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RESEARCH ARTICLE OPEN ACCESS

Diversity and Metabolic Potential of Earthworm Gut Microbiota in Indo-Myanmar Biodiversity Hotspot

Vabeiryureilai Mathipi[1], Surajit De Mandal[1], Zothansanga Chawngthu[2], Ruth Lalfelpuii[1], Nachimuthu Senthil Kumar[1] and H. Lalthanzara[2]*

[1]Department of Biotechnology, Mizoram University, Aizawl - 796 004, Mizoram, India.
[2]Department of Zoology, Pachhunga University College, Aizawl - 796 001, Mizoram, India.

Abstract

Earthworms are important members of the soil macrofauna that play a significant role in soil structure and fertility. However, there is scanty information on the earthworm gut microbial flora and their metabolic potential. In the present study, the diversity of the microbial community and their metabolic potential from the gut content of four different earthworm species from Indo-Myanmar Biodiversity Hotspot were collected and identified by standard methods. The microbial diversity and their metabolic potential were assessed by high throughput sequencing of V3-V4 region of 16S rRNA using Illumina technology. Analysis of microbial diversity was performed by QIIME software package v.1.8.0 with their metabolic potential by PICRUSt (v1.1) software package. A total of 3,36,047 processed sequences were obtained that generated 3686 operational taxonomic units (OTUs). Major bacterial phyla identified were Proteobacteria (47.1%), Firmicutes (38.9%), Actinobacteria (6.3%), Bacteroidetes (3.6%) and Cyanobacteria (1.1%). The abundant genera were *Lysinibacillus* (26.9%), *Acinetobacter* (21.2%), *Pseudomonas* (4.7%), *Bacillus* (3.8%), *Staphylococcus* (3.5%), *Stenotrophomonas* (1.1%) and *Ralstonia* (1%). The functional annotation of the metagenome revealed abundance of bacterial community associated with amino acid, carbohydrate as well as energy metabolism. Furthermore, the presence of enzymes involved in the process of denitrification and methanogenesis were also identified. This study gives insight into the gut microbial composition and their putative functional roles in the gut of tropical hilly earthworms. The study on forest and garden soil earthworm gut microbiomes might help us understand the role of these organisms in their respective ecosystems.

Keywords: Earthworm gastrointestinal, microbial, Mizoram, Next Generation Sequencing, soil

***Correspondence:** hzara.puc@gmail.com; +91-7005902282

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INTRODUCTION

Earthworms are known as 'keystone species' in soil food webs and play an important function in soil nutrient cycling[1]. The major portion of the invertebrate biomass present in the soil is contributed by earthworm[2]. During earthworm's feeding, the microorganisms were also ingested along with organic matter for their nutrients[3]. The microbiota present inside the earthworm gut is the main driver of their beneficial activities[4-5]. Most of the gut microbes harboured by earthworms are acquired from their surrounding habitats and perform different activities including fermentation and denitrification[6-8]. The capacity of the microorganisms to endure the enteric condition of the gut of earthworm is very important[3]. The ingestion of microorganism's populations plays a major role in earthworm's nutrition by facilitating in the decomposition of organic materials, mostly the constituents which are the earthworms cannot utilize in their natural condition[9]. The oxygen-limited environment prevalent in the earthworm gut allows the ingested anaerobes to grow and secrete exoenzymes which help the degradation process of the complex organic materials[7,10].

The relationship between earthworms and microorganisms is at the level of their digestive tract, castings, and burrow walls[2]. They maintain mutualism that exists between earthworms and microorganisms[11] along with higher microbial activity in earthworm castings. The plant nutrient availability in soil is likely relying on the activity of earthworm gut microflora and the diversity of the microorganisms depends on the biotic environment along with various features such as temperature, humidity, apparent density, pH, and organic matter which form part of their nourishment[12]. The member of the Rhizobiale bacteria was involved in the denitrification process and produces N_2O gas inside earthworm gut[5,7,13]. Earthworms are known to have an association with free-living soil bacteria and constitute the drilosphere[14-15]. The gut of earthworms *Lumbricus terrestris* and *Aporectodea caliginosa* are reported to comprise a higher number of aerobes compared to the soil[16].

Majority of the gut metagenomic studies focus on the epigeic species, whereas anecic earthworms gut microbial study is still scanty[10]. In the Indian subcontinent, only a handful of work has been carried out on microbes of earthworm gut content. A good number of papers regarding bacterial diversity of earthworm alimentary canal was published[17-20]. The report on the taxonomic with functional annotation of gut microbial communities of two epigeic earthworm species (*Eisenia foetida* and *Perionyx excavatus*)[21] as well as an exploration of epigeic earthworm gut microbiome in respect to detoxification of nanoparticles in soil system are also published[22]. Isolation and characterization of gut microflora of an epigeic *P. excavatus* was reported from West Bengal[23]. The common Indian earthworm *Lampito mauritii* was also assessed for its gut bacteria community[24]. The recent study on microbial diversity of an earthworm gut was done from the surface dwellers region only (epigeic species) like *E. fetida, Eudrilus eugeniae* and *P. excavatus* at the global level, and an epi-anecic species like *L. terrestris* and *A.caliginosa* of the temperate region only[25,26]. There is no work on gut biota of tropical anecic earthworms.

Considering the scarcity of information on the earthworm gut microbial communities in tropical hilly regions and in particular from Indo-Myanmar Biodiversity hotspot region, the present study aims to characterize the microbial diversity, community profile and their putative metabolic potentials of the gut microbiota of an anecic and an epi-endogeic species of earthworms.

MATERIALS AND METHODS

Collection, identification and extraction of earthworm gut content

The earthworm sampling was done by digging and hand sorting method. The earthworm specimens were collected from brownish-grey loamy and light grey sandy garden soil with slightly acidic pH (5.8 – 6.4) and 22-25% moisture content in Aizawl city in Mizoram, northeast India (Indo-Myanmar biodiversity hotspot) and the worms were transported to the laboratory for identification.

The earthworm specimens were identified by Lalthanzara in consultation with monographs[27-29]. One individual each of four species of earthworms such as *Eutyphoeus gigas* (Stephenson)*, Eutyphoeus* sp.*, Amynthas alexandri* (Beddard) and *Metaphire houlleti* (Perrier) were selected (Table 1). For gut content analysis, the live

earthworms were washed thoroughly, sacrificed and the skin was cut open to expose the gut from the dorsal site without puncturing the alimentary canal. The intestine was carefully cut open from post-clitellum to posterior end with a fine sterile blade following dissection method[23,30-31]. Maximum precaution was taken to avoid any contamination with tissues and other contaminants. The intestinal content was collected into a sterilized Eppendorf tube and refrigerated at -20˚C for further microbial analysis.

Isolation of metagenomic DNA and sequencing

The metagenomic DNA was isolated from gut samples by using the Fast DNA spin kit (MP Biomedical, USA) and measured quantitatively by a microplate reader (Spectra Max 2E, Molecular Devices, USA). We employed high throughput Illumina sequencing and software tools to survey the microbial community and its potential putative functions of the earthworm species along the elevation gradient of a mountainous place in the Indo-Myanmar biodiversity hotspot region. The V3-V4 hyper variable region of the 16S rRNA gene was sequenced using Illumina MiSeq (Illumina Inc., San Diego, CA, USA).

The QIIME software package (v.1.8.0) was used to process and analyze raw fastq sequences[32-33]. Sequences quality score <25 with reading length <200 bp were filtered and chimeric sequences were removed using USEARCH[34-35]. Preprocessed V3-V4 sequences were assembled into operational taxonomic units (OTUs) using the Uclust (similarity cutoff = 0.97)[36]. Each OTU of their representative sequence was classified using Green genes database[37-38]. PICRUSt (v1.1) (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used for the identification of phylogenetic differences among the microbial communities of earthworms on the metabolic potential following the recommended workflow. The functional composition of the metagenome was predicted by using marker gene data (16S rRNA) along with the genomes reference database[39].

RESULTS

Characterization of microbial diversity

A total of 3,36,047 filtered sequences are obtained from the raw sequences of four earthworm species and a total of 3686 OTUs were obtained. The good's coverage of the samples for 16SrRNA amplicon was found to be 99.71% (PUCZM375A), 99.84% (PUCZM351), 99.86% (NRY1) and 99.92 % (EWBK2) (Mean ± SD) which implied that most of the diversity were captured. The estimation of alpha diversity indices revealed a significant difference in bacterial communities of earthworm gut samples (between pH, habitat type and altitude) (Fig. 1). The species richness was highest in PUCZM375A (1060.686) followed by PUCZM351 (981.647), NRY1 (7584.264) and EWBK2 (527.882). A significant variation was also detected in the non-parametric Shannon index for microbial population and ranged from PUCZM351 (2.338) PUCZM375A (3.154), EWBK2 (4.882) and lowest in NRY1 (6.990) (Fig. 1). In this study, we found a significant effect of the type of earthworm species on the beta diversity of the microbiome, which was evident using UniFrac distances (Fig.2).

Bacterial community profile

16S rRNA gene amplicon sequencing yielded more than 20 bacterial phyla in the complete dataset (Fig.3). Major bacterial phyla identified were Proteobacteria (47.1%), Firmicutes (38.9%), Actinobacteria (6.3%), Bacteroidetes (3.6%) and Cyanobacteria (1.1%). The other phyla include Chlamydiae, Chloroflexi, Fusobacter, Verrucomicrobia, Fusobacteria, Planctomycetes, Chloroflexi, Fusobacteria, Gemmatimonadetes, NKB19, Nitrospirae, OD1, Planctomycetes, TM6, TM7, Tenericutes, Verrucomicrobia and Thermi. Proteobacteria is dominant in EWBK2, NRY1 and PUZ375A but Firmicutes dominate in PUC351. Bacteroidetes is comparatively low abundant in both habitats. Other phyla such as Actinobacteria, Chlamydiae, Chloroflexi, Fusobacter, Verrucomicrobia, Fusobacteria, Planctomycetes, Chloroflexi, Fusobacteria, Gemmatimonadetes are prominent in home garden habitat compare to forest type. The distributions of bacterial family level were *Planococcaceae* (27.4%), Enterobacteriaceae (4.1%), Staphylococcaceae (3.5%), Aeromonadaceae (5.6%), Bacillaceae (4.4%), Comamonadaceae (2.4%), Oxalobacteraceae (1.2%), Caulobacteraceae (1.2%) and Enterococcaceae (1.1%). Distribution of bacterial genera were *Lysinibacillus* (26.9%), *Acinetobacter* (21.2%), *Pseudomonas* (4.7%), *Bacillus* (3.8%),

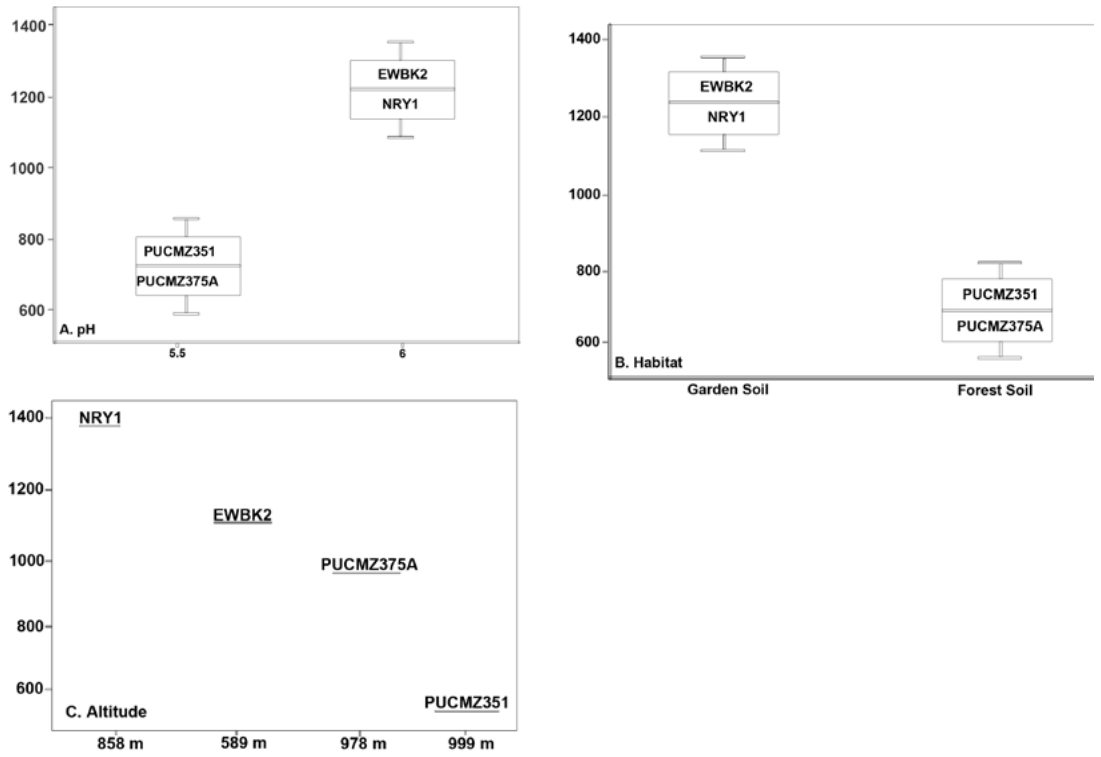

Fig. 1. Alpha diversity measurements of the Metagenome among the earthworm samples (EWBK2-*Amynthas alexandri*, PUCZM375A-*Metaphire houletti*,PUCZM351-*Eutyphoeus sp*, NRY1-*Eutyphoeus gigas*) around Aizawl city, Mizoram during 2017.
Box plot displaying the diversity difference between (A) pH; (B) Habitat; (C) Altitudinal variations.

Staphylococcus (3.5%), *Stenotrophomonas* (1.1%) and *Ralstonia* (1%).

It was observed that phylum Proteobacteria and Firmicutes are the most abundant in both epigeic and anecic earthworms contributing more than 86% of the total microbial community in both followed by Actinobacteria (Supplementary Fig. 1). Home garden revealed the richer diversity as compared to forest habitat, which might be due to the availability of a variety of food.

Metagenomics analyses of the earthworm gut microbiome: Metabolic potential

Extensive *in-silico* analysis using the PICRUSt provides the metabolic composition of the earthworm gut microflora. The present work predicts a large number of functional genes involved in various biochemical cycles. It was observed that metagenomic analysis of four species of earthworm gut microbiome revealed the major metabolic substances such as alanine, aspartate and glutamate, amino sugar and nucleotide sugar, arginine and proline, benzoate, butanoate, cysteine and methionine, glycine, serine and threonine, glyoxylate and dicarboxylate,

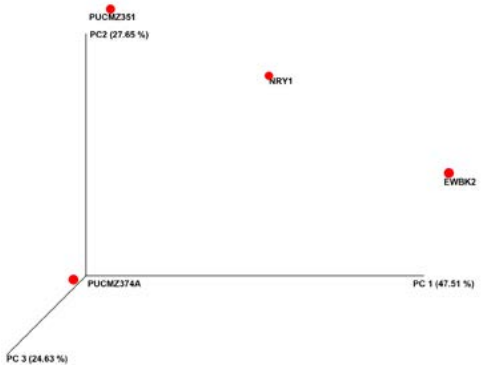

Fig. 2. Beta diversity analysis of the gut microbiota of different earthworm.
EWBK2, *Amynthas alexandri* PUCZM375A, *Metaphire houlleti*; PUCZM351, *Eutyphoeus sp.* NRY1, *Eutyphoeus gigas*.

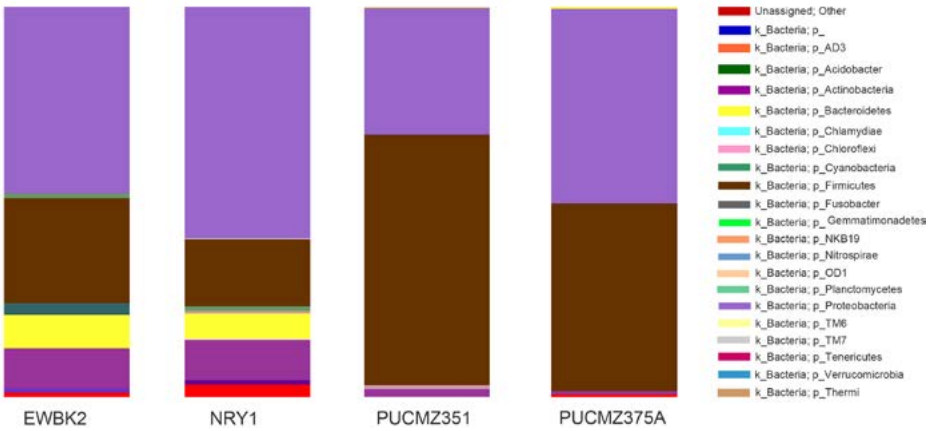

Fig. 3. Taxa distributions at phylum level of individual earthworm species.
EWBK2, *Amynthas alexandri*; PUCZM375A, *Metaphire houlleti*;
PUCZM351, *Eutyphoeus sp.* NRY1, *Eutyphoeus gigas.*

lysine, methane, nitrogen, propanoate, and pyruvate based on the KEGG pathway analysis. Our result revealed the evidence for enrichment of pathways related to nitrate reductase which involved in the nitrate to nitrite. Furthermore, two enzymes such as cytochrome c-type protein and glucosamine-6-phosphate deaminase were identified which participate in the conversion of nitrite to nitric oxide (Fig. 4).

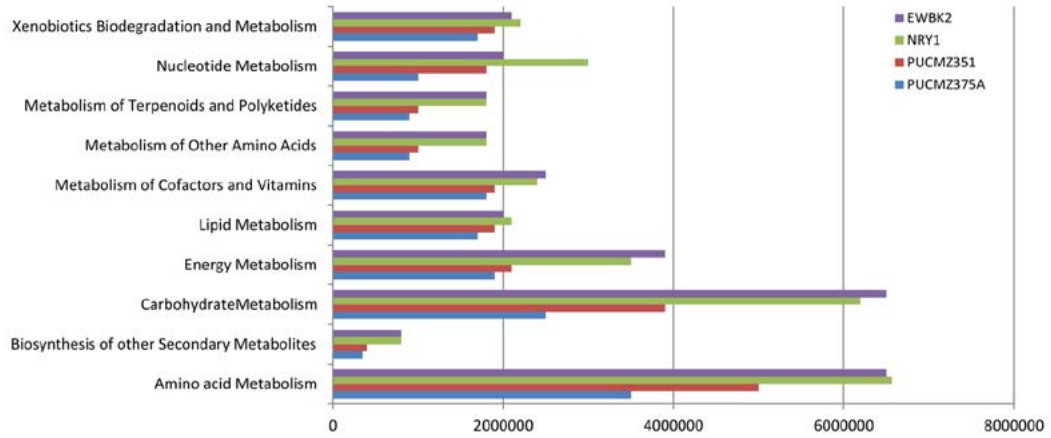

Fig. 4. Predicted metabolic profiles of the earthworm gut microbiome.
EWBK2, *Amynthas alexandri*; PUCZM375A, *Metaphire houlleti*;
PUCZM351, *Eutyphoeus sp.*; NRY1, *Eutyphoeus gigas*.

DISCUSSION

The present investigation of the earthworm gut bacterial community of Mizoram, northeast India,is the first systematic approach in entire northeast India which emphasizes the gut-bacterial communities of the hilly mountainous biodiversity-rich region.

Earthworm gut contains a unique anaerobic environment and the microbiome is adapted to perform functions including decomposition of nutrients, production of greenhouse gases etc[5,8]. Factors such as vegetation, pH and altitude play role in the richness and diversity of earthworm species (Fig. 1).The degrees of Alpha diversity in the present study were comparable with other studies investigating the earthworm gut microbiota[40]. The significant alterations in the observed OTUs and the richness (Chao1 index) suggest that differences between the bacterial communities are mainly compelled by the

low abundance OTUs, which is supported by the UniFrac analysis. Our finding revealed a reduction in microbial diversity in the epigeic species. The low bacterial diversity in surface dwellers might be due to the presence of diverse digestive enzymes which allows them to digest microorganisms as well as plant debris[25,41]. Quantitative changes were noted for the occurrence of bacterial taxa among the samples which could be due to the differences in the feeding substrates. Different substrate components are known to lead to a different microbial community structure[42]. Deciphering the microbial community would be useful in understanding the ecological function of these earthworms[43].

The earthworm gut is inhabited by diverse bacterial communities[18-20,39]. The present study reveals complex bacterial communities present in the four earthworm samples collected from different places. We found that 10 bacterial genus *Bradyrhizobium, Methylobacterium, Mesorhizobium, Rhizobium*, *Sinorhizobium*, *Rhodopseudomonas, Oligotropha, Pseudomonas* and *Mycobacterium* were involved in the process of N_2O emissions. A similar finding was reported[8] from the study on earthworms of Brazil.

Another abundant genus was identified as *Streptomyces*. Members under this genus were capable of producing cellulase and thereby helping the host to degrade plant materials[43]. Another dominant genus *Pseudomonas* possess genes involves in terpene metabolism and thereby participate in plant litter decomposition[45]. One of the stimulating observations was the high abundance of the bacterial genus *Lysinibacillus*. Members under this genus participate in polyethylene degradation[46-50] and xylan biodegradation could be an appropriate candidate for forest-based waste degradation[51]. Thus, this earthworm species can be further explored for forest waste biomass vermicomposting.

Stenotrophomonas is a common soil microbe, it plays a central role in nitrogen fixation as well as cellulolytic activity[52-54]. Another dominant genus, *Acinetobacter* is a common intestinal species found to be involved in the decomposition of catechin (plant secondary metabolite)[55]. The genus *Acinetobacter* is enhanced during fermentation. The abundance of this genus *Acetobacter* was possibly due to the anoxic environment and high organic substrates existing in the gut stimulate of the earthworm species[56-57]. We also identified the genus *Methylococcus*, belonging to the bacterial group Methylotrophs which capable of utilizing reduced one-carbon compounds, methanol or methane as the carbon source, and multi-carbon compounds such as dimethyl ether and dimethylamine[58].

Due to the variation in location as well as feeding habits, different groups of earthworms harbour different bacterial communities. Our result indicates that major differences observed within bacterial phyla Firmicutes, Actinobacteria and Bacteroidetes. Firmicutes have significantly decreased in surface dwellers (epigeic) species but increased in deep soil inhabitants (anecic worms). Actinobacteria and Bacteroidetes were increased in sub-surface earthworms (endogeic) compared to anecic species[59]. Previous studies reported that the gut metagenome of the anecic earthworms was dominated by the Beta proteobacteria and Gammaproteobacteria[59]. The present study is also in line with this report in having proteobacteria as the dominant phylum. However, Firmicutes[60] is also a dominant phylum in our study, which is not following the temperate region. This may be attributed to geographical differences in the two studies.

The metabolic activity of the earthworm gut microbial community of the present study showed an abundant representation of genes which represent pathways related to metabolism including the metabolism of amino acid, carbohydrate, energy, cofactor and degradation of xenobiotics. The abundance of these metabolic modules may be due to the feeding habitat of the analyzed earthworms. The metabolic profile of the species EWBK2 and NRY1 were similar compared to other species. This probably is due to the forest environment which allows them to ingest a large amount of plant material which is normally rich in cellulose, hemicellulose and soluble carbohydrates[61]. In conformity with the taxonomic characterization, we found the representative genes involved in denitrification, methanogenesis. This is an important function played by the gut bacterial community which helps in recycling nitrogen waste of the host[62].

CONCLUSION

Illumina sequencing reveals the diversity of bacterial communities in earthworm gut micro-biota from Indo-Myanmar Biodiversity Hotspot and predicts their imputed metabolic profiles. This bacterial diversity can be attributed to differences in the environmental condition such as pH, altitude, and type of forest. Overall, this study gives insight into the gut microbial composition and their putative functional roles in the gut of tropical hilly earthworms. Further studies on the functional roles of these microbes will enlighten the ecosystem functioning and geo-biochemical cycles.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial contribution to the work and approved it for publication.

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ETHICS STATEMENT

This work does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

The sequences obtained from NGS were submitted to NCBI which are available under Bio Project ID PRJNA376467.

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