GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 9, NO. 2, MARCH 2003: 171 -175
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A PRELIMINARY SURVEY OF MICRO-ORGANISMS IN THE GUT AND PELLETS OF A TROPICAL MILLIPEDE *DORATOGONUS UNCINATUS ATTEMS* (DIPLOPODA, SPIROSTREPTIDA, SPIROSTREPTIDAE)

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(Received 10 May 2002; Revision accepted 3 January 2003).

ABSTRACT

Millipede gut microbiology and decomposition of faecal pellets over a period of eight weeks were studied in the laboratory. Bacterial numbers, carbon and nitrogen content, pH and weight loss were monitored. Heterotrophic bacteria were the most abundant and reached a peak in the first two weeks of decomposition. The amount of carbon was constant while ammonium nitrogen decreased from 1.51 % to 0.03 % after eight weeks. The pH of the pellets was slightly acidic and did not change much during the course of decomposition. A succession of micro-organisms was observed on decomposing pellets. Zygomycetes were replaced by Ascomycetes after 20 days of decomposition. Decomposition was significantly affected by temperature. The rate of decomposition was highest at 35°C.

Key words: decomposition, pellets, bacteria, fungi, succession

INTRODUCTION

Millipedes are detritivores that eat dead plant material and fungal fruiting bodies (Blower, 1985). According to Blower (1985) millipedes prefer to feed on leaf litter which has been decomposed by micro-organisms and contains large populations of micro-organisms.

Anderson & Bignell (1980) reported large number of micro-organisms in the hind gut of a litter feeding millipede, *Glomeris marginata*. In decomposition studies of millipede faecal pellets and food litter Mwabvu (1996) attributed high mass loss observed in pellets (compared to food litter) to the large numbers of micro-organisms that may pass out of the gut with faecal material during defaecation. Griffiths & Wood (1985) also reported greater density of micro-organisms in the hind gut of an isopod, *Oniscus asellus* than the food litter

Hackstein & Stumm (1994) reported that methanogenic bacteria are present in the hindgut of nearly all the representatives of tropical millipedes, cockroaches, termites and beetles. It has been reported that species of micro-organisms present in the alimentary canal of earthworms are also the same as those found in the soil in which they live (Edwards & Lofty, 1977). According to Lathum (1979) all the vertebrate herbivores as well as many invertebrates such as termites and woodlouse rely on gut micro-organisms for the degradation of certain structural components present in the leaf litter they ingest. The faecal pellets of millipedes contain lignin, cellulose and various imperfectly digested matter, soil organisms, fine organic matter and mineral particles (Tajovsky *et al*, 1992).

During decomposition the physio-chemical and biochemical nature of the substrate continuously change such that its suitability as food for organisms changes (Mason, 1976). These changes may affect microbial biomass and/or diversity resulting in apparent succession. The changes in the substrate environment may be brought about by the organisms themselves, these changes may include reduction in nutrients, oxygen levels or changes in pH, other changes may be due to climatic factors such as temperature (Mason, 1976).

Organisms colonising a substrate may utilise it in many ways. Microbes may be involved in the direct decomposition by utilising the obtained energy for growth, parasitize other organisms or utilise their refuse for survival or use the substrate as an attachment site while the source of food is neither the substrate or the saprophytes (Mason, 1976).

Although many micro-organisms are known to be associated with guts of invertebrates, no attempts have been made to characterise micro-organisms occurring in the gut and faecal pellets of tropical millipedes. The objectives of the present study was to identify the micro-organisms that are found in the gut and pellets of a tropical millipede,

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Doratogonus uncinatus and record the changes that occur in the pellet after deposition

MATERIALS AND METHODS

The tropical millipede *D. uncinatus* and leaf litter were collected near Mazowe dam, Zimbabwe during the peak of the summer rainfall. The site was dominated by the tree species *Combretum* sp. and *Celtis africana*. The millipedes were maintained at 25°C in glass tanks (length 75 cm, width 35 cm, depth 20 cm) and fed moist leaf litter and soil taken from the site. Freshly collected specimens weighing approximately 12 g were dissected to expose the gut.

Homogenate of gut and gut contents was prepared following the methods by Anderson & Bignell (1980).

Pellet collection and sampling

The experiment required many pellets in a similar state of decomposition. To generate enough pellets 10 millipedes were placed in a glass tank (length 30 cm, width 30 cm, height 20 cm). A total of 7 glass tanks were used. The millipedes were fed leaf litter mixed with soil in the ratio 1:1 by mass. Pellets were collected and discarded for four days and on the fifth day the pellets were collected and stored at -2°C to suppress microbial activity.

After generating 600 pellets equal numbers were put into 4 petri dishes (85 mm diameter) and moistened using sterile distilled water. These petri dishes were divided into four portions for later sampling. The petri dishes were placed in an aerated chamber underlain by a moist filter paper to maintain high moisture levels and kept at 25°C. Samples for determining changes in pH, carbon (C), nitrogen (N), bacterial counts and fungal characterisation were taken on days 0, 2, 5, 7, 14, 28, 42, and 56. Samples were drawn by taking an equal number of pellets from each portion of the petri dishes. The pellets were dried for 72 hours at 37°C.

Enumerating bacteria from the millipede gut

20 millipedes were weighed and dissected to expose the gut. The guts were transferred into McCartney vials (diameter 3 cm) containing 10 ml sterile peptone water. The vials were shaken vigorously for 5 min to disperse the gut contents before filtering through a 0.5 mm sieve. Dilutions were made to make viable plate counts possible. Selective media were used to grow the different groups of micro-organisms.

Heterotrophic bacteria were grown using Nutrient agar (Oxoid) with cycloheximide at a concentration of 100 mg/ml to suppress fungi. Water agar (Oxoid) was used to grow and obtain viable counts of oligotrophic bacteria. Rogosa agar (Biolab) was used for lactic acid bacteria and McConkey agar (Difco) was used to obtain viable counts for enteric bacteria.

In order to grow gut microflora 0.2 ml of the homogenate was plated on potato dextrose agar (Oxoid) and malt extract agar (Oxoid). 10 replicates were prepared. The plates were incubated at 25°C for 72 hours and basic light microscopy was used to identify the fungi after staining with lactophenol.

Enumerating bacteria from faecal pellets

Dried pellets weighing 300 mg were aseptically transferred to a test tube containing 9 ml of sterile Ringer's solution. The pellets were homogenized by a sterile glass rod. This dilution was called 10^{-1}, further dilutions were prepared up to 10^{-9}. The three highest dilutions were used to obtain viable plate counts of bacteria (heterotrophs, enteric bacteria, lactic acid bacteria and oligotrophs). Six plates were prepared at each dilution and were incubated at 25°C for 72 hours.

Characterising fungi from faecal pellets

The homogenate of pellets from the least dilute tube was used to plate out fungi on potato dextrose agar and malt extract agar. 0.2 ml of the pellet homogenate was transferred onto petri dishes containing potato dextrose agar and malt extract agar. Five plates were prepared for each media and were incubated at 25°C for five days.

Determining pH of faecal pellets

Since adding a small amount of water does not affect the pH of the pellet, pH determination was carried out by homogenising a pellet sample of weight 300 mg in 10 ml of distilled water. A pH meter which had been calibrated by buffers of pH 4 and 7 at 25°C was used in all the experiments requiring pH determination. Samples used for determining pH were taken on the same days when samples for bacterial counts were taken.

Carbon determination (Organic carbon) in faecal pellets

A sample of ground pellets weighing 0.4 g pellet was transferred into a labelled 100 ml conical flask. 10 ml 5 % potassium dichromate solution was added to the sample and allowed to wet the pellet grains and to dissolve the standards. 20 ml sulphuric acid from a fast burette was added to the sample and the standards. The mixture was gently

swirled to allow mixing. After cooling, 50 ml of 0.4% barium chloride was added and gently swirled. The mixture was allowed to stand overnight.

An aliquot of the clear supernatant solution was transferred into a colorimeter and the absorbance was measured at 600 nm for the sample and the standards.

A graph of absorbance against standard concentration was plotted and used to determine the concentration of the sample. The mean concentration of the blanks was subtracted from the sample to give the corrected concentration, K. Percentage carbon was then calculated using the following formula.

$$\% \text{ organic carbon} = K * 0.1/W*0.74$$

where W is the weight of the sample.

Nitrogen determination in faecal pellets

Ammonium nitrogen was determined following the method by Bremner (1982).

Ammonium nitrogen was first extracted from the pellets by grinding a sample weighing 0.3 g. The ground sample was transferred to a 100 ml conical flask. 100 ml of 0.5M potassium chloride was added to the sample. The sample was vigorously agitated for an hour. 0.1 ml of each standard and sample were transferred into suitably marked test tubes. 5.0 ml of the reagent N1 was added to each test tube and left for 15 min after mixing well. 5.0 ml of the reagent N2 was added to each test tube and left for an hour to allow full colour development. The absorbance of the standards and the samples was read at 655 nm.

A graph of absorbance against standard concentration was plotted. Solution concentration for each unknown was determined using the graph.

Using the corrected concentration, C, % nitrogen was calculated using the following formula.

$$\% N = C*0.1/W$$

Decomposition of pellets

Pellets were collected and samples weighing 300 mg were placed in thirty sterile petri dishes (85 mm diameter). Samples were moistened with 5 ml sterile distilled water once every week.

A completely randomised design was used to assign a set of 10 petri dishes to their respective treatments. The treatments were 15°C, 25°C and 37°C. After eight weeks the pellets were dried at 37°C for 72 hours and re-weighed.

RESULTS

Four groups of bacteria namely, heterotrophic, lactic acid, enteric and oligotrophic bacteria, and nine fungal general were isolated from the gut. There were no protoctists in the gut or pellets of the millipede.

Heterotrophic bacteria were the most abundant group of bacteria in the millipede gut on each day, followed by the enteric bacteria, the lactic acid bacteria were the least abundant (Table 1). The number of heterotrophs was about 10 000 times more than that of the lactic acid bacteria. All the bacterial counts were expressed as per gram of the pellet's dry weight.

The fungi that were isolated included the saprophytic *Rhizopus* sp., *Mucor* sp., *Penicillium* sp., *Aspergillus* sp. and *Alternaria* sp., and the potentially parasitic *Ambrosiella* sp., *Paecilomyces* sp., *Metarrhizium* sp. and *Deightoniella* sp. Basidiomycetes were absent from both the millipede gut and faecal pellets. Unlike the potentially parasitic fungal genera, *Ambrosiella*, *Metarrhizium* and *Deightoniella* which were confined to the gut, all genera which appeared in the faecal pellets were also found in the millipede gut. A succession from Zygomycetes to Ascomycetes was observed on the pellets.

The pH of pellets from day 0 to day 56 at 35° C was slightly acidic (mean = 6.14, sd = 0.35). On fitting a regression line on pH against time, the slope of the regression was not significant ($p = 0.33$).

The amount of carbon expressed as a percentage of the pellet's dry weight was relatively constant from day 0 to day 56 (Table 1). The mean percentage carbon was 19.6 with a standard deviation of 1.78.

The amount of ammonium nitrogen decreased from day 0 to day 56 (Table 1).

The mean weight loss for pellets decomposing at three different temperature levels (15°C, 25°C and 37°C) was significant ($p < 0.001$). Weight loss of 3.4 % at 15° C, 12.2 % at 25° C (Table 1) and 21.0% at 35° C were recorded.

DISCUSSION

It is likely that the numbers of bacteria would vary in the gut depending on a number of factors, such as, the type of food ingested and /or its state of decomposition. Results of this study show differences in numbers of heterotrophic, lactic acid oligotrophic and enteric bacteria in the gut. The micro-organisms may be involved in digestion as

well as decomposition of faecal material after defaecation (Griffiths & Wood, 1985). It is however unfortunate that the bacteria could not be identified to genera.

Although this study does not show exactly where the bacteria and fungi are concentrated in the gut, it is unlikely that the anterior section of the gut would have a greater diversity of micro-organisms than the posterior. However, not all micro-organisms occurring in the millipede gut will be important in digestion. The fungal genera, *Ambrosiella, Paecilomyces, Metarrhizium* and *Deightoiella* sp. may contain parasitic species.

The large numbers of bacteria observed in millipede gut suggest a greater importance of bacteria in digestion than fungi. Fungi may only become prominent in decomposition of faecal material after defaecation.

The micro-organisms in the millipede gut may be the major source inoculum of bacteria and fungi which were responsible for decomposing millipede faecal pellets. This is supported by the fact that the four groups of bacteria and fungi (except *Ambrosiella, Metarrhizium and Deightoniella*) that were isolated from the gut were the same groups present in the faecal pellets. The Zygomycetes, *Mucor* and *Rhizopus* were the first group of fungi to colonise the millipede pellets followed by Ascomycetes as conditions in/on the pellets changed because of the activities of the micro-organisms.

The presence of oligotrophs indicated the availability of inorganic nutrients as sources of energy. Oligotrophs are able to survive in the absence of organic substrates such as carbohydrates (Lynch, 1979) by exploiting inorganic nutrients that are released after the mineralization of organic compounds by heterotrophs.

The presence of enteric bacteria in the gut may be important in digestion of food litter and later in pellet decomposition. Enteric bacteria found in the gut could have been ingested with food litter. This is supported by Stanier *et al.* (1986) who reported the presence of enteric bacteria, *Enterobacter* sp., *Secratia* sp. and *Proteus* sp. in soil.

The peak in activity of these heterotrophs in the first two weeks of decomposition shows that they were utilising readily available metabolisable sources of carbon such as carbohydrates. The decrease in their number after 14 days was followed by a decrease in the amount of ammonium nitrogen in the pellets. At the end of 56 days ammonium nitrogen content was low. The production of ammonium nitrogen is a microbial process which occurs when proteins are decomposed leading to the production of nitrogenous ammonia (Stanier *et al.*, 1986). Ammonium nitrogen was therefore a index showing the activity of micro-organisms with time. Micro-organisms were thus effective in breaking down nitrogen containing compounds which were present in millipede pellets.

Percentage loss of nitrogen from day 0 to day 56 was above ninety. The results/patterns are consistent with those reported by Tajovsky *et al.* (1992).

Enteric bacteria and oligotrophs had a slight peak of activity at day 42 which corresponded to the

Table 1: Chemical and microbial changes (Log 10) in decomposing millipede faecal pellets at 25°C

	Days of Decomposition							
	0	2	5	7	14	28	42	56
pH	6.4	5.6	5.8	6.5	5.9	6.5	6.2	6.4
Heterophic bacteria	11.8	11.2	11.3	11.0	12.0	9.0	9.3	8.0
Oligotrophic bacteria	9	9	9.8	8.7	9.9	7	6.3	7
Enteric bacteria	3.1	7.8	7.6	5.3	7	8.4	5.4	5
Lactic Acid bacteria	10.3	8.3	7.2	7.2	5.8	7	5	7
% Carbon	18	20	23	17	16	17	21	18
% Nitrogen	2	2.5	2	1.7	1	0.3	0.4	0
% Weight Loss	0	4	4.8	7.3	8.3	9.2	9	12.2

decline in the numbers of the heterotrophs, suggesting a succession. The succession on *D. uncinatus* pellets closely resembles that which was observed on the faecal pellets of the temperate millipede, *Glomeris marginata* (Nicholson *et al*, 1966) although of course the numbers of bacteria were lower in the temperate millipede. This could be explained by the lower experimental temperatures used and the smaller size of the pellets, as a potential food source, in temperate areas. It appears therefore that competition for nutrients, specific substrate and other interactions between the various groups of bacteria and fungi were important factors during the decomposition of pellets. Weight loss was highest at 35° C, thus highlighting the importance of temperature in influencing the rate of decomposition and the exploitation of pellets as food/nutrient sources by micro-organisms. The absence of basidiomycetes may have been caused by insufficient food resources in faecal pellets to support their growth.

The determination of organic carbon was not useful since it took into consideration all the organic matter present in the pellets including bacterial cell walls, fungi and plant litter. This explains why the amount of organic carbon was relatively constant throughout the study.

The results of this study show that millipede gut micro-organisms are the same as those that initiate decomposition in the pellets. However, work needs to be done on the relative densities and distribution of micro-organisms in the gut of tropical millipedes. The role of the different types of micro-organisms in digestive processes also need study in order to understand the evolution of such associations. Although micro-organisms are known to enhance digestive efficiency in many invertebrates the nature of the relationships is not clear. Micro-organisms may themselves be used as a food resource (Hassall & Rushton, 1985) besides being agents of cellulose digestion in the gut.

ACKNOWLEDGEMENTS

We would like to thank the technical staff in the Department of Biological Sciences for their assistance during this study and the department for making research material available.

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