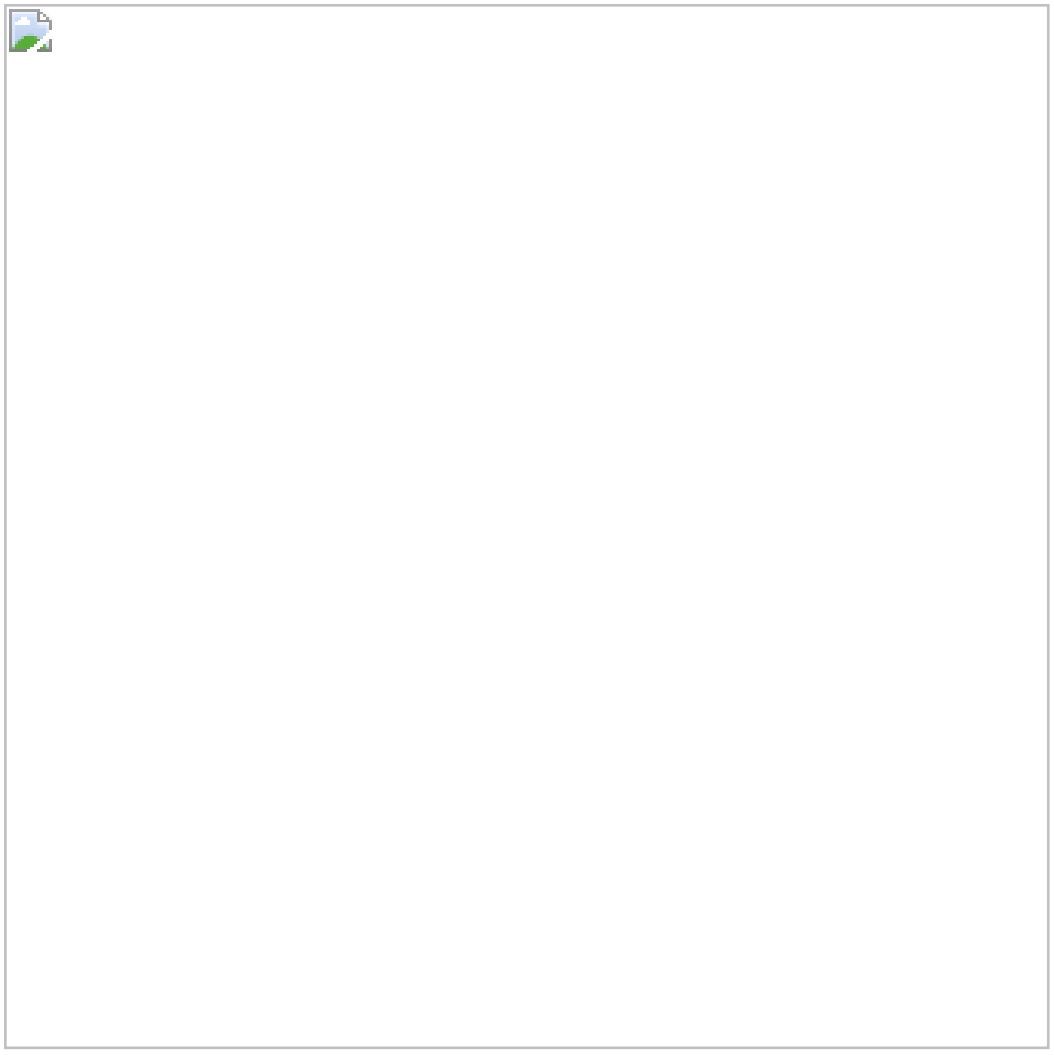

Microbial Diversity in the Gut of Cashew Stem Girdler, Analeptes trifasciata Fabricius (Coleoptera: Cerambycidae), in Ibadan, Nigeria

Authors: Oyedokun, A.V., and Adeniyi, D.O.

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Microbial Diversity in the Gut of Cashew Stem Girdler, *Analeptes trifasciata* Fabricius (Coleoptera: Cerambycidae), in Ibadan, Nigeria

A.V. Oyedokun[1] and D.O. Adeniyi[2]

[1]Entomology Section, Crop Protection Division, Cocoa Research Institute of Nigeria, Idi Ayunre, Ibadan, Oyo State, Nigeria. [2]Pathology Section, Crop Protection Division, Cocoa Research Institute of Nigeria, Idi Ayunre, Ibadan, Oyo State, Nigeria.

ABSTRACT: The cashew stem girdler, *Analeptes trifasciata*, is a major insect pest of cashew in Nigeria causing economic damage in cashew plantations even at low density. In this study, newly emerged adults of *A. trifasciata* reared from field-infested cashew stems were collected from the rearing cages, sexed, and dissected to reveal the internal structures of the insects. The gut was excised and separated into the foregut, midgut, and hindgut. The dissected gut compartments were blotted dry by sandwiching in sterile Whatman No. 1 (150 mm) filter paper for a minute. The inoculated gut parts showed the presence of eight fungi flora, namely, *Aspergillus repens*, *Trichoderma* spp., *Fusarium verticillioides*, *Lasiodiplodia theobromae*, yeast, *Aspergillus niger*, *Fusarium* spp., and *Rhizopus stolonifer*. The frequencies of occurrence of bacteria in the gut compartments of *A. trifasciata* were *Enterobacter* spp.: 83.33%; *Escherichia coli* and *Streptococcus* spp.: 55.56% each; *Staphylococcus* spp.: 44.44%; *Klebsiella pneumonia*: 50% and *Salmonella shigella*: 11.11%, while each of *Serratia marceascea*, *Pseudomonas* spp., and *Micrococcus lutea* had 5.56% occurrence. The occurrence of mycoflora and microbiota species varied in the gut compartments of *A. trifasciata*, indicating the role of these microorganisms in metabolic and other bioprocesses of *A. trifasciata* during digestion and synthesis of complex food substances from the cashew stem substrate. This study would provide basic information for enzymatic studies of *A. trifasciata* with a view to developing an integrated pest management (IPM) protocol for managing the pest in cashew plantations.

KEYWORDS: *Analeptes trifasciata*, mycoflora, microbiota, microbial population, gut compartment

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CORRESPONDENCE: gokman.dee@gmail.com

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Introduction

A myriad of insect pests attack the cashew plant, *Anacardium occidentale* (leaf, stem, flower, and fruit—the pseudo apple), on the field, and prominent among them is the cashew stem girdler, *Analeptes trifasciata*. This pest causes economic damage to the cashew plant via some of its biological activities (especially feeding and reproduction) on the cashew stem, and it poses serious threat to sustainable cashew production in Nigeria if uncontrolled. The pest is widely distributed in most of the cashew-producing states in Nigeria, and despite being a low-density insect, its damage profile on cashew stem is enormous.[1]

Generational success of insects has depended, in part, on the vast relationships with beneficial microorganisms that aid digestion of nutrient-poor diets and recalcitrant food components; protect from predator, parasites, and pathogens; aid the inter- and intraspecific communication,[2–4] and govern mating and reproductive systems.[5] Similarly, insect guts contain symbiotic protozoans, mycoflora, and/or microbiota populations specialized for performing different functions in insect bioactivities, including nutrient provision (amino acids),[5–7] lignocellulose digestion,[8,9] protection against parasites,[4,7,8] fermentation, and other pertinently prominent roles in the digestive tract of an insect, where they may serve as key mediators of the varied lifestyle of insect host.[2–4,6–13]

Microbial colonization depends on the physicochemical conditions of the gut compartments, which could be exhibited in varied pH, availability of particular substrates, and redox potential.[4] Insect guts with large microbial communities have actively high microbial metabolism that determines the condition of different compartments of the gut. For instance, in scarab beetle, *Pachnoda ephippiata*, there was abundant but varied microbial fermentation products such as acetate, formate, and lactate in the midgut and hindgut of the insect, signifying the specialized activities of microbial communities in the gut compartment of the insect.[14]

Since *A. trifasciata* is a cashew stem girdler, a wood-feeding insect at larval and adult stages, it is believed that it will harbor gut microbial species, especially fungi and bacteria, that are involved in cellulose degradation during metabolism as reported earlier on pine engraver *Ips pini*,[10] wood-boring beetles—*Anoplophora glabripennis* and *Saperda vestita*,[15]—cricket,[16] termites and cockroaches,[17] and *Bombyx mori*.[18] However, the microbial communities existing in the gut of this pest (*A. trifasciata*) are not yet known. The objective of

this study was to provide basic and preliminary information on the population diversity of the microbes in the gut of *A. trifasciata* in Nigeria.

In a bid to control *A. trifasciata*, chemical control option had been the main viable strategy presently adopted by farmers in Nigeria. This control option has attendant shortcomings such as resurgence of the insect pest 24 hours after the knockdown effect of the available chemicals at the manufacturer's recommended dosage. With the usage of higher concentrations above manufacturers' dosage rate, more insecticides are being used by farmers to control this pest at an extra cost, resulting in other risk factors such as killing of nontarget organisms and also environmental pollution through pesticide residues. Therefore, this study preliminarily explored the bioecology of *A. trifasciata* via the assessment of the microbial species diversity in the gut of the pest with a view to identifying some potential antagonist microorganism(s) that can alter and/or disrupt the bioactivities of symbiotic microbes in the gut of *A. trifasciata*, especially during metabolism as an environment-friendly control option.

Materials and Methods

Laboratory rearing of *A. trifasciata*. Adult emergents of *A. trifasciata* were obtained from a culture from the fallen infested host plant (cashew stem) samples. As described by Asogwa et al,[1] field-collected cashew stems with signs of infestation and egg laying spots of *A. trifasciata* were collected from cashew plots in Cocoa Research Institute of Nigeria, Ibadan, and cultured in rearing cages (62 cm length × 62 cm breadth × 115 cm height) at ambient tropical temperature (27 ± 2°C) and relative humidity (70 ± 5%). The infested cashew stems were left in the cages undisturbed until adults started emerging at about 55–60 days postcollection into the rearing cages in the Entomology Section, CRIN, Ibadan. Fresh cashew stems with no signs of infestation were supplied weekly as food substrate and laying site for newly emerged *A. trifasciata* adults.

Dissection of *A. trifasciata*. Newly emerged adults (1–3-day old) *A. trifasciata* (three males and three females) that had been feeding on the cashew stems immediately after emerging as adults were collected from the rearing cages, sexed, surface sterilized in 70% alcohol for one minute and rinsed in sterile water, and dissected to reveal the internal structures of the insects. Under a computer-connected Celestron USB Microscope (20×–800× magnification), the gut was excised and dissected in sterile distilled water using sterile scalpel and forceps into three parts (foregut, midgut, and hindgut) following the description by Chapman et al[19] for each sex to have the following: foregut male ($FGM_{1–3}$), midgut male ($MGM_{1–3}$), hindgut male ($HGM_{1–3}$), foregut female ($FGF_{1–3}$), midgut female ($MGF_{1–3}$), and hindgut female ($HGF_{1–3}$). The dissected parts were kept separately in sample bottles containing deionized water before inoculation on growth media. The experiment was replicated three times per sex.

Experiment 1: culture and isolation of the mycoflora species. Each of the dissected parts was blotted dry by sandwiching in sterile Whatman No. 1 (150 mm) filter paper for one minute. The potato dextrose agar (PDA) was routinely prepared in the laboratory, sterilized at 121°C for 15 minutes, allowed to cool to about 45°C on the bench, and poured into Petri dishes. The gut parts of *A. trifasciata* were aseptically inoculated into the acidified PDA in Petri dishes in triplicates and incubated at 28 ± 2°C for five to seven days for the isolation of mycoflora in the gut. The mixed culture growth was subcultured to obtain pure cultures of incidence organisms. The microbial identifications were made on the observed morphological features and characteristics of the isolates with reference to published identification manuals such as Barnett and Barnett and Hunter among others.

Experiment 2: culture and isolation of the microbiota species. The routinely prepared nutrient agar and MacConkey agar were used for the bacteriological assay of the gut of *A. trifasciata*. The gut parts were separately inoculated into the media and incubated at 37°C for 24 hours. The colonies were counted, occurrence of the associated bacteria was recorded, and gram staining was performed.

Data analysis. Data collected were rated in percentage and frequency for occurrence, while colonies were counted visually to determine the incidence using standard pathological procedures.

Results

Experiment 1: mycoflora diversity in the gut parts of *A. trifasciata*. The inoculated gut parts showed the presence of eight fungi flora, namely, *Aspergillus repens, Trichoderma* spp., *Fusarium verticillioides, Lasiodplodia theobromae,* yeast, *Aspergillus niger, Fusarium* spp., and *Rhizopus stolonifer* (Table 1). Each of the gut parts of *A. trifasciata* had two or more mycoflora present, each of HGF and MGF had two fungi, and both had *L. theobromae* and either of *Trichoderma* spp. or yeast, but three fungi were recorded in each of FGM, HGM, FGF, and MGM. *A. repens* was common to FGM and HGM, only MGM had *F. verticillioides* and *R. stolonifer,* and yeast was common to MGF and FGF.

The assay of the fungi present in the gut of *Analeptes* showed that *L. theobromae* was present in four (HGM, HGF, MGF, and FGF) of the six dissected gut parts and likewise was *Trichoderma* spp. cultured from four others (FGM, HGM, HGF, and MGM). *Fusarium* spp. and *A. niger* were only found in FGM and FGF, respectively. The combination of the highest occurring fungi (*L. theobromae* and *Trichoderma* spp.) was recorded in HGM and HGF (Table 1).

Experiment 2: microbiota diversity in the gut parts of *A. trifasciata*. The bacterial assay of the gut of *A. trifasciata* is shown in Table 2, comprising nine bacterial genera. The occurrence of the bacteria varied in the gut parts, the *Enterobacter*

Table 1. Incidence, fungi colony count, and the mycoflora species in the gut of *A. trifasciata*.

GUTS OF *Analeptes trifasciata*	FUNGI COLONY COUNT	MYCOFLORA	INCIDENCE
FGM_1	3	*Aspergillus repens*, *Trichoderma* spp., *Fusarium* spp.	1, 1, 1
FGM_2	3	*Trichoderma* spp.	3
FGM_3	3	*Trichoderma* spp.	3
MGM_1	1	*Fusarium verticillioides*	1
MGM_2	1	*Rhizopus stolonifer*	1
MGM_3	2	*Trichoderma* spp.	2
HGM_1	3	*A. repens, Lasiodiplodia theobromae*	2, 1
HGM_2	1	*L. theobromae*	1
HGM_3	1	*Trichoderma* spp.	1
FGF_1	3	*A. niger, L. theobromae*	2, 1
FGF_2	3	Yeast	3
FGF_3	3	Yeast	3
MGF_1	2	Yeast, *L. theobromae*	1, 1
MGF_2	3	Yeast, *L. theobromae*	2, 1
MGF_3	3	*L. theobromae*	3
HGF_1	3	*L. theobromae*	3
HGF_2	3	*Trichoderma* spp.	3
HGF_3	3	*Trichoderma* spp.	3

Abbreviations: $FGM_{1–3}$, foregut male: 1–3; $MGM_{1–3}$, midgut male: 1–3; $HGM_{1–3}$, hindgut male: 1–3; $FGF_{1–3}$, foregut female: 1–3; $MGF_{1–3}$, midgut female: 1–3; $HGF_{1–3}$, hindgut female: 1–3.

spp. recorded 83.33%, *Escherichia coli* and *Streptococcus* spp. had 55.56%, while each of *Serratia marceascea, Pseudomonas* spp., and *Micrococcus lutea* had 5.56%. A comparison of the incidences of the nine bacterial species is shown in Figure 1. *E. coli* was present in either one or two replicates of all the gut parts of *A. trifasciata* except in the midgut of the male ($MGM_{1–3}$), *Salmonella shigella* was found in HGF_3 and MGF_1, and *S. marceascea* was present only in FGM_2. Both *Streptococcus* spp. and *Staphylococcus* spp. were absent in FGF_2 and $MGM_{1–3}$, *Klebsiella pneumoniae* was absent in $FGM_{1–3}$ and FGF_1, while *Enterobacter* spp. was found in all the gut parts. MGF region recorded the highest number of bacteria, followed by FGM, HGM, and HGF regions that recorded five bacterial isolates, but MGM region had only *K. pneumonia* and *Enterobacter* spp.; *Staphylococcus* and *Streptococcus* were commonly cultured from all gut parts except FGF and MGM, while *E. coli, K. pneumonia,* and *Enterobacter* spp. were isolated from HGM, MGF, and FGF regions, but *E. coli* and *Enterobacter* spp. occurred together in all gut parts, except one (Table 2).

Discussion

This study has revealed the diversity in the mycoflora and microbiota communities existing within different gut compartments of *A. trifasciata* in Nigeria. From this study, only 17 microbial species were identified in the gut of *A. trifasciata*, and it consists of nine bacteria and eight fungal species. This is in consonance with earlier reports[4,20] that most insect guts contain relatively few microbial species as compared with mammalian guts. The foregut of male *A. trifasciata* consists mainly of *Trichoderma* species (78%), which is known to be parasitic to other fungi species, and this may suggest the protection role it plays in the foregut of *A. trifasciata* in order to maintain balance by killing other harmful fungi species ingested by the insect.[2,4,21–23] Other fungi species in the foregut of *A. trifasciata* include *A. repens* and *Fusarium* sp., but they are negligible in incidence rating with 11% each. The *Aspergillus* species (*niger* and *repens*) produce citric and gluconic acids,[4,16,17,21,22] which might be playing significant roles in the digestion of food materials in the gut of *A. trifasciata*. This corroborates the possibility of ingestion of environmental fungal spores such as *Aspergillus* species by the insect that might be playing significant roles in some biological processes of the insect[2,4,24] and that there are antagonistic interactions between different gut microorganisms in insects.[4]

There was a reduction in the percentage occurrence of *Trichoderma* spp. in the midgut of male *A. trifasciata* from 77% to about 50% that occurred in the foregut. The midgut of male *A. trifasciata* had 25% each of *F. verticillioides*—a fungus symbiont that is a source of detoxification enzyme[21]—and *R. stolonifer*; whereas, the female midgut had mainly yeast (37.5%) and *L. theobromae* (62.5%). Similarly, reduction trend in *Trichoderma* sp. along the gut compartments of male *A. trifasciata* was repeated in the hindgut with 20% occurrence, while other species such as *A. repens* and *L. theobromae* had 40% occurrence in all the colonies. This suggests how the gut

Table 2. Percentage occurrence of bacteria species in the gut compartments of *A. trifasciata.*

GUTS OF *Analeptes trifasciata*	*Escheria coli*	*Klebsiella pneumonia*	*Enterobacter* spp.	*Serratia marceascea*	*Salmonella shigella*	*Pseudomonas* spp.	*Staphylococcus* spp.	*Micrococcus lutea*	*Streptococcus* spp.
FGM_1	–	–	–	–	–	–	+	–	+
FGM_2	+	–	+	+	–	–	+	–	–
FGM_3	–	–	–	–	–	–	–	–	+
MGM_1	–	+	+	–	–	–	–	–	–
MGM_2	–	+	+	–	–	–	–	–	–
MGM_3	–	+	+	–	–	–	–	–	–
HGM_1	+	+	+	–	–	–	+	–	+
HGM_2	+	+	+	–	–	–	+	+	+
HGM_3	–	+	+	–	–	–	+	–	+
FGF_1	+	–	+	–	–	+	–	–	–
FGF_2	–	+	+	–	–	–	–	–	–
FGF_3	+	+	+	–	–	–	–	–	–
MGF_1	+	–	+	–	+	–	–	–	–
MGF_2	+	+	+	–	–	–	+	–	+
MGF_3	+	–	–	–	–	–	+	–	+
HGF_1	–	–	+	–	–	–	–	–	+
HGF_2	+	–	+	–	–	–	–	–	+
HGF_3	+	–	+	–	+	–	+	–	+
% occurrence	55.56	50	83.33	5.56	11.11	5.56	44.44	5.56	55.56

Abbreviations: FGM, foregut male; MGM, midgut male; HGM, hindgut male; FGF, foregut female; MGF, midgut female; HGF, hindgut female.

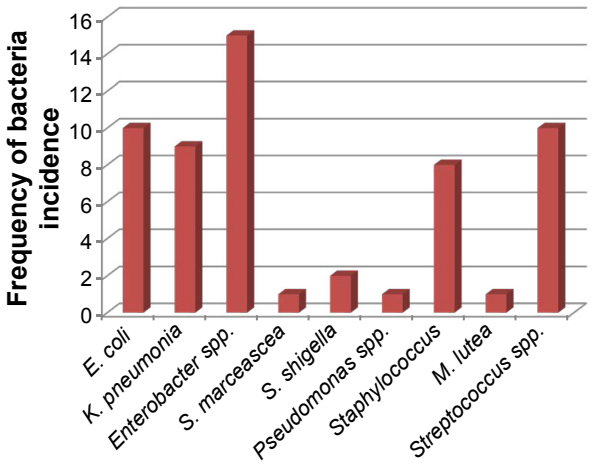

Figure 1. Isolated bacteria species in the guts of *A. trifasciata*.

compartments of this insect harbor varied symbiotic mycofloral communities specific for different functions or roles.[22,25,26] However, there was no incidence of *Trichoderma* species in the foregut of female *A. trifasciata*, but *A. niger*, *L. theobromae*, and yeast had 22.22%, 11%, and 66.67%, respectively. This suggests the varied digestion processes in the sexes of *A. trifasciata*, indicating different nutritional requirements for other bioactivities such as mating, egg formation, and laying.[27–30] The hindguts of male and female *A. trifasciata* presented varied fungi species with no regular pattern. For instance, the HGM had *Trichoderma* spp. (20%), *L. theobromae* (40%), and *A. repens* (40%), while HGF had only *L. theobromae* (33.33%) and *Trichoderma* sp. (66.67%). The occurrence of 40% *A. repens* in the HGM suggests incomplete digestion of lignocellulose ingested by male *A. trifasciata* in the midgut compartment and that further digestion and/or reabsorption of food materials via citric and gluconic acid production might be taking place in the HGM; whereas the HGF had no *A. repens,* suggesting complete digestion of food materials in the MGF. Presence of *L. theobromae* in HGM and HGF indicates the introduction of the pathogen into the environment (soil) via its excreta and might be indicative for the spread of *L. theobromae* that is implicated for infections in plant materials.

The species richness of bacteria in the gut compartments of *A. trifasciata* was low with *E. coli* existing in the representative samples of FGM, HGM, FGF, MGF, and HGF except in MGM where *E. coli* was not isolated. The absence of *E. coli* in MGM might be a factor responsible for the presence or absence of some symbiotic fungi in the gut compartments of the insect as recorded in this study. This corroborates earlier studies[7,16,29] that bacterial mutualists in insect guts play important roles in protecting the host from other potentially harmful microbes and may as well engage in opportunistically harmful interactions with the host. The FGM and HGF recorded 0% occurrence of *K. pneumoniae,* whereas *K. pneumoniae* was isolated from other gut compartments of *A. trifasciata*. Some species identified in the gut compartment of *A. trifasciata* from this study had been identified in other studies and/or organisms (adults or developmental stages) and include the following: *Pseudomonas* sp. and *Streptococcus* sp. in *Ips pini*;[10] *Enterobacter* sp., *Staphylococcus* spp., and *Streptococcus* spp. in *Agrilus planipennis*;[5] *S. marceascea* and *Enterobacter* sp. in *Longitarsus* flea beetle, and *Microbacterium* sp. in *M. lutea*.[7] All these gut microbial species of *A. trifasciata* play significant roles in its ability to digest the food substrate (cashew stem) containing complex compounds, and this needs further investigations to ascertain the specific role and/or function of the microbiota and mycoflora communities in the gut compartments of cashew stem girdler.

Conclusion

Identification of the 17 gut microbial populations (nine bacteria and eight fungi species) of *A. trifasciata* forms the basis for investigating the general and specific functions of the fungal and bacterial species associated with the insect, in relation to its generational success in terms of food digestion and utilization, secretion of enzymes, and its reproductive capacity. This information can be very useful in developing entomopathogenic organisms as control options in managing this economic pest of cashew.

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Author Contributions

Conceived and designed the experiment and cultured and dissected the insects: AVO. Isolated and identified associated microorganisms in the gut of the insect: DOA. Contributed the reagents: AVO and DOA. Wrote the paper: AVO and DOA. Both authors reviewed and approved of the final manuscript.

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