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Microbial Dynamics in Food, Intestine and Fecal Pellets of Two Endemic Pill-Millipedes (*Arthrosphaera*: Sphaerotheriida) of the Western Ghats

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Abstract Assessment of microbial communities in food, gut and feces as a consequence of ingestion and egestion of organic matter by saprophagous fauna assumes importance in elemental recycling and improvement of soil fertility. This study addresses the dynamics of six microbial communities in food (native leaf litter), intestine (gut contents) and fecal pellets (manure) of two endemic giant pill-millipedes of the Western Ghats (*Arthrosphaera fumosa* and *A. magna*). Load of four bacterial communities (heterotrophic bacteria, actinomycetes, phosphate solubilizing bacteria and rhizobia) showed siginficantly increasing trend from leaf litter to gut content to feces, while it was opposite for filamentous fungi as well as yeast. The overall microbial dynamics was similar between pill-millipedes in spite of their occurrence in different habitats of the Western Ghats. Microbial dynamics in pill-millipedes has been compared with other millipedes and earthworms. A combination of pill-millipedes and earthworms has been proposed for the production of quality organic manure.

Keywords: Pill-millipedes, mesocosm, leaf litter, gut microbiota, manure

Introduction

Millipedes are the major saprophagous fauna widely distributed in tropical, subtropical and temperate regions playing an important role in the improvement of soil fertility (Hopkin and Read, 1992). They ingest the leaf litter along with soil particles, metabolize up to 0.3–7% of the ingested material by significant increase of surface area, which promotes microbial growth and decomposition through gut passage and fecal pellets (Byzov *et al*., 1996). Up to 90% of the fed litter mass will be egested as faecal pellets serving as reservoir of minerals and different microbes. The organic matter ingested by millipedes will be processed.

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by a variety of bacteria and fungi in their gut (Pherson and Beattie, 1979; Szabo *et al*., 1990; Rossi and Weir, 1998; Oravecz *et al*., 2002). Although the gut microbiota of millipedes differ from those inhabiting in leaf litter and soil (Byzov *et al*., 1996), millipedes distributed in different habitats are known to harbor more or less similar gut bacterial communities (Szabo *et al*., 1990).

Millipede feeding activities stimulate soil microbes leading up to 90% of chemical breakdown (Anderson and Bignell, 1982). However, they are devoid of lignocellulose degrading microbes in their gut and primarily depend on litter and soil microbes for degradation of lignocellulosic substrates (Oravecz *et al*., 2002). Besides, the litter bacterial communities like *Arthrobacter* spp. and *Streptomyces* spp. serve as nutritional source of millipedes (Byzov *et al*., 1996). Many microbes are unique to the intestine of millipedes are not inhabitants in leaf litter or soil (Chu *et al*., 1987). Some microbes exist as endosymbionts with millipedes and transmitted through eggs to the next generation (Buchner, 1965). Knowledge on the occurrence, function and dissemination microbes by millipedes are essential for understanding their role in soil fertility (Hanlon, 1981).

The giant pill-millipedes of the genus *Arthrosphaera* endemic to Southern India, Sri Lanka and Madagascar are known to harbor a variety of actinobacteria, bacteria and fungi (Kämpfer *et al*., 2001, 2006, 2009; Sridhar and Kadamnnaya, 2009). The fungus, *Triainomyces hollowayanus* belonging to Laboulbeniales has been isolated from pill-millipedes (*Procyliosoma tuberculatum*) in New Zealand (Rossi and Weir, 1998). Although pill-millipedes represent a reservoir of microbes, their role in mineral cycling, manure quality and plant growth promotion are yet to be investigated. Compared to temperate pill-millipedes (e.g. *Glomeris*), assessment of microbial components of tropical pill-millipedes (e.g. *Arthrosphaera*) have not been well documented. The present preliminary study envisaged to assess the dynamics of selected microbes (bacteria and fungi) in the food, intestine and feces of two endemic pill-millipedes (*Arthrosphaera fumosa* and *A. magna*) of the Western Ghats of India.

Materials and methods

Pill-millipedes and mesocosm

Two dominant pill-millipedes, *Arthrosphaera fumosa* (12.9-13.6 g) and *A. magna* (9.2-9.8 g) were collected from the forests and plantations of the Western Ghats (Karike: 12°45'N, 75°38'E; Adyanadka: 12°41'N., 75°6'E), respectively during post-monsoon season (July-August 2012) (Pocock, 1899;

Attems, 1936; Kadamannaya and Sridhar, 2009). *Arthrosphaera fumosa* are dominant in the evergreen and semi-evergreen forests of high altitude regions of the Western Ghats of Karnataka and also recovered from Coimbatore region of Tamil Nadu (Kadamannaya and Sridhar, 2009). *Arthrosphaera magna* are dominant in foothill locations of the Western Ghats and well adapted to the organically managed mixed plantations. Besides, they are known from Khandala Hills (Maharashtra) and Shevaroy Hills (Tamil Nadu). Along with pill-millipedes, their preferred mixed leaf litter from their native locations were sampled in sterile polythene bags. Pill-millipedes (4-6 individuals) were maintained in the laboratory mesocosm in clean plastic containers (30 × 15 × 15 cm; with holes on the top and sides for aeration) kept in horizontal position and offered pieces of native mixed wet leaf litter from their respective locations (Ambarish and Sridhar, 2013a). Three replicate mesocosms were maintained and pill-millipedes were allowed to feed the pieces of leaf litter for three days to assess microbial dynamics in food, intestine and fecal pellets.

Analysis of microbiota

Pieces of wet leaf litter offered to millipedes in mesocosms were sampled for microbial analysis. Three-days fed millipedes in mesocosms were transferred to sterile polythene bags and accumulated fecal pellets were sampled for microbial analysis. To ascertain the microbial load in the gut, millipedes were washed in sterile distilled water followed by 70% ethanol and sterile distilled water to avoid external contamination. They were sacrificed and the gut content was collected into phosphate buffered saline (NaCl, 8.01 g/l; KCl, 0.2 g/l; Na_2HPO_4 2 H_2O, 1.78 g/l; KH_2PO_4, 0.27 g/l; pH, 7.4). Unit wet weight of churned leaf litter, gut content and fecal pellets were serially diluted in phosphate buffer saline up to 10^{-7}. An aliquot (0.1 ml) was spread on the surface of different media in standard Petri plates. Three replicates of each sample were maintained per treatment drawn from three independent mesocosms.

Aerobic heterotrophic bacteria were enumerated by plating on nutrient agar (Allen, 1959), actinomycetes on Ken Knight's agar (Allen, 1959), phosphate solubilizing bacteria on Pikovskaya agar (Pikovskaya, 1948) and rhizobia on yeast extract mannitol agar media ATCC, 1992). Phosphate solubilizing bacteria were enumerated based on formation of halo zone surrounding the colonies on tricalcium citrate amended Pikovskays agar medium. Filamentous fungi were assessed using Martin's rose Bengal agar medium (Martin, 1950) and yeasts on yeast extract malt extract agar medium (Galloway and Burgess, 1962). The results of the bacterial and fungal

population are expressed as cfu/g fresh weight of samples. The cfu represents the mean of nine replicates drawn from three replicates each of three independent mesocosms.

Data analysis

The differences in the population of bacteria and fungi in leaf litter vs. gut, leaf litter vs. fecal pellets and gut vs. fecal pellets were assessed by One-way ANOVA (Origin pro 8.1).

Results and discussion

Microbial population

Microbial population was differentially altered from food to gut to fecal pellets amongst the two *Arthrosphaera* species studied has been represented in qualitative and quantitative barcodes (Fig. 1). The population of aerobic heterotrophic bacteria was highest among the bacteria and fungi (Table 1). They steadily increased from leaf litter to gut content ($4–6 \times 10^6$ vs. $7–11 \times 10^6$ cfu/g) and leaf litter to feces ($4–6 \times 10^6$ vs. $21–26 \times 10^6$ cfu/g: $p < 0.05$), which was more pronounced in *A. magna* than in *A. fumosa*. The population of rhizobia was next to heterotrotrophic bacteria, but its increase from leaf litter to feces was higher in *A. fumosa* ($7–21 \times 10^4$ cfu/g: $p < 0.01$) than in *A. magna* ($4–18 \times 10^4$ cfu/g: $p < 0.01$). Third highest population was actinomycetes and its increase from leaf litter to feces was similar to rhizobia (*A. fumosa*: $9–14 \times 10^3$: $p < 0.05$; *A. magna*: $2–7 \times 10^3$ cfu/g: $p < 0.01$). The least among the bacteria studied was phosphate solubilizing bacteria and its dynamics was similar to rhizobia and actinomycetes (*A. fumosa*: $2–7 \times 10^3$ cfu/g: $p > 0.05$; *A. magna*: $1–6 \times 10^3$ cfu/g: $p > 0.01$). Filamentous fungi were higher than the yeasts and their population substantially decreased from leaf litter to feces unlike bacteria (fungi: *A. fumosa*, $5–1 \times 10^3$ cfu/g: $p < 0.05$; *A. magna*, $6–2 \times 10^3$ cfu/g: $p < 0.01$) (yeast: *A. fumosa*, $3–0 \times 10^1$ cfu/g; *A. magna*, $1–0 \times 10^1$ cfu/g).

Millipede gut provide unique ecological niche for many microorganisms, which are unable to survive outside (Márialigeti *et al*., 1985). Such microbial communities play a significant role in the breakdown of ingested plant biopolymers (Oravecz *et al*., 2002). Besides, millipedes increase the surface area of detritus, which enhances the microbial colonization and rate of breakdown (van der Drift, 1951; Anderson and Bignell, 1980). The aerobic cellulolytic bacteria provide utilizable substrates to the millipedes, in turn millipedes supply constant flow of substrate for degradation in addition to

creating regulated environment (e.g. moisture, temperature and pH). In the present study, to simulate the natural conditions, wet mixed litter collected from native habitats of pill-millipedes were offered in mesocosms. As pill-millipedes prefered mixed leaf litter over monolitter, a variety of microbes seem to involve in conversion of ingested lignocellulosic materials (Ashwini and Sridhar, 2005; Ambarish and Sridhar, 2013b). The overall microbial dynamics between *A. fumosa* and *A. magna* in the present study is comparable in spite of occurrence pill-millipedes in different habitats.

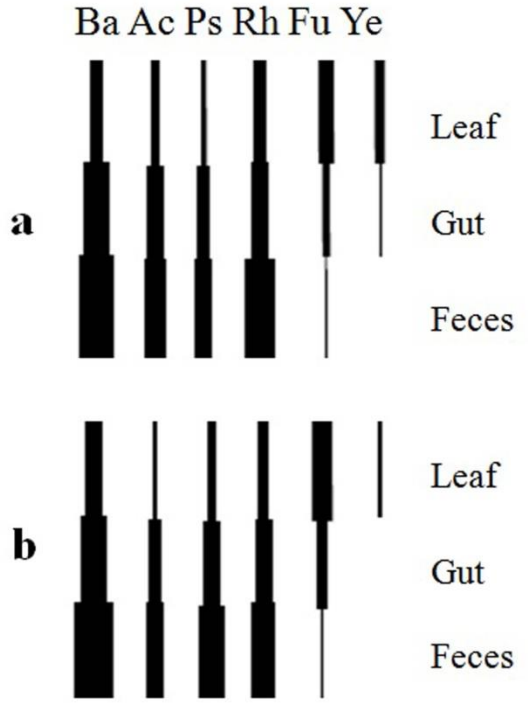

Figure 1. Qualitative barcoding microbial dynamics during native leaves processed by *Arthrosphaera fumosa* (a) and *A. magna* (b) (Ba, Bacteria; Fu, Fungi; Ye, Yeast; Ac, Actinomycetes; Ps, Phosphate solubilizers; Rh, Rhizobia).

Bacteria and fungi

Microbial colonization of leaf litter influences the palatability of temperate pill-millipede *Glomeris marginata* (Anderson and Ineson, 1983; Bignell, 1984, 1989). The viable count of bacteria was higher in the gut and feces compared to the ingested leaf litter. The hindgut consists of bacteria, actinomycetes and fungi and maximum bacterial population was found in feces as proliferation of bacteria takes place in the hindgut (Gebhardt *et al*., 2002). Increase of bacterial population in feed to fecal pellets ranged between 10 and 100 folds, whereas fungal population decreased up to 3.5 folds (Anderson and Bignell, 1980). As seen in *Glomeris*, the overall results of present survey support the view that the gut passage of leaf litter to fecal pellets increases the bacterial population, while decreases the fungal population (Anderson and Bignell, 1980; Anderson and Ineson, 1983; Anderson *et al*., 1983; Maraun and Scheu, 1996).

Table 1. Changes in population of microbiota in food (leaf litter), intestine (gut content) and faces (manure) of *Arthrosphaera fumosa* and *A. magna* (cfu/g fresh weight; mean n=9).

	Bacteria				Fungi	
	Heterotrophic Bacteria ($\times10^6$)	Actino-mycetes ($\times10^3$)	Phosphate solubilizers ($\times10^3$)	Rhizobia ($\times10^4$)	Filamentous fungi ($\times10^3$)	Yeast ($\times10^1$)
Arthrosphaera fumosa						
Leaf litter	4^{a}	9^{a}	2^{a}	7^{a}	5^{a}	3^{a}
Gut content	7^{ac}	11^{ac}	3^{b*c}	14^{b*c}	3^{b*c}	1^{a}
Feces	21^{b*d*}	14^{b*d*}	7^{b*d*}	21^{b**d**}	1^{b*d*}	0
Arthrosphaera magna						
Leaf litter	6^{a}	2^{a}	1^{a}	4^{a}	6^{a}	1
Gut content	11^{ac}	4^{ac}	3^{ac}	7^{ac}	5^{ac}	0
Feces	26^{b*d*}	7^{b**d**}	6^{ad**}	18^{b**d**}	2^{b**d**}	0

Data across the rows between leaf litter vs. gut content and feces and gut content vs. feces with different alphabets are significantly different (*$p < 0.05$, ** $p < 0.01$) (One-way ANOVA)

Bacteria like *Pseudomonas putida* and *P. stutzeri* survived the gut passage of millipede *Pachyiulus flavipes* and their population increased in fecal pellets (Byzov *et al*., 1996). However, *P. flavipes* exhibited selective and rapid killing of bacteria and yeast during digestion. Aerobic heterotrophic bacteria significantly increased from feed to feces between 4.5 and 5.5 folds in the

present study. Significant increase from food to feces was also seen in rest of the bacteria (actinomycetes, phosphate solubilizing bacteria and rhizobia) in both millipedes. Studies on aerobic bacteria of two desert millipedes (*Orthoporus ornatus* and *Comanchelus* sp.) revealed cellulose and hemicellulose degradation takes place in the midgut, while pectin degradation in the hind gut (Taylor, 1982). Being saprophytes, the potential of gut enzymes of diplopods involve in digestion of microbes and their mechanism of action are unclear. Several bacterial groups like alpha-proteobacteria (*Methylobacterium populi* and *Novosphingobium subarcticum*), beta-proteobacteria (*Cupriavidus basilensis* and *Pseudomonas putida*), gamma-proteobacteria (*Citrobacter amalonaticus, Klebsiella pneumoniae, Pantoea agglomerans* and *Pseudoxanthomonas koreensis*) and bacilli (*Bacillus arsenicus, B. licheniformis* and *B. subtilis*) were reported from the fecal pellets of *A. magna* (Sridhar and Kadamannaya, 2009) indicates the richness of bacteria. From fecal pellets, three new species of bacteria were also described recently (*Chitinophaga skermanii, Chryseobacterium arthrosphaerae* and *Microbacterium arthrosphaerae*) (Kämpfer *et al*., 2001, 2006, 2009).

Actinomycetes such as cellulomonads, nocardias and streptomycetes (e.g. *Cellulomonas, Leifsonia* and *Streptomyces*) harbored in the intestine of millipedes are known to possess good cellulolytic activity (Byzov *et al*., 1996). The gut nocardiforms of millipedes relay on forest leaf litter represent taxonomically heterogeneous group (Chu *et al*., 1987). It is also known that many actinomycetes (e.g. *Arthrobacter, Nocardia* and *Rhodococcus*) have the capability to metabolize aromatic compounds and some are pioneers in decomposition of lignin (Márialigeti *et al*., 1985). Like fungal hyphae, vegetative biomass of actinomycetes will also be ingested along with the food and digested in the intestine. Gram-positive nocardias found in the gut as well as fresh faecal pellets of *Glomeris hexasticha* seem to be obligate intestinal symbionts, which are absent in the leaf litter and soil. *Streptomyces* and *Promicromonospora* are known to produce fungal cell wall digesting enzymes in the gut (Márialigeti *et al*., 1985). Many actinobacteria like *Gordonia terrae, Microbacterium imperiale, M. trichotecenolyticum, Rhodococcus fascians* and *Streptomyces griseodotifer* have also been isolated from the fecal pellets of *A. magna* indicates their major role in the intestine of pill-millipedes (Kadamannaya and Sridhar, 2009).

There seems to be selective assimilation and elimination of microbes passing through the gut of invertebrates (Hand *et al*., 1988; Bignell, 1989 Byzov *et al*., 1993). Unlike bacteria, decreased ergosterol content from feed to gut to fecal pellets of *Glomeris marginata* reveals intense digestion of fungi in the gut passage (Maraun and Scheu, 1996). Similarly, the gut passage of beech

leaf litter considerably decreased the fungal biomass in *G. marginata* (446 vs. 131 μg/g) (Anderson *et al*., 1983; Maraun and Scheu, 1996). In the present study, although yeasts were present in the leaf litter, they were absent in fecal pellets of both pill-millipedes corroborate earlier observation in diplopods by Byzov *et al*. (Byzov *et al*., 1998). The fungus, *Triainomyces hollowayanus* (Laboulbeniales) has been isolated from pill-millipede *Procyliosoma tuberculatum* (Sphaerotheriidae) in New Zealand and more than 50% of millipedes preferred feeding ascospore-releasing structures of fungi (Rossi and Weir, 1998) denotes the assimilation of calcium by diplopods. Diplopods assimilate high calcium content (122 mg/g dry mass) and transform into calcium oxalate and calciferous tergites (Carter and Grag, 1976). Earlier reports also showed the oxalate decomposing microbes in the gut of macro arthropods (Cromack *et al*., 1977) and their role in conversion of calcium oxalate and oxaloacetic acid into ionic calcium.

Comparison between millipedes and earthworms

Microbial dynamics differed between pill-millipedes fed with native leaf litter compared to earthworms (*Eudrilus* sp.) grown on coconut leaf litter with cow manure (10:1, w/w) in tropical condition (Gopal *et al*., 2009). The aerobic heterotrophic bacteria gradually increased from feed to gut to manure in pill-millipedes, but they were high in the gut of earthworms rather than the feed and vermicompost. Although dynamics of actinomycetes was almost similar to heterotrophic bacteria in pill-millipedes, it was reverse in earthworms. However, the phosphate solubilizing bacteria was highest in fecal pellets of pill-millipedes as well as vermicompost. Rhizobial population also showed highest population in pill-millipede fecal pellets similar to heterotrophic bacteria, actinomycetes and phosphate solubilizing bacteria. Except for *Azotobacter*, the population of free-living nitrogen fixers, *Azospirillum, Nitrosomonas* and *Nitrobacter* reached the highest level in vermicompost. The fungal load was highest in feed and it was least in fecal pellets of millipedes, while they were least in the gut and highest in the vermicompost, however the fungi *Trichoderma* spp. although survived in the gut passage of earthworms, vermicompost was devoid of them. Decrease in fungal population in the gut of pill-millipedes compared to feed reveals their dependence on fungi for calcium replenishment.

There are some reports on feeding and incorporation of millipede fecal pellets of *Glomeris marginata* into soil by some earthworms (e.g. *Lumbricus castaneus* and *Octolasion lacteum*) (Scheu and Wolters, 1991; Bonkowski *et al*., 1998). It is evident that the coconut leaf litter amended with 10% cow manure

produced vermicompost by *Eudrilus* sp. with highest population of plant beneficial microorganisms (Gopal *et al*., 2009). Coconut leaf litter was also one of the preferred leaf litter by *A. magna* in mesocosms and field experiments (Ashwini and Sridhar, 2005, 2006). Unlike pill-millipede compost, vermicompost produced by coconut leaf litter resulted in significantly high population of free-living nitrogen fixers, phosphate solubilizers and fungi (Gopal *et al*., 2009). On the contrary, the coconut leaf litter (with amendment of 10% cow manure) also resulted in preponderance of aerobic heterotrophic bacteria and actinomycetes. Besides, *Azotobacter* and *Trichoderma* spp. were detected only in vermicompost derived from coconut leaf litter amended with cow manure than in vermicompost derived only from coconut leaf litter.

Conclusions

Millipede gut-adapted microbes capable to degrade complex organic matter to provide simple desired substrates to the millipedes, in turn millipedes constantly supply organic matter to gut microbes with suitable conditions. In the interest of organic agriculture, it is worth considering the association of pill-millipedes with other microbes like plant growth promoting bacteria (e.g. *Azotobacter, Azospirillum* and *Beijerinckia*), fungi (mycorrhizas) and plant protectants (e.g. *Trichoderma*). Hoffmann *et al*. (1998) reported that the intestine of soil invertebrates facilitates horizontal gene transfer in bacteria. *Bacillus* sp. endosymbiotically associated in the hind gut of *Glomeris* is known to produce a strong antifungal metabolite bacillomycin D (Gebhardt *et al*., 2002), which may also be one of the reasons for low fungal population in the pill-millipede gut. Pill-millipede manures produced by offering mixed leaf litter to *A. fumosa* and *A. magna* resulted in increased nitrogen and phosphorus in appreciable quantity within 2-4 weeks denotes the impact of nitrogen fixers and phosphate solubilizers in manures (Ashwini and Sridhar, 2006; Ambarish and Sridhar, 2013a). Being Gondwanan origin, *Arthrosphaera* seems to harbor rare and extremophilic microbiota involve in plant growth promotion, production of secondary metabolites and production of organic manure as alternative to vermicompost (Sridhar and Ambarish, 2013). Within three weeks of composting mixed leaf litter using pill-millipedes, eggs of earthworms likely inoculants from decomposing mixed leaf litter hatched out and the juveniles were active showing the compatibility of pill-millipedes and earthworms in manure production (Ambarish and Sridhar, 2013b). Thus, two step composting using *Arthrosphaera* followed by earthworms may be more profitable and provide new insight into the organic manure production and utilization.

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