higher concentration of bacteria in non-forest species than in forest species (Mohd-Taib et al., 2018). This was anticipated as non-forest species were more exposed to human settlements. Diagne et al. (2017) discovered that individual host factors, such as body mass and sex, were crucial in driving rodent bacterial communities. In conclusion,

comparing these results with existing literature, it becomes evident that the oral microbiota of black rats (*Rattus rattus*) exhibits unique characteristics, while some similarities exist with the oral microbiota of other mammals. This study provides valuable insights into the oral microbiota of *Rattus rattus*, contributing to our understanding of the microbial communities that inhabit the oral cavity of these rodents. The dominance of Proteobacteria, especially Gammaproteobacteria, and the prevalence of specific orders and genera highlight the complexity and uniqueness of the oral microbiota in *Rattus rattus*.

Conclusion

It can be concluded that the high prevalence of potentially pathogenic bacteria like Pseudomonas suggests a risk of zoonotic diseases that could affect both local wildlife and human populations. Future studies should focus on identified bacterial communities in other rodent species and small mammals to compare their roles in disease ecology. This could identify specific species that are particularly significant in zoonotic transmission.

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