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Occurrence and Distribution of Microflora in the Gut Regions of the Variegated Grasshopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae) during Development

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Kehinde O. Ademolu and Adewunmi B. Idowu (2011) Occurrence and distribution of microflora in the gut regions of the variegated grasshopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae) during development. *Zoological Studies* **50**(4): 409-415. The variegated grasshopper *Zonocerus variegatus*, like other insects, harbors microorganisms in its alimentary canal system. In this study, we investigated the occurrence and distribution of microflora in the gut regions of *Z. variegatus* during post embryonic development (1st instar to the adult stage) and food plants it consumed using a pour-plate method. The colony forming units (cfu) of bacteria, yeasts, and molds increased from the 1st instar stage to the adult stage, with the midgut having the highest occurrence of the 3 gut regions. *Staphylococcus aureus* was the most widely distributed bacteria in the gut while *Klebsiella* spp. were the least. In the mold category, *Penicillium* sp. and *Aspergillus niger* had the widest distributions, whereas *Candida* sp. was the only isolated yeast. There was a positive relationship (r^2 = 0.4797) between bacterial cfu and the stage of development of the insect. Similarity was observed in the species of organisms isolated from the gut regions and food plants consumed by the insects. The relevance of gut microflora to the insect hosts and their mode of transmission are further discussed.
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Key words: Gut microflora, *Zonocerus variegatus*, Food plant, Transmission mode.

Zonocerus variegatus is a polyphagous species capable of consuming most of the plant species in its surroundings (Toye 1982), and is reported to consume more than 250 plant species among 71 families (Chiffaud and Mestre 1990), among which are many crops, including citrus, cocoa, banana, and vegetables, such as cassava.

The early stages of development of *Z. variegatus,* particularly the 1st-3rd instars, survive on weeds (Toye 1974) such as *Chromolena odorata*, *Ageratum conazordes*, *Talinum triangulare*, *Aspilla lacifolis*, *Asp. africana*, and *Tridax procumbens*, whereas later instars (4th-6th instars) and adults prefer cassava leaves (*Manihot esculenta*). However, not all food plants contribute to the survival and development of *Z. variegatus*. For instance, cassava leaves and *Vernonia amygdalina* (Tamu 1990) were shown to support the growth and development of *Z. variegatus*. On the other hand, *C. odorata* and *Asp. africana* do not support the growth of the insect.

The alimentary canal system of *Z. variegatus* consists of the foregut, midgut, and hindgut. However, the midgut is the main active site of digestion. Insects like other animals are known to accommodate microorganisms in their alimentary canal systems which aid digestion (Mead et al. 1988) and also contribute to the nutrition of the host. Similarly, bacteria in the digestive tract of *Locusta migratoriodes* convert lignin to locustol (5-ethyl guanol), a pheromone involved in aggregation (Dillon and Dillon 2004).

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The alimentary canal of *Z. variegatus* was reported to harbor a variety of microorganisms, mainly bacteria, fungi, and molds. The transmission of gut microbes can be either by (1) vertical transmission, that is, from mother to egg, or (2) horizontal, that is, uptake by the host via a food source. The microbial count increased from the 3rd instar to the adult stage, which is a reflection of an increased size of the gut (Idowu and Edema 2004). However, no attempt was made to enumerate the microbial activity in the gut of 1st and 2nd instars during postembryonic development, thus leaving in doubt the actual origin of these microbes.

A literature review revealed scanty information on the microbial flora of the earliest instars (1st and 2nd) of *Z. variegatus,* thus making it impossible to know the actual source of these microbes. Information is needed in order to understand the mode of transmission of these microbes and to use this knowledge to synthesize possible biological control for the insect. The objective of this study was to examine the gut microflora of *Z. variegatus* in all postembryonic developmental stages (1st instar to the adult stage) in order to ascertain the mode of transmission. The pest status of *Z. variegatus* was established and confirmed by the report of Toye (1982). It was reported that it consumes and destroys both food and cash crops in West African countries of Nigeria, Benin and Cameroun.

MATERIALS AND METHODS

Thirty-five individual *Z. variegatus* nymphs and adults were used for this experiment (5 insects for each developmental stage). Before dissection, each insect was surface-sterilized by swabbing with iodine followed by 70% ethanol.

Dissection of the insects for a gut examination was carried out following the method described by Youdeowei (1974). The body cavity was opened by a ventral longitudinal cut which exposed the alimentary canal system. The gut was separated from adjoining tissues like fat bodies and malpighian tubules.

The gut was partitioned into 3 parts by flamed forceps: the foregut, midgut, and hindgut. The gut contents of the various parts were emptied into labeled Petri dishes, while the wall was thoroughly washed with distilled water to free any adhering material from it. Using a sterile mortar and pestle, each gut section was homogenized in 1 ml of sterile distilled water. The homogenate was decanted into labeled bottles containing 9 ml of sterilized water; 1 ml of a sample was homogenized in 9 ml of sterile diluted water, and 6-fold serial dilutions were made. Aliquots of 1 ml of 4-6 fold dilutions were plated in duplicate by a pour-plate technique using the following media: potato dextrose agar (PDA) was used for fungal enumeration; while nutrient agar (NA) and de Man, Rogosa, and Sharpe medium (MRS) (sigma, Oxford, UK) were respectively used for the bacterial and lactobacillus enumeration.

PDA plates were incubated at 30°C for 5 d, while NA and MRS were incubated at 37°C for 48 h. After 48 h, the colony forming units (cfu) were determined by visual counting. Purified colonies were grouped according to their colony morphology and cell characteristics. Yeasts and molds were identified after staining with cotton blue lactophenol. Further identification was carried out according to Kreger-venrij (1984) by pseudomycelium formation and patterns of sugar fermentation (glucose, galactose, maltose and lactose). Bacterial isolates were identified using *Bergey's Manual of Systematic Bacteriology* (Sneath et al. 1986) and methods of Hugh and Leifson (1963) and Harrigan and MacCance (1970).

The above procedures were also used for the microbiological analysis of the food plants consumed by the insects during the study. Ten grams of macerated leaves was put into 90 ml of sterilized distilled water, and 6-fold serial dilutions were made.

Statistical analysis

The cfu from the various gut regions of different developmental stages were analyzed by a one-way analysis of variance (ANOVA), and where significant means existed, they were separated by the Student Newman-Kuel (SNK) test. A regression analysis was also used to determine relationships between the cfu and developmental stages.

RESULTS

Results of the microbial load count of the gut and gut wall showed that no bacterium was found in the gut or gut wall of 1st instars of *Z. variegatus*. The cfu values indicated that there was a gradual rise in numbers from the 2nd to 5th instars until

a decrease was observed at the 6th instar, which then increased again in the adult stage (Table 1). It was observed that the midgut had the highest cfu followed by the hindgut, while the foregut had the smallest number.

The bacterial count for the gut walls did not follow a regular pattern, although a regular trend was noted in earlier instars (2nd-5th) which became irregular in the 6th instar and adult stage (Table 1). There were no significant differences ($p > 0.05$) between the total microbial counts of the 2nd and 3rd instars in any of the 3 gut regions.

Molds were not detected in the 3 sections of the 1st instar gut. However, the midgut recorded the highest cfu value followed by the hindgut, and the least value was the foregut (Table 1) during the 2nd and 3rd larval stages. The cfu values of the gut and its walls did not follow any particular trend.

Yeast cells were not detected at all in any regions of the gut except in the midgut of the 6th instar (Table 1). Similar observations were made in the wall except that the foregut walls of the 4th, 5th, and 6th instars recorded respective cfu values of 1.4×10^4, 2.1×10^4, and 2.5×10^4.

The regression analysis of the cfu (bacteria) and stages of development (Fig. 1) revealed a positive linear relationship between them ($r^2 = 0.4797$). Similarly, a positive linear relationship existed between the cfu (bacteria) and the length of the gut (Fig. 2).

Table 1. Colony-forming units (cfu × 10^4) of the gut regions and gut walls of *Zonocerus variegatus*

	Bacterial load					
Stage	Foregut	Foregutwall	Midgut	Midgutwall	Hindgut	Hindgutwall
1st	ND	ND	ND	ND	ND	ND
2nd	20 ± 0.1^{e}	24 ± 0.9^{c}	50.8 ± 0.01^{c}	25 ± 0.4^{c}	56 ± 0.01^{b}	16 ± 0.1^{d}
3rd	25 ± 0.2^{d}	26 ± 0.5^{c}	52.8 ± 0.11^{c}	21 ± 0.2^{c}	66 ± 0.7^{a}	21 ± 0.23^{d}
4th	60 ± 0.1^{c}	72 ± 0.6^{a}	50 ± 0.3^{c}	50 ± 0.36^{b}	60 ± 0.05^{b}	30 ± 0.4^{c}
5th	75 ± 0.2^{a}	75 ± 0.1^{a}	90 ± 0.5^{a}	53 ± 0.2^{b}	65 ± 0.12^{a}	37 ± 0.6^{b}
6th	23.5 ± 0.4^{d}	38 ± 0.8^{b}	86 ± 0.5^{a}	71 ± 0.1^{a}	28 ± 0.3^{c}	54 ± 0.01^{a}
Adult	65.0 ± 0.22^{b}	19 ± 0.1^{d}	70 ± 0.2^{b}	21 ± 0.3^{c}	15 ± 0.11^{d}	13 ± 0.6^{de}

	Mold load					
Stage	Foregut	Foregut wall	Midgut	Midgut wall	Hindgut	Hindgut wall
1st	ND	ND	ND	ND	ND	ND
2nd	1.5 ± 0.2	5.5 ± 0.22^{c}	2.3 ± 0.5^{c}	1.3 ± 0.1	2.0 ± 0.2^{c}	2.0 ± 0.2
3rd	1.7 ± 0.4	9.0 ± 0.8^{a}	7.0 ± 0.2^{a}	1.8 ± 0.2	1.1 ± 0.5^{c}	3.0 ± 0.2
4th	2.5 ± 0.01	3.0 ± 0.5^{cd}	7.0 ± 0.7^{a}	1.0 ± 0.5	2.0 ± 0.1^{c}	2.0 ± 0.5
5th	1.7 ± 0.31	2.5 ± 0.7^{d}	1.2 ± 0.1^{c}	5.0 ± 0.2	7.0 ± 0.9^{a}	4.5 ± 0.6
6th	2.5 ± 0.1	2.5 ± 0.1^{d}	4.0 ± 0.9^{b}	3.5 ± 0.01	3.5 ± 0.1^{b}	4.0 ± 0.2
Adult	1.0 ± 0.2	7.0 ± 0.5^{b}	1.5 ± 0.2^{c}	1.2 ± 0.5	2.1 ± 0.1^{c}	1.9 ± 0.5

	Yeast load					
Stage	Foregut	Foregut wall	Midgut	Midgut wall	Hindgut	Hindgut wall
1st	ND	ND	ND	ND	ND	ND
2nd	ND	ND	ND	ND	ND	ND
3rd	ND	ND	ND	ND	ND	ND
4th	ND	1.4 ± 0.3	ND	1.7 ± 0.7	ND	ND
5th	ND	2.1 ± 0.1	ND	ND	ND	ND
6th	ND	2.5 ± 0.1	1.2 ± 0.1	ND	ND	ND
Adult	ND	ND	ND	ND	ND	ND

[abc]mean values in the same column having the same superscript are not significantly different ($p > 0.05$) (SNK).

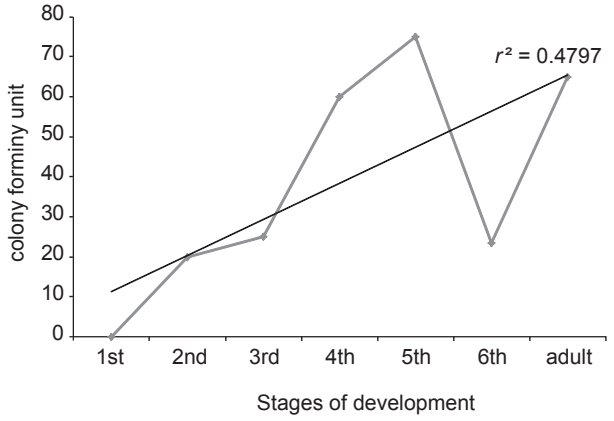

Fig. 1. The relationship between bacteria colony forming unit and the stages of delopment of *Z. variegatus*.

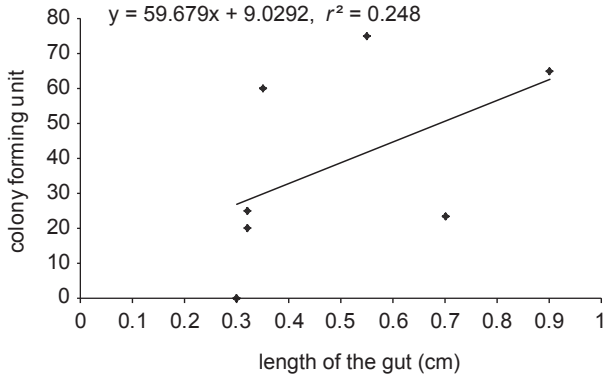

Fig. 2. The relationship between bacteria colony forming unit and the length of the gut of *Z. variegatus* during development.

Characterization of the microflora

Only 1 yeast species was isolated from the gut examination and was identified as *Candida* sp. On the other hand, 6 genera of molds were isolated: *Rhizopus stolonifor*, *Pennicillum* sp., *Fusarium* sp., *Aspergillus niger*, *Geotrodium* sp., *Muco mucede*, and *Aspergillus flavus*. Gram-positive and -negative non-motile rods and cocci were isolated and identified in the gut regions: *Staphylococcus aureus*, *Sta. epidermis*, *Klebsiella* sp., *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas* sp., *Micrococcus* sp., *Bacillus subtilis,* and *Proteus microbilis*.

The list of isolated microflora from the different regions of the insect gut is given in table 2.

Distribution of the microflora

Table 3 shows the distributions of the various yeast, mold, and bacterial isolates from the gut regions of *Z. variegatus*. Among the bacteria isolated, *Sta. aureus* was the most widely distributed in all gut regions and gut walls, followed by *B. subtilis,* while the least distributed was *Klebsiella* sp. For molds, *Penicillium* sp. and *A. niger* had the widest distributions, while *Fusarium* sp. was least distributed.

Food plant microflora

During the course of the experiment, *Z. variegatus* was maintained on leaves of *C. odorata* and *M. esculenta*. The organisms isolated from leaves of these 2 plants consumed by the insects

Table 2. Organisms isolated from the gut regions

Bacteria (B)	Molds (M)	Yeast (Y)
I. *Staphylococcus aureus*	I. *Rhizopus stolonifor*	I. *Candida* sp.
II. *Staphylococcus epidermidis*	II. *Penicillium* sp.	
III. *Klebsiella* sp.	III. *Fusarium* sp.	
IV. *Streptococcus faecalis*	IV. *Aspergillus niger*	
V. *Streptococcus* sp.	V. *Geotrichum* sp.	
VI. *Escherichia coli*	VI. *Muco mucede*	
VII. *Pseudomonas* sp.	VII. *Aspergillus flavus*	
VIII. *Micrococcus* sp.		
IX. *Bacillus subtilis*		
X. *Proteus mirabilis*		

I-X, numbers of different organisms isolated from the gut used in table 3.

were *Mucor* sp., *Aspergillus* sp., *Sta. aureus*, *Bacillus* sp., *Rhizopus* sp., *E. coli*, and *Proteus* sp. (Table 4).

DISCUSSION

The present study shows that microorganisms (bacteria, fungi, and yeast) are present in the gut of *Z. variegatus*, and as a phytophagous insect, it has associations with microorganisms (Campbell 1990) and thus it possesses an open system that is suitable for different kind of organisms.

No microorganism was isolated or detected in the gut of 1st instars. Chapman (1990) earlier reported that the alimentary canal of grasshoppers is sterile when the instar hatches from the egg, but soon acquires a bacterial flora which increases in number and species throughout life. DeVries et al. (2001) likewise examined the gut of western flower thrips *Frankliniella occidentalis* and discovered that most very young 1st instar larvae were not infected with gut bacteria. This might probably be a result of the non-feeding habit of freshly hatched 1st instar nymphs which still depend on nutrient reserves from the egg, thus the channel of gut infection is not yet established. This observation suggests that microorganisms are not vertically transmitted from the parent to offspring via the egg.

No significant differences were observed between total microbial counts of the 2nd and 3rd instars in any gut region. This parallels observations by Ademolu et al. (2009) who detected no significant difference in enzyme activities of femoral muscles of 1st and 2nd instar stages of *Z. variegatus*. This is a reflection of the common diets eaten by these instars. The 1st-3rd instars of *Z. variegatus* prefer *C. odorata*, while 6th instars and adults show a preference for cassava, *M. esculenta* (Chapman et al. 1986). The highest cfu values for bacteria and molds were recorded in the midgut. This is possibly due to the characteristics of the midgut. Rost-Roszkowska and Udrul (2008) and Rost-Roszkowska et al. (2010) observed that the midgut of insects is composed of epithelial and regenerative cells which are responsible for digestion, secretion, and absorption.

There was a similarity in the species of microorganisms isolated from the food plants and those isolated from the gut regions of insects that consumed them. This indicates that the microorganisms are actually from the food plants eaten. This is consistent with Dillon's (2001) assumption that locusts *Schistocerca gregaria* derive their microbiota from ingested food plants. Locusts possess a locally indigenous microbiota composed of species commonly encountered in their environment (Hunt and Chmley 1981). In a similar study by Mead et al. (1988), *Enterococcus* spp. and *Enterobacter agglomerans* isolated from gut regions of the migratory grasshopper *Melanoplus sanguinipes* were similarly present on the bran fed the grasshoppers, suggesting that the gut flora was directly derived from the diet.

The microbial load in the gut of *Z. variegatus* increased as the age of the insect increased, except in the 6th instar. Similarly, a positive linear relationship existed between the microbial load

Table 3. Distribution of the microflora in the gut regions

	1st	2nd	3rd	4th	5th	6th	Adult
Foregut	Nd	BIX, BI, MII, MIII, MV	BI, BIX, MIV, MVI	BI, BVIII, BIX	BII, BV, MII, MIII	BVIII, BIX, MII, MIV	BI, BV, BVIII, MIV, MVI, MVII
Midgut	Nd	BI, BV, MII, MIV	BI, BV, BIX, MIV, MI	BI, BVI, BIX, MII, MIV, MV	BI, BIV, MI, MII, MIV	BI, BII, BIX, MIV, YI	BIV, BVIII, MII, MIII, MIV, MVII
Hindgut	Nd	BVI, BIX, BX	BVI, BIX, MIV, MVI	BI, BVI, BX, MI, MV	BIII, BVI, BVII, MII	BIII, BVI, BVIII, MII, MIV, MV	BIII, BIV, BVI, BVII, MIV, MVI, MVII
Fore wall	Nd	BI, BII, BIX, MIV, MVII	BI, BIX, MIV, MVII	BI, BIX, MII, YI	BI, BII, MI, MIV, MVII, YI	BI, BII, BVIII, YI	BI, BIV, MIV, MVII
Mid wall	Nd	BI, BVI, MVI	BI, BV, MVII	BI, BVI, BVIII, MIII, MIV, YI	BI, BII, BIV, MVII, MII	BI, BIX, MI, MIV	BI, BIV, MIV, MVII
Hind wall	Nd	BV, BVI, MVII	BV, BVI, MIV, MVII	BVI, BVIII, BX, MI, MV, YI	BI, BIII, BIV, MII, MIII	BIII, BVI, MI, MII	BIII, BVI, BVII, MI, MII, MIV, MVI

B, bacteria; M, mold; Y, yeast. Nd, not detected.

count and the age of the insects. This corroborates DeVries (2001) findings that bacteria grow exponentially in the thrip gut. Recently, Idowu and Edema (2004) ranked adult *Z. variegatus* as having the greatest and the 3rd instar as having the smallest microbial loads in the gut. This can be explained by the increase in gut size as the insect ages and the increase in food consumption as the insect grows to meet its metabolic needs. Higher cfu values were recorded on the gut wall than in the gut contents. Idowu and Edema (2004) made similar observations for *Z. variegatus* instars and adults. Although the reason for these observations could not be ascertained at present, it could be that the gut wall offers a better and more-stable habitat for microbes to thrive than the gut contents that are transient.

It was observed that the microbial load of 6th instars of *Z. variegatus* was lower than those of earlier instars. The 6th instar stage is the penultimate stage which undergoes a final molt, and during this process, the perithrophic membrane and the gut system itself change (Moritz 1986).

Results of the microbial load of the gut indicated that more bacteria than molds and yeasts were found in the gut. This is likely due to the characteristics of the organisms. Bacteria are known to be ubiquitous, living in nearly all environments, while fungi and yeasts are more selective in their choice of hosts (Martin and Kukor 1984). This is agrees with Chapman's (1990) findings that the most commonly occurring microorganisms in insects are bacteria and bacterium-like organisms.

The roles played by microorganisms in insect digestion are highly significant. In scarabaesid larvae, microorganisms ferment the wood, and without them, the larvae would be unable to utilize the cellulose of the wood (Chapman 1990). Microorganisms supply essential vitamins and other substances, hence change a poor diet into an adequate one. Furthermore, ingested microorganisms liberate enzymes that remain active in the gut surroundings and thus expand or extend the digestion and metabolic capabilities of organisms that harbor them (Martin and Kukor 1984). Also, microbial products play subtle roles in the life of the insect, being involved in the digestion of refractory food and detoxification of secondary plant compounds (Dillon and Dillon 2004).

The process of hydrolyzing cyanide present in the food plant *M. esculenta* eaten by *Z. variegatus* is still not clear. However, microorganisms isolated from the gut, like *Proteus* sp. are known to produce proteolytic enzymes. In a recent study, Idowu et al. (2009) discovered that the majority of bacterial isolates from the gut of *Z. variegatus* were able to degrade linamarin and cellulose substitutes, indicating linamarase and cellulase activities. Hence, the presence of these enzymes produced by bacteria may be the means by which the insect degrades cyanoside present in its food, *M. esculenta*.

Table 4. Microflora of the food plants consumed by *Zonocerus variegatus*

Food plant	Isolated organisms
A) *Chromolaena odorata*	*Mucor* sp. *Aspergillus* sp. *Staphylococcus aureus*
B) *Manihot esculenta*	*Bacillus* sp. *Rhizopus* sp. *Escherichia coli* *Streptobacillus* sp. *Proteus* sp.

The organisms were isolated different food plants with no overlap.

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