BEFORE THE NATIONAL GREEN TRIBUNAL

WESTERN ZONE BENCH, PUNE

ORIGINAL APPLICATION NO. 193 OF 2024 (WZ)

[Earlier Original Application No. 1157/2024 (PB)]

New item titled "Gujarat: Mugger Crocodile in Vadodara's polluted Vishwamitri are stressed, those in rural charotar are not, finds study:, Appearing in Down To Earth dated 14.08.2024

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1.		Affidavit-In-Reply on behalf of Respondent No. 4 Chief Wildlife Warden, Gujarat.	
	A	A Copy of Report.	

Regd. No.: 354
Date : 7-2-2025.

BEFORE THE NATIONAL GREEN TRIBUNAL
WESTERN ZONE BENCH, PUNE

ORIGINAL APPLICATION NO.193 OF 2024 (WZ)
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In the matter Of:

News item titled "Gujarat: Mugger Crocodiles in Vadodara's polluted Vishwamitri are stressed, those in rural charotar are not, finds study", Appearing in Down To Earth dated 14.08.2024

AFFIDAVIT-IN REPLY ON BEHALF OF RESPONDENT NO. 4
CHIEF WILDLIFE WARDEN, GUJARAT

I, Agneeshwar Vyas (IFS), Age: 41 years, serving as Deputy Conservator of Forest, Gujarat, authorized signatory of Respondent No.4, having address at Office of the Dy. Conservator of Forests, Social Forestry Division Vanbhavan, 1st Floor, B/H Raopura Police Station, Vadodara, do hereby state oath and make this affidavit as under :-

1. I have read and understood the Order dated 12.09.2024 issued by the National Green Tribunal Principal Bench, New Delhi, in reference to the News Item titled "News item titled "Gujarat: Mugger Crocodiles in Vadodara's polluted Vishwamitri are stressed, those in rural Charotar are not, finds study", Appearing in Down To Earth dated 14.08.2024. I am competent to file this affidavit as I am well acquainted with the issues highlighted by the Hon'ble Tribunal regarding prolonged high levels of stress hormones leading to long term issues such as reproductive failure and weekend immune function.

2. I submit that the Vishwamitri River, which primarily flows through the western part of Vadodara city, is not designated as part of any

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Protected Area (such as a Wildlife Sanctuary or National Park), and the Forest Department does not have legal jurisdiction over the land.

However, the river serves as a habitat for the Marsh Crocodile, a Schedule I species under the Wildlife Protection Act. As such, the Social Forestry Division of the Gujarat Forest Department in Vadodara is responsible for the rescue and rehabilitation of these crocodiles. Data of rescue of animals done in past 5 years is tabulated as below.

Sr. No	Year	Crocodile rescue (In nos.)
1	2020-21	143
2	2021-22	118
3	2022-23	134
4	2023-24	113
5	2024-25	218

3. I submit that as observed over the past few years, there is an indication of an upward trend in the crocodile population. Moreover, there has been no noticeable change observed in their breeding or behavior patterns. The last crocodile census of the year 2015 & 2020 shows the number of crocodiles is increased over last few years.A tabular chart indicating the same is as under:

Crocodile Census: Year of 2015 & 2020

Sr. No.	Year	No. of Crocodile (Day)	No. of Crocodile (Night)
1.	Jan-2015	164	248
2.	Feb-2020	220	287

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4. I submit that, according to the research article ***'Gut microbiota analysis of marsh crocodiles (Crocodylus palustris) of Vishwamitri river'*** by Shreya Kalariya, Dr. Jigna R. Desai, and Vishal Thakur, published in 2024, the gut microbiome plays a critical role in the health of the host. A metagenomic analysis of the gut microbiota of marsh crocodiles (Crocodylus palustris) revealed a dominance of the phylum *Firmicutes*, followed by *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. Within the *Firmicutes* phylum, classes such as Bacilli, Gammaproteobacteria, and Clostridia were prevalent, along with a significant presence of fecal coliforms in their aquatic habitat. Notably, despite constant exposure to potential pathogens in their environment, crocodiles seem to be resistant to infections—both systemic and through skin wounds or lesions. This suggests that crocodiles may possess robust antimicrobial properties, either within their immune system or through their gut microbiome. A Copy of the aforesaid report is annexed herewith and marked as **Annexure: A.**

5. I submit that the research report published in ***Down To Earth*** on 14.08.2024 mentions the presence of elevated stress hormone levels in the faecal samples of marsh crocodiles. However, it does not conclusively provide information regarding the impact of these elevated hormone levels on the physiological or behavioral aspects of the free ranging Crocodiles. Therefore, further research is necessary to determine how these elevated levels may affect the crocodiles. It is suggested that this matter be referred to an expert institution, such as the Wildlife Institute of India, to conduct a more comprehensive study on the potential long term impact of these elevated stress hormone levels along with suitable mitigation measures.

6. I submit that, based on our field observations, population estimates, and the number of rescues conducted in the area, we have not

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observed any significant decline in the crocodile population or any notable alterations in their behavior. Therefore, we request that further research on these findings be conducted to suggest any potential mitigation measures, if required, from our end.

7. I further submit that field survey for the population estimation exercise for the Crocodile in the Vishwamitri river, Vadodara city has been undertaken by GEER foundation [Gujarat Ecological Education and Research Foundation], Gandhinagar from 4th to 6th February, 2025. I further submit that the aforementioned report is still awaited.
8. The answering respondent craves liberty of the Hon'ble Tribunal to file additional reply, in future, if required.

9. In view of submissions made hereinabove, it is respectfully submitted hat this Answering respondent shall abide by any order(s) or direction(s) passed by this Hon'ble tribunal in the present application.

Solemnly affirmed at _____ on this _____ day of February 2025.

Deputy Conservator of Forest
Extension Division
Vadodara
Deponent

VERIFICATION

Verified at _____ on this day of _____ that the contents of the above reply are correct and true. Nothing has been concealed therefrom and mis-stated.

NOTARY MEENA B. GONDALIYA Area : Vadodara City R. No.: 12857/17 GOVT. OF INDIA

NOTARY MEENA B. GONDALIYA Area : Vadodara City R.No.12857/2017 Expiry Date : 07-02-2027 GOVT. OF INDIA

Deputy Conservator of Forest
Extension Division
Vadodara
Deponent

ATTESTED
Gondaliya M. B.
M. B. GONDALIYA
NOTARY (Govt. of India)

Deputy Conservator of Forest
Extension Division
Vadodara

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Research Article

Gut microbiota analysis of marsh crocodile (*Crocodylus palustris*) of Vishwamitri River

Shreya Kalariya[1*], Jigna Desai[2], Vishal Thakur[3]

[1]Department of Bioscience, Veer Narmad South Gujarat University, Surat, India
[2]Department of bioscience, veer narmad south Gujarat university, surat, India
[3]Canine Group Association, Vadodara

**Corresponding Author: kalariyashreya82@gmail.com*

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Abstract—A study was conducted to establish baseline information regarding the microbiome composition of the Marsh crocodile (Crocodylus palustris) gut flora through DNA isolation and sequencing of scat samples. Metagenomics, focusing on microbial communities within ecosystems, was employed to analyze the diverse microbiota, including bacteria viruses, archaea, and eukaryotes. These microorganisms are ubiquitous and inhabit various environmental niches, such as soil, air, and water, and also form symbiotic relationships with host organisms, including their skin, lungs, and gastrointestinal tracts. Traditional bacterial biochemical identification approaches were found to be labor-intensive due to the inability to culture certain microbes. The use of One Codex software facilitated the analysis of the Marsh crocodile scat community, revealing a rich diversity comprising 84 phyla, 75 classes, 150 orders, 326 families, 1104 genera, and 3025 species. The gut microbiome is recognized for its significant impact on host health. In this study, Firmicutes predominated, comprising 50.07% of the phylum, followed by Proteobacteria, Actinobacteria, and Bacteroidetes. Notably, Firmicutes, including Bacilli, Gammaproteobacteria, and Clostridia, were associated with high levels of fecal coliforms in the aquatic environment inhabited by Marsh crocodiles.
Keywords: gut flora, microbiome, Firmicutes, Marsh crocodile, DNA isolation.

1. Introduction

India, known for its cultural icons such as snake charmers and elephants, has recently garnered attention as a nation significantly populated by crocodiles. Among the three crocodilian species inhabiting India, the Mugger or Marsh crocodile (Crocodylus palustris) is the most prevalent.[1]Crocodylus palustris, widely distributed across the country, is of particular interest compared to the other two species, Crocodylus porosus (Estuarine or Saltwater Crocodile) and Gharial (Gavialis gangeticus), due to its characteristic features. All three species enjoy legal protection in India, though they face various threats such as habitat loss, pollution, and human activities like hunting and the pet trade, leading to environmental degradation.[2] Despite these challenges, crocodilian populations remain substantial in India and neighboring countries like Pakistan, Nepal, Sri Lanka, Bangladesh, Bhutan, and Myanmar. However, Bhutan and Myanmar have reported their extinction due to habitat loss[3]. Recent reports from Bangladesh indicate the persistence of Mugger populations in their native habitats[4,5,6].

Metagenomics, a discipline investigating microbial diversity within ecosystems, encompasses the study of microbiomes comprising bacteria, viruses, archaea, and eukaryotes.[7,8] These microorganisms are omnipresent, existing in diverse environmental niches such as soil, air, water, and within mixnismal body parts including the skin, lungs, and gastrointestinal tract. Understanding microbial diversity and functional capabilities within specific habitats is pivotal for elucidating microbial evolution and ecology[9]. Traditional approaches to microbial diversity studies, involving bacterial biochemical identification and culture-dependent methods, pose significant scientific and technological limitations, including laborious processes and challenges associated with identifying non-culturable bacteria[10]. Consequently, researchers have endeavored to develop novel methods for studying microbial diversity[11].

The term "metagenome" encompasses two main approaches: structural metagenomics, which examines the structure of uncultivated microbial populations to understand interactions between individual components, and functional metagenomics, which identifies genes through the generation of expression libraries followed by activity-based screenings.[12,13]. Studies often employ 16S rRNA gene investigations as a metagenomic approach, which can elucidate metabolic pathways and the functional potential of microbiomes. Sanger sequencing technology has historically facilitated microbial diversity analysis in metagenomic studies[14]. Environmental factors such as temperature,

salinity, water quality, and dissolved oxygen significantly influence fish biological functions, including reproduction and growth.[15] Organisms adapt to environmental variations by altering protein-coding DNA sequences, thereby affecting gene expression and individual fitnes[16].

2. Materials and method

Sample collection The Crocodile scat sample was collected in an air-tight plastic bag from the Vishwamitri river bank. Sample fig.1 Scat analysis Scat analysis was done by the proper hand shorting method. During analysis, different type of material was observed and collected from the scat. DNA extraction Sample preparation

1. Add 0.8 ml of 1XSS buffer to 1 ml of sample and mix. 2. Centrifuge for 1 minute at 12000 rpm in a microfuge tube and discard 1 ml of supernatant. 3. Add 1 ml of 1XSSC buffer again, vortex, and centrifuge at 12000 rpm for 1 minute, and remove all of the supernatant. 4. Add 370 microliter of 0.2 M sodium acetate to each pellet and mix by inverting the tube briefly. 5. Add 25 microliter of 10% SDS and 5 microliter of proteinase K (20 mg/ml). mixed by inverting the tubes briefly and incubated for 1 hour at 55°C. 6. Add 100 microliter of phenol-chloroform-isoamyl alcohol and mix for 30 seconds. 7. Centrifuged for 2 minutes at 12000 rpm in a microcentrifuge tube. 8. Collect the aqueous layer (top layer) carefully in a new microcentrifuge tube, add 1 ml of cold 100% ethanol, mix, and incubate for 15 minutes at -20°C. 9. Centrifuged for 2 minutes at 12000 rpm in a microcentrifuge. The supernatant was removed by draining the tubes. 10. Add 180 microliters 1 X TE buffer, vortex, and incubate at 55°C for 10 minutes. 11. Add 20 microliter of 2 M sodium acetate and mix. 12. Add 500 microliter of cold 100% ethanol mixed. Centrifuged for 1 minute at 12000 rpm in a microcentrifuge. 13. Decant supernatant and rinsed the pellet with 1 ml of 70% ethanol. Centrifuged for 1 minute at 12000 rpm in microcentrifuge. 14. Decant supernatant, dry the pellet in a speed-vac for 10 minutes, or until dry at room temperature. 15. Resuspend the pellet by adding 200 microliter of 1X TE buffer. Incubate overnight at 55°C, vortex periodically to dissolve the genomic DNA.

3. Results and Discussion

Result DNA Visualization isolated DNA was visualized by 0.8% Agarose gel electrophoresis (Bio-Rad) using 0.5 X TEA buffer at 60-65 voltage for approximately 45 minute to 1 hour. The Image of the gel was captured in the gel documentation system to check the quality of the DNA. Fig.-2. 16s metagenomics report of marsh crocodile scat sample: Qualitative and quantitative analysis of gDNA. DNA was isolated from given sample by Qiagen gDNA kit[17]. The quality of gDNA was checked on 0.8% agarose gel (loaded 5□l) for the single intact band. The gel was run at 110 V for 30 mins. 2□l of the sample was loaded in BioTek Epoch to determine the A260/280 ratio. The DNA was quantified using a Qubit dsDNA HS Assay kit(life Tech). 1□ of each sample was used for determining concentration using a Qubit 2.0 Fluorometer. Preparation of libraries for run chemistry: the amplicon library was prepared as per the 16s Metagenomics sequencing library preparation protocol. primer for amplification of the hyper-variable region of 16rDNA gene of bacteria and archaea:primer set V2-4-8.primer set V3-6,7-9 software for community metagenomics analysis by One Cordex: The one cordex data platform works with the dual goals of analyzing microbial reference data against the largest possible collection of microbial reference genomes, as well as presenting those results in a formate that is consumable by applied end-users.one codex identifies microbial sequences using a “k-mer based” taxonomic classification algorithm through a web-based data platform, using a reference database that currently includes approximately 40, 000 bacterial, viral,fungal,protozoan genomes. The data were analyzed with standard instructions provided by the developer (Minot et al.,2015) DNA isolation from the crocodile scat sample was done by using the 1X SS buffer method. DNA visualization the isolated DNA from the crocodile scat sample was visualized as a single compact DNA band by using the 0.8% agarose gel electrophoresis. Fig.3 Quantification of gDNA using Qubit Flurometer dsDNA Assay lane sample Concentration No. (ng/□l) 1 Crocodile scat sample 40 community metagenomic study with one Codex: one codex database classified the sequence into 84 phyla, 75 classes, 150 orders, 326 families, 1104 genera, and 3025 species. community metagenomices study with one codex : all results of the metagenomices study with one codex are given in Fig. 5,6,7,8,9,10. The community of crocodile scat samples was analyzed by the softwere one codex. the phylum lavel analyzed showed 87 phyla of which the highly abundant phyla was Firmicutes 596522 (50.07%), followed by proteobacteria 268260 (22.52%), Actinobacteria 55653 (4.67%) and Bacteroidetes 5290 (0.44%). The Class level analysis showed a high abundance of Bacilli 479480 (40.25%), followed by Gammaproteobacteria 244885 (20.55%), Clostridia 87946 (7.38%), and Actinobacteria 46456 (3.9%). The Order level analysis reported 150 Orders in which the most abundant was Bacillales 453906 (38.1%), followed by Enterobacterales 114056 (9.57%), Pseudomonadales 110728 (9.29%) and Clostridiales 87295 (7.33%). At the family level, a total of 326 Families were identified, and from that the highly abundant was Bacillaceae 175461 (14.73%) followed by Pseudomonadaceae 103920 (8.72%), Enterobacteriacesae 46062 (3.87%), and Morganellaceae 44764 (3.78%). At the genus level high abundance of Bacillus 146362 (12.28%) followed by Pseudomonas 103454 (8.68%), Proteus 40575 (3.41%), and Clostridium 38845 (3.26%). At the species level, Escherichia coli was reported most abundant from the total 3025 species followed by Bacillus sp.UFRGS-B20 9088 (0.76%), Clostridioides dif cile 4813 (0.4%), and Salmonella enterica 2470 (0.21%). Krona-based interactive chat was generated by one codex software for in-depth analysis of data. The one codex generated the Krona-based interactive chart for depth analysis of the relative abundance and confidence within the complex hierarchies of all the microbes present in the Crocodile scat sample. Fig.13,14,15

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Figures and Tables

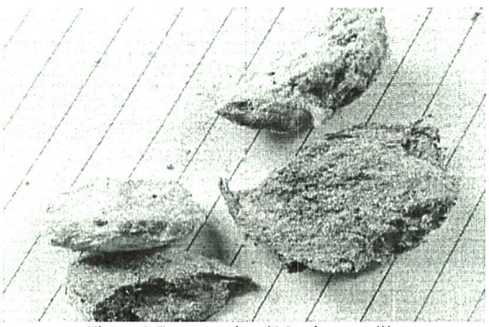

Figure 1 Scat sample of Marsh crocodile

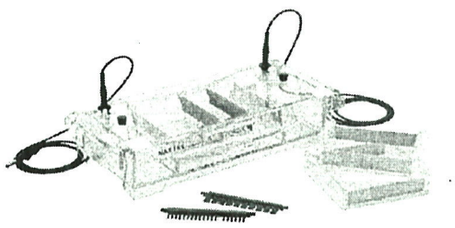

Figure 2 Gel electrophoresis

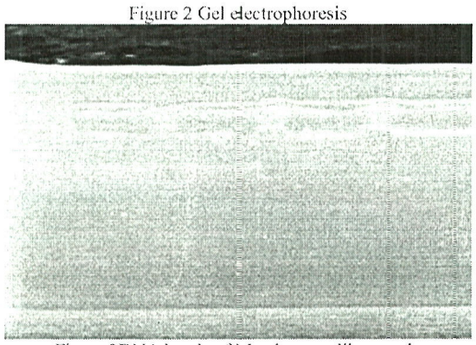

Figure 3DNA bands of Marsh crocodile sample

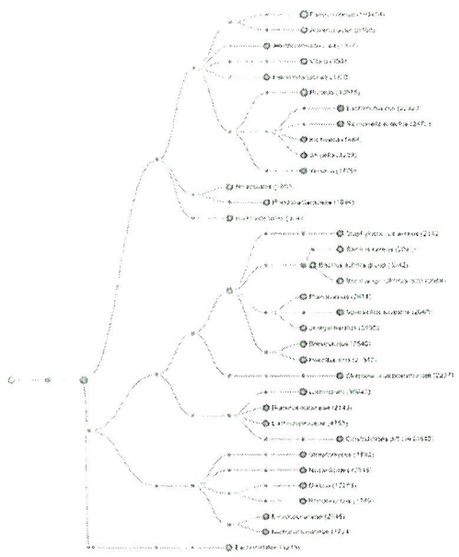

Figure 4 Taxonomic chart of classified read

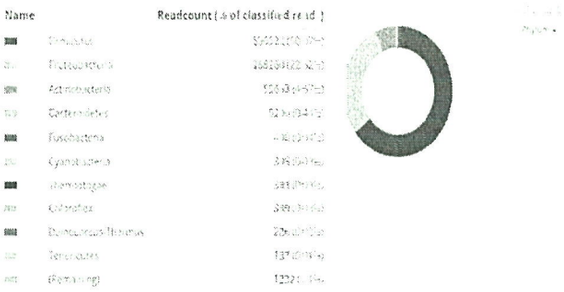

Figure 5 % classified reads of phyla from sequence

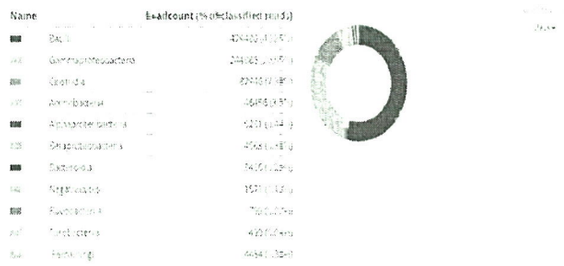

Figure 6 % classified reads of Class from sequence

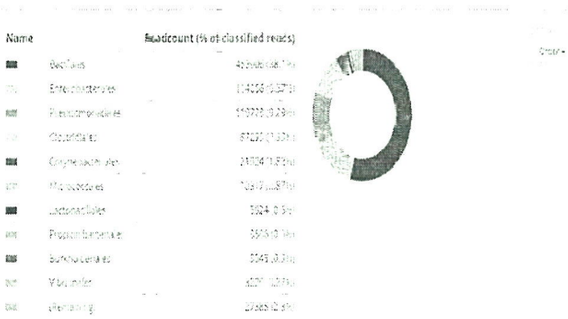

Figure 7 % classified reads of Orders from sequence

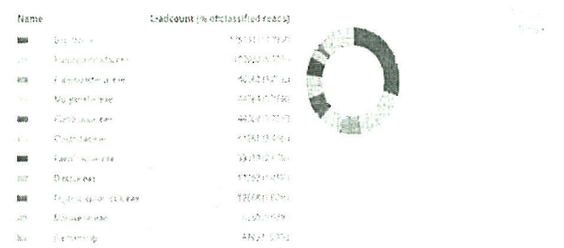

Figure 8 % classified reads of Family from sequence

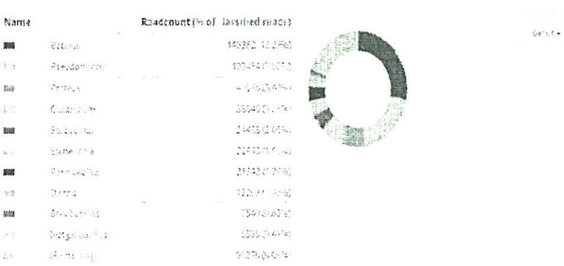

Figure 9 % classified reads of Genera from sequence

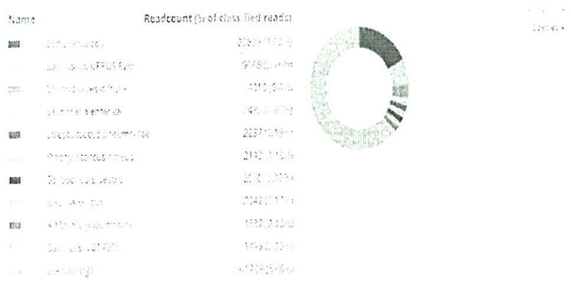

Figure 10 % classified reads of Species from sequence

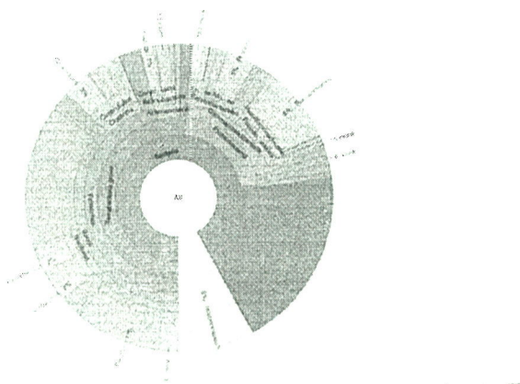

Figure 11Top Kingdom Diversity Classification in Sample

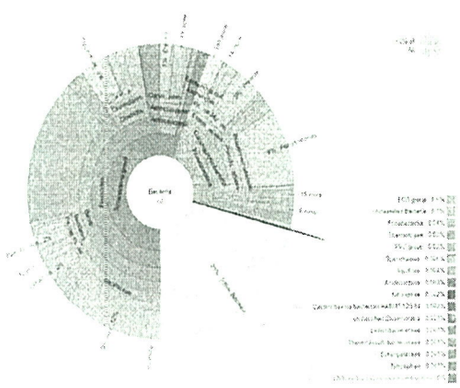

Figure 12 Top Phylum Diversity Classification in Sample

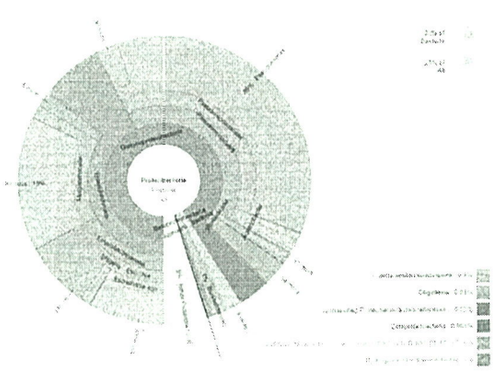

Figure 13 Proteobacteria class Diversity Classification in Sample

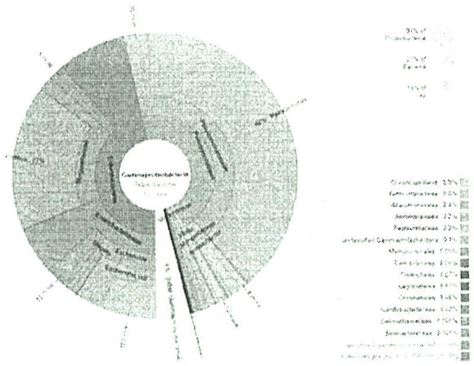

Figure 14 Gammaproteobacteria class diversity classification of sample

4. Conclusion and Future Scope

EThe gut microbiome was accepted to play a crucial role in the health of the host. This study of metagenomics evaluation scat of a Crocodile (Crocodylus palustris) revealed that the microbiota of the gut of a Crocodile (Crocodylus palustris) was dominated by phylum Firmicutes, followed by Proteobacteria, Actinobacteria, and Bacteroidetes mainly. Which, Firmicutes phylum consists of Bacilli, Gammaproteobacteria, and Clostridia across a high load of fecal coliforms in their aquatic environment. Nonetheless, despite constantly being exposed to many potential pathogens, crocodilians do not seem to be susceptible to infection by these organisms, either systemically or through their skin, via lesions or wounds suggesting that these species possess potent antimicrobial abilities in their immune system or gut microbiome.

Data Availability
None

Conflict of Interest
None

Funding Source
"This work had no funding source."

Authors' Contributions

Author-1 conducted a literature review and conceptualized the study. Author-2 contributed to protocol development, obtained ethical approval, and conducted data analysis. Author-3 drafted the initial manuscript. All authors critically reviewed, edited, and approved the final version of the manuscript.

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AUTHORS PROFILE

Shreya Kalariya earned B,Sc.from junagadh., and M.Sc.from veer narmad south Gujarat University In bioscience science, she is currently working as a research chemist at Zymo cosmetic. She does research in the agriculture field and the conservation of wildlife.

Dr. Jigna R. Desai working as an associate professor at the bioscience department of Veer Narmad South Gujarat University Surat. jigna does research in environmental pollution, biodiversity and wildlife conservation, animal cell culture, and cytotoxicity.

Vishal Thakur is a wildlife conservationist and wildlife camp organizer at Vadodara. He is a director of canine group of Association, Vadodara.