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Identification of the Bacterial Community in the Gut of Millipedes

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Ecosystem engineers influence the structure and function of soil food webs through non-trophic interactions. The activity of large soil dwellers, such as earthworms, has a significant impact on the soil microarthropod community. However, the influence of millipedes on soil microarthropod communities remains largely unknown. Bacterial strains from the gut of two different millipedes, *Arthospheara magna* and *Alacobolus newtoni*, were isolated. After culturing on the described media, the bacterial isolates were identified through phenotypic, biochemical, and molecular analysis. Six bacteria were isolated, and molecular analysis revealed that nucleotide sequence similarity was seen with Salmonella bongo, *Erwinia papaya, Citrobacter portucalensis, C. freundii, Heyndrickxia oleronia,* and *Klebsiella oxytoca*. It is confirmed by the sequence similarity search BLAST tool. These isolates might play an important role to increasing the quality and fertility of the soil.

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Keywords: Arthospheara magna; Alacobolus newtoni; Citrobacter freundii; Heyndrickxia oleronia; Klebsiella oxytoca; BLAST.

1. INTRODUCTION

"Millipedes are known to be macro detrivores terrestrial arthropods feeding on decaying vegetable matter and mineral soil and are represented by more than 80,000 species. They are essentially soil-dwelling and in some ecosystems, they are more important than worms as agents of soil and nutrient turnover. They are representatives of the soil macro fauna that live in the deciduous forest litter and play an important role in the decomposition processes of decaying plant material" [1]. "The food source of the millipedes is the forest litter. The digestion of leaf litter and decaying matter is mainly due to the micro biota residing in the gut of the millipedes. The gut of millipedes may represent a reservoir of bacterial and fungal species. The intestinal microbiota community plays a significant role in the breakdown of plant polymers" [2].

"Interaction between millipedes and microorganisms are very important aspect of the decomposition process. It is known, for example, that the comminution of plant material by millipedes as well as by other soil-feeding animals increases the surface area available for the microbiota colonization" [3] and "the intestines of these animals act as a favorable environment for the bacterial growth" [4,5]. The presence of gut bacteria in the millipede is also a possible source of the amino acid detected in the fecal pellets due to the secretion of the gut lining and associated symbiotic microorganism. The degradation of glucose, predominantly sourced from cellulose indicates the presence of significant cellulolytic activity in the gut of the millipede which is likely associated with either endogenous cellulose or symbiotic gut bacteria capable of cellulose degradation.

"It is estimated that the millipedes assimilate only a – tenth of their total feed intake and 90% of the leaf litter fed was egested out as fecal pellets. Millipedes transform plant material into fecal pellets that affect important physiochemical properties, specifically by decreasing the carbon and nitrogen ratio and in the subsequent process of decomposition" [6-7]. "Millipedes are not able to digest the leaf litter or feed intake on the whole and are not well equipped with specialized enzymes that enable to digest, so they are enhanced by the microbial population that is accommodated in the gut of the animals. The food intake is grained by the mandibles and then subjected to enzyme action from the salivary glands, midgut epithelium and finally the most important role played by the microbial community in the digestive tract of the millipede. It is suspected that micro- organisms in the alimentary canal play a crucial role in the digestion of food and indirectly influence the fluxes of nutrients" [2-4]. "The transformation of leaf litter into feces by saprophagous macroarthropods can influence the decomposition process in several ways" [2]. "Microorganisms as endosymbionts in the gut of millipedes play an important role in improving the digestibility of plant materials. Symbiotic microflora in the gut of litter feeding animal convert decaying organic matter into assimilable energy" [8]. Anderson and Bignell illustrated this by showing that millipedes are not directly responsible for more than 10 percent of chemical decomposition. Nonetheless, because of their feeding activity the microorganisms can carry out approximately 90 percent of the chemical breakdown. The goal of this paper is to describe the majority bacteria that have been isolated and characterized from the guts of two different species of millipedes *A. magna* and *A. newtoni* and the hypothesis presented in this paper is concise, contemporary, and supports the notion that millipedes are creatures that only break down plant litter; rather, the bacterial population living in their intestines helps the animals function as effective detrivorous arthropods.

2. MATERIALS AND METHODS

2.1 Sample Collection

The Millipedes were collected near the reserve forest of Alagar Hills (10°0′-10°30′N and75°55′-78°20′E), in Madurai district. A random sampling method was followed for collecting the Millipedes and brought to the laboratory and acclimatized. Millipedes were anesthetized by gradual cooling, and the whole gut was dissected out with sterile scissors. The whole gut was dissected and homogenized using 1mL of physiological saline and serially diluted. The dilutions 10^{-3} to 10^{-6} were plated in LB agar following the spread plate method and incubated at 37 ºC for 18-24 hours. After 24hrs.of incubation individual colonies were picked based on their morphological features. The isolated strains were pure cultured for further analysis. The CFU/ml was calculated.

2.2 Biochemical Characterization

A total of fifteen tests were carried out to assess the biochemical characteristics of the gut isolate of two millipede species, A*. magna* and *A. newtoni*. The selected bacterial strains of two species of millipede were identified using standard biochemical tests described in Bergey's Manual of Systematic Bacteriology [9].

2.3 Molecular Characterization

DNA was extracted from the bacterial sample by Phenol: Chloroform: Isoamyl alcohol method. The DNA extracted was quantified using Nano drop (Thermo Scientific Company). The quality and intactness of DNA was checked electrophoretically using 0.8% Agarose Gel. The extracted DNA were amplified using universal primer 27 Forward (5'AGAGTTTG ATCCTGGCTCAG3') and 1492 Reverse (5'GGTTACCTTGTTACGACTT3') in a 23 thermocycler (Eppendorf company) (Fietto *etal*., 2004). The PCR product is 1500bp in size and it is then quantified electrophorectically in 1% agarose gel stained with ethidiumbromide (Working concentration1mg/ml). The gel was visualized using Ultra Violet light trans-illuminator or GEL documentation system (Biorad-2017) to visualize the DNA bands. The obtained PCR amplicon were verified using 1kb ladder (BIO-HELIX – DMO15-R500). Then the amplified PCR product was transferred into a new 1.5ml tube (required volume) and sent to Medauxin, Hyderabad, India for 16SrRNA sequencing. The sequences were identified with the help of the NCBI database using BLAST tool and analyzed.

3. RESULTS

The total bacterial count in the gut was remarkably higher in *A. magna* ($3.9X10^{11}$ CFU g^{-1} dry wt.) than *A.newtoni* ($2.89X10^{11}$ CFU g^{-1} dry wt.). The results were observed after the respective days of incubation. Different types of bacteria were observed (Fig. 1). First, they were characterized to identify them as a part of Millipede's gut microbial population. A total of six bacteria were obtained (R1, R2, R3, R4, R5, R6) identified depending on their morphological and biochemical characteristics. Concerning the Six identified bacteria, five were Gram negative and only one was Gram positive (Table 1). All the isolates were rod shape bacteria. All the isolates gave positive result for Motility test, oxidize test, Amylase test and Protease test. For indole test R2 and R3 isolates were shown as positive result. All the isolates were negative for Voges Proskauer and hichrome coliform test. All the isolates were negative for catalase activity except bacterium R5 (Table 1). The DNA was quantified using Nano-drop; the results are represented in the Table 2. Strain R3 & R4 had higher amount of Nucleic acid (775.7 & 481.6) compared than other Strains. Extracted genomic DNA was subjected to PCR Amplification by using 16s rRNA (Figs. 1 & 2). The sequences were analyzed using NCBI Blast and the species similarity is mentioned in the Table 3. Through this BLAST analysis 6 strain were identified as *Salmonella bongor* (R1), *Erwiniapapaya* (R2), *Citrobacter portucalensis* (R3), *Citrobacter freundii* (R4), *Heyndrickxia oleronia* (R5), *Klebsiella oxytoca* (R6).

Table 1. Characteristics of gut bacteria of *A. magna* and *A. newtoni*

Biochemical Test	R1	R2	R3	R4	R5	R6
Gram's Staining	-	-	-	-	+	-
Morphology	Rod	Rod	Rod	Rod	Rod	Rod
Motility Test	+	+	+	+	+	+
Indole test	-	+	+	-	-	+
Methyl Red Test	+	+	+	+	-	+
Voges Proskauer Test	_	_	_	_	_	_
Citrate utilization Test	-	+	-	+	-	+
Macconkey Agar Test	-	+	+	-	-	+
EMB Agar Test	+	+	+	+	_	_
Hichrome Coliform Test	-	-	-	-	-	-
Rapid Hicoliform Agar Test	+	+	+	+	-	+
Protease	+	+	+	+	+	+
Amylase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	-	-	-	-	+	-

Table 2. DNA quantification of nano-drop

SampleID	Nucleic Acid	Unit	A260	A280	260/280	260/230	Sample Type	Factor
R1	203.4	ng/µl	4.068	2.571	1.58	0.95	DNA	50
R2	775.7	ng/µl	15.515	10.191	1.52	0.99	DNA	50
R3	481.6	ng/µl	9.631	5.806	1.66	0.85	DNA	50
R4	220.5	ng/µl	4.409	2.926	1.51	0.64	DNA	50
R5	97.5	ng/µl	1.95	1.217	1.6	1.6	DNA	50
R6	45.3	ng/µl	0.907	0.535	1.69	0.9	DNA	50

Table 3. Sequence analysis by BLAST tool

SampleID	QueryLength	OrganismMatched	PercentageSimilarity	Accession No
R1-F	518	*Uncultured organism*	94.30%	MG202000.1
R1-R	1173	*Salmonella bongor*	83.74%	CP053336.1
R2-F	523	*Erwiniapapayae*	95.30%	MT322787
R2-R	355	*Uncultured organism*	79.44%	HQ793390
R3-F	1022	*Citrobacterportucalensis*	97.76%	CP039327
R3-R	1559	*Citrobacterfreundii*	87.04%	OQ381203
R4-F	441	*Citrobacterfreundii*	98.62%	MT471000
R4-R	761	*Citrobacterfreundii*	82.11%	CP070559
R5-F	807	*Heyndrickxiaoleronia*	97.14%	CP079720
R5-R	1005	*Heyndrickxiaoleronia*	88.90%	KY773585
R6-F	923	*Klebsiellaoxytoca*	98.15%	KT895294
R6-R	997	*Klebsiellaoxytoca*	83.49%	KX212257

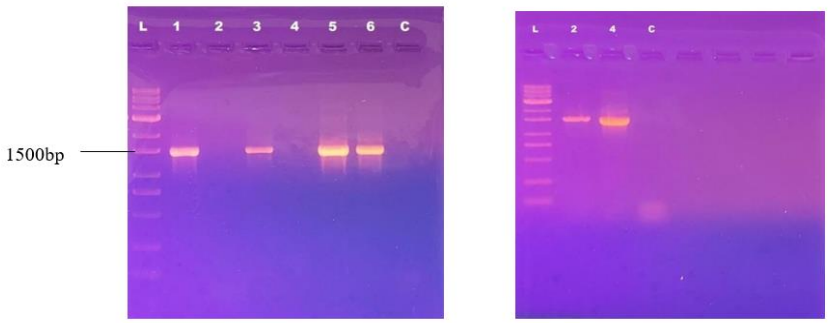

Fig. 1. Universal primer PCR L-1kb ladder, *Salmonella bongor*-1, *Erwinia papaya*-2, *Citrobacter portucalensis*-3, *Citrobacter freundii*-4, *Heyndrickxia oleronia*-5, *Klebsiella oxytoca*-6, No template control-C

Fig. 2. Results of PCR amplification (25µl reaction) L-1kb ladder, *Salmonella bongor*-1, *Erwinia papaya*-2, *Citrobacter portucalensis*-3, *Citrobacter freundii*-4, *Heyndrickxia oleronia*-5, *Klebsiella oxytoca*-6, No template control-C

PCR Amplicon size-1500bp, Forwardprimer–1microliter, Reverse primer 1microliter, Sample 1microliter, MilliQ 2microliter, Mastermix 5microliter, overall, 10microliter reaction.

4. DISCUSSION

"Earthworm populations are low in tropical forest; Millipedes play an important role in facilitating decomposition of the leaf litter. Millipedes are major consumers of organic debris in decompose and tropical hardwood forests, where they feed on dead vegetative matter. Millipedes are associated with many other organisms that also inhabit in soil surface and subterranean environments" [10]. "These include, but are not limited to bacteria, fungi, nematodes, nematomorphs, annelids, insects and mites. In the soil system, there is close contact between many of the inhabitants, both directly and indirectly through their activities. The actual decomposition of complex molecules from fragmented leaf litter and wood is accomplished almost exclusively by microorganisms that reside in the soil system, especially bacteria which are the predominant organisms possessing the enzymes capable of breaking down the complex compounds produced by plants. They produce many enzymes for the decomposition of plant compounds such as cellulases, hemicellulases and other cellulolytic enzymes" [11-12].

The presence of gut bacteria in the millipede is also a possible source of the amino acids detected in the fecal pellets due to the secretion of the gut lining and associated symbiotic microorganisms. The degradation of glucose, predominantly sourced from cellulose suggests the presence of appreciable cellulolytic activity in the gut of the millipede, associated with either endogenous cellulases or symbiotic gut bacteria capable of cellulose degradation. Bacteria are more abundant in the gut of the millipede and in the fecal matter than in leaf litter that they feed upon [13] while the presence of cellulolytic enzymes in millipedes capable of cellulose degradation has been suggested [14].

The total heterotrophic bacteria from the millipedes *Arthrosphaera magna* and *Aulocobolus newtoni* from Alagar hills of Tamil Nadu were characterized [15]. It is recorded that six strains were identified as *Salmonella bongor* (R1), *Erwinia papaya* (R2), *Citrobacter portucalensis* (R3), *Citrobacter freundii* (R4), *Heyndrickxia oleronia* (R5), *Klebsiella oxytoca* (R6). Similarly, the species *Schizophyllum sabulosum* is the host for the following bacterial community genera *Klebsiella, Sarcina, Bacillus and Corynebacterium* [16-17]. "The millipede, *Glomeris marginata* contains bacterial populations like *Pseudomonas alcaligenes*, *Klebsiella pneumoniae"* [18-19]. The species *Ommatoiulus sabulosus* contains *Escherichia coli*, *Enterobacter aggomerans*, *Klebsiella, Pseudomonas fluorescence, Sarcina* [20-22]. The bacterial community *Proteus mirabilis and Citrobacter freundii* are the dwelling place in the gut of the millipede *Xenobolus carnifex* [23]. "The gut of millipede *Cylindroiulus caeruleocinctus* is a habitat for *Citrobacter freundii, Pantoea agglomerans, Serratia marcescens, Raoultella planticola and Salmonella arizonae"* [24]. "The following bacterial species such as *C. freundii, P. agglomerans, R. planticola and Xanthomonas maltophilia* are found in the gut of the millipede *Ommatoiulus sabulosus"* [22].

"Bacterial counts showed that bacterial growth was enhanced in the guts and feces of these animals as a habitat for soil bacteria has received little attention. The feeding activities of soil invertebrates can cause gross shifts from fungal to bacterial activity in litter and soils" [14,22]. "The mid gut of millipede is the main site for the degradation of cellulose and hemicelluloses and the amount of pectin degradation in hindgut is considerably high" [8,25]. "The gain of the millipede from the microbial lignocellulose degradation in the gut, and consequently the mutualistic status of the relationship between the millipede and its cellulolytic gut bacteria, depends on the ability of the millipede to take up microbial metabolites as nutrients through the hindgut wall. Enzymes expressed in the intestine can degrade all components of lignocellulose except lignin" [26]. "Millipedes distributed among different habitats are known to harbor more or less similar gut microbial communities. Microbial population was differentially altered from food to gut to fecal pellets amongst the Arthrosphaera species. The population of aerobic heterotrophic bacteria was highest" [27-28].

"The findings of the present study suggest that symbiotic bacteria of two millipede species are involved in metabolic process, such as synthesis of essential enzymes for digestion of plant litter for example cellulose, chitin, xylan, strach and some proteins" [29]. As the millipede species is essential components of the forest ecosystem,

the gut bacteria of millipedes can able to synthesize many enzymes for digestion of plant material, some essential nutrient are derived from these materials after the action of gut bacteria of millipedes. The raw materials of plants cannot directly utilize the plants, hence it is complex and unassimable forms. The gut bacteria of millipedes act on complex plant substances and convert them into simple assimable nutrient form. Moreover, millipede is a predominant organism for enrich the fertility of soil and recycling the plant material in ecosystem with the association relationship of gut bacteria. Findings of the present study suggest that two different millipede species is well equipped to handle plant materials and utilizing other source of food. The presence of special carbohydrases such as amylase, Cellulase, chitinase and xylanase apart from protease contributed by the gut of bacterial constitute an important adaptive resource for *A.magna* and *A. newtoni* because they allow the millipede to effectively to utilize plant detritus in the ecosystem. Furthermore, millipede species and their endosymbiotic bacteria play a crucial role in enriching the soil fertility of Alagar Hill.

5. CONCLUSION

The presence of gut bacteria in the millipede is also a possible source of amino acids detected in the fecal pellets due to the elimination of the gut lining and associated symbiotic microorganisms. The bacteria in the foregut were probably present as a result of ingestion and are therefore not part of the resident lora. During winter, the number of foregut bacteria varies greatly from individual to individual, whereas the midgut and hindgut of all individuals retain large populations The forest ecosystem's most essential component is the millipede, which is a predominant organism laying a major role in enriching soil fertility, recycling of plant material in ecosystem in relation with gut bacteria. Millipedes change the chemical composition of the leaf material during ingestion and favor the establishment of soil bacterial population. It is likely to be believed that the microorganisms in the alimentary channel play a crucial role in the digestion of such food materials.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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