Gut digestive enzymes and bacterial and fungal diversity of *Apis mellifera adansonii* (Hymenoptera: Apidae) from three ecological zones of Nigeria

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**ABSTRACT**

Gut digestive enzymes and microbial diversity of worker honeybees, *Apis mellifera adansonii* L. from rainforest, guinea savannah and derived savannah zones of Nigeria during the onset of rain, the wet season, and dry season was evaluated. The pour-plate method was used for microbial analysis while spectrophotometric method was used to determine activities of gut digestive enzymes. Gut activities of lipase and proteinase were not significantly different in the honeybees from the three ecological zones during the seasonal periods. However, amylase and α-glucosidase were significantly affected by seasonal periods and ecological zones. Bacterial count was higher on the body surface than in the gut of the honeybees. The lowest (gut and body surface) bacterial count was recorded during the dry season period. Fungal load counts on the body surface and gut were significantly different between the ecological zones during each of the seasonal periods. Bacterial species varied in the honeybees from the ecological zones and this was higher on the body surface than in their gut. Yeast isolate of the genus Candida was the only identified yeast on the body surface and in the gut of the honeybees. The gut of honeybees could selectively harbour microbes while performing digestive roles.

**Introduction**

Honeybees are social insects and economically important insects due to their roles in pollination and honey production [1]. They pollinate a large number of crops in orchards and other agricultural systems [2–4]. Honeybee colony is active all year round in the beehive while their population size as well as the level of colony activities varies with different seasons [5,6]. However, foraging activities of the honeybees reach its peak during the period of increased blooming of food crops [7].

Research on honeybee health is on the increase, focusing on their association with beneficial and harmful symbionts such as fungi, yeasts, bacteria, mites, protozoans, and viruses [8,9]. However, more research on the microbial association of the honeybees submitted that microorganisms are detrimental to the honeybee colony. For example, 10, reported that microorganisms such as fungi, bacteria could cause infections in bees and lead to its colony collapse.
Some of the microorganisms associated with the honeybees include spore-forming bacteria, *Paenibacillus larvae* [11], non-spore-forming bacteria *Melissococcus* (Streptococcus) *plutonius* [12], *Rhizopus sp, Baccillus sp* and *Mucor hiemalis* [10], *Mucor hiemalis, Aspergillus*, and *Rhizopus sp* [13]. According to these authors, the association of these microbial species with honeybees was associated with bee diseases and colony collapse.

On the other hand, gut microbial association in honeybees has been identified with their potential metabolic roles in enzymes expression responsible for polysaccharides breakdown in diets [14,15]. Additionally, some bacterial strains in honeybee gut could competitively exclude pathogens in-vitro [16,17]. Therefore, colonization of the honeybee by lactic acid bacteria could help to prevent disease [18–20]. However, microbial community in the gut of honeybees have been suggested to be transmitted socially within the colony through interactions with the different hive components as well as fecal materials [21,22]. 23, also suggested that bacterial species are transmitted horizontally from crop species to honeybee hives during foraging.

The importance of beekeeping to society is enormous. According to 24, the primary insect species responsible for crop plant pollination around the world is *Apis mellifera*, which is also domesticated for the production of wax, honey, and royal jelly. Beekeeping has been practiced in different parts of Nigeria. However, there have been speculations that the continued existence of Nigerian honeybee colonies is related to the environment they live in. Similarly, bacterial and fungal associations with honeybees have been used as an indication of how healthy the honeybee colony is [13]. The report of 25, also affirmed that gut microbial load count and digestive enzymes are useful tool of evaluating the health status of invertebrate organisms. Therefore, in an attempt to monitor the health of the Nigerian honeybee, *Apis mellifera adansonii*, gut digestive enzymes and gut and body surface microbial (fungal and bacterial) association of honeybee workers from the rainforest, guinea savannah, and derived savannah zones of Nigeria were evaluated in this study.

**Materials and methods**

**Study locations**

This study was conducted on three major ecological zones of Nigeria. These are the Derived savannah zone (Kwara state), rainforest zone (Ogun state) and Guinea savannah zone (Oyo state). A total of six apiaries consisting of two apiaries each from the three ecological zones were selected and used for this study. These were based on the suitability of apiary to the desired ecological zone and approved consent by the apiary owners. Suitability was used to denote a fully established apiary with active colonies and situated within the desired ecological zone. The map showing the study apiaries are shown in (Figure 1).

**Samples collection**

A modified method of 13, was used for honeybees’ collection. The collection was made using a round-shaped 1-liter volume plastic container with a diameter of 14.5 and 7.5 cm height. Honeybee hives were gently tapped and the plastic container was used to trap the honeybees from the entrance of the hive as the honeybees rushed out of the hives. About two hundred honeybees were collected and transported to the laboratory in ice box. Collected honeybee samples were sorted carefully to remove any incidence of non-worker castes. A total of fifty-four (54) honeybee colonies were used in this study, comprising of eighteen (18) colonies each from the rainforest, guinea savannah and derived savannah zones of Nigeria during the onset of rain, wet season and dry season periods. Gut digestive enzymes
and microbial analyses were conducted separately per colony of honeybees from each of the ecological zones during each of the study periods.

**Gut enzymes analysis of the bees**

The gut of honeybees was dissected using sterile scissors following the method described by 26. In order to prepare the enzyme extract, 1 g of honeybee guts was grounded in ceramic mortar at 5 °C on ice with 20 ml 1/10 M sodium acetate buffer pH 5.0 the buffer extract centrifuged at 2°C for 30 minutes at 18,000 g. From enzyme extracts, activities of amylase were determined using the methods of 27 and 28. Lowrey folin-ciocalteu method (29) was used to determine proteinase activity while 30, method was used for lipase activity determination. The method used by 31, was adopted in the quantification of α – glucosidase activity.

**Microbial analysis**

**Body surface**: One gram (1 g) of whole body (with all appendages intact) of honeybees was carefully weighed into sterilized bottles containing 9 ml of sterilized water. One gram of whole body samples of honeybees contained about 10 to 15 honeybee individuals depending on the ecological zone and season of collection. These were properly shaken. The mixture was further processed into the serial dilution folding for the microbial evaluation using pour plate method of the honeybee body surface.

**Gut**: The body surface of each honeybee was disinfected by swabbing with iodine (25 mg/L) followed by 70% ethanol to avoid contamination of the gut with external microbes [13]. The dissection of the honeybee gut was carried out with sterile scissors following the method previously described by 26. Total gut of two honeybees were homogenized in 1 ml of sterilized water and carefully
transferred into a sterilized bottle containing 9 ml of sterilized water. Gut microbial analysis was conducted in three replications per honeybee colony in each of the ecological zones.

All glassware including Petri-dishes, conical flasks, test tubes, beakers, pipette, and bottles used for the experiment were thoroughly washed with detergents and sterilized in an autoclave at 121°C for 15 min. Work bench was also frequently sterilized with 90% ethanol. Potato dextrose agar, plate count agar, and nutrient agar were used and prepared based on the manufacturers’ prescription. Serial dilution methods, bacteria and fungi identification methods used by 25, were also adopted for this study.

**Statistical analyses**

Data analyses were done using SPSS (Statistical Package for Social Sciences) version 20.0 [32]. Means were compared using the multivariate analysis of variant (MANOVA). Ecological zones and the seasonal periods were used as grouping factors. Results were presented as mean±standard deviation. Post hoc test was done using the Student-Newman-Keuls (SNK). Probability value less than 0.05 (p < 0.05) was considered to be statistically significant.

### Results

**Gut digestive enzymes activity**

Results showed that gut activities of amylase and α-glucosidase were not significantly different in the honeybees from the three ecological zones during the onset of rain period (Table 1). On the other hand, gut activities of amylase and α-glucosidase were significantly lower in the honeybees from the rainforest zone than those from the guinea savannah and derived savannah zones during the wet season period. During the dry season, however, honeybees from the guinea savannah zone had significantly higher gut activities of amylase and α-glucosidase. These were not significantly different in the honeybees obtained from the rainforest and derived savannah zones. Gut activities of lipase and proteinase were however not significantly different in the honeybees from the three ecological zones during the onset of rain, the wet season, and dry season periods. Also, seasonal interactions only showed a significant effect on the gut activities of amylase and α-glucosidase of the honeybees.

**Microbial association of the gut and body surface**

**Microbial load count**

Bacterial count on the body surface and gut of the honeybees from the three ecological zones

| Table 1. Activities of some digestive enzymes (g/100 g) in the honeybees from three ecological zones of Nigeria. |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Amylase         | α-glucosidase   | Lipase          | Proteinase      |
| Onset of rain                    |                 |                 |                 |                 |
| RF                               | 45.56 ± 1.66a   | 36.49 ± 3.29a   | 13.97 ± 0.91a   | 21.58 ± 0.09a   |
| GS                               | 45.46 ± 4.63a   | 36.87 ± 3.15a   | 14.11 ± 1.77a   | 21.59 ± 0.74a   |
| DS                               | 44.54 ± 1.21a   | 35.69 ± 2.94a   | 14.02 ± 0.33a   | 22.64 ± 0.29a   |
| Wet season                       |                 |                 |                 |                 |
| RF                               | 44.62 ± 0.43b   | 33.31 ± 0.21b   | 12.64 ± 0.27a   | 19.85 ± 0.29a   |
| GS                               | 47.61 ± 1.46a   | 38.03 ± 1.37a   | 13.90 ± 0.62a   | 21.25 ± 0.70a   |
| DS                               | 47.38 ± 1.29a   | 36.82 ± 2.22a   | 13.85 ± 0.61a   | 21.10 ± 0.68a   |
| Dry season                       |                 |                 |                 |                 |
| RF                               | 42.24 ± 0.59b   | 30.91 ± 0.98b   | 16.53 ± 0.41a   | 20.92 ± 0.75a   |
| GS                               | 46.43 ± 1.46a   | 35.78 ± 3.39a   | 14.19 ± 1.80a   | 21.73 ± 1.92a   |
| DS                               | 42.29 ± 1.21b   | 31.53 ± 1.16b   | 15.67 ± 1.63a   | 21.79 ± 0.81a   |
| Seasonal interactions            |                 |                 |                 |                 |
| F value                          | 8.307           | 8.877           | 3.221           | 2.594           |
| P value                          | 0.01*           | 0.01*           | 0.06            | 0.08            |

*Means (±Standard deviation) in the same column for each of the seasonal periods having similar superscripts are not significantly different at p < 0.05; RF = Rainforest; GS = Guinea savannah; DS = Derived savannah; *Interaction significant at p < 0.05
was observed to be lowest during the dry season period (Table 2). The gut microbial load was also significantly higher in the honeybees from the rainforest zone during the onset of rain, the wet season, and dry season periods. On the other hand, no fungal count was recorded in the gut of the honeybees from the rainforest zone during the wet season period. Also, fungal load count was lowest in the gut of the honeybees from the rainforest zone during the onset of rain, the wet season, and dry season periods. Results also showed significant fluctuations in the fungal count load on the body surface of the honeybees from the three ecological zones during each of the onset of rain, the wet season, and dry season periods. However, statistical analysis showed no significant interaction between the seasonal periods and the gut and body surface microbial load counts of the honeybees.

**Bacterial diversity**

Diversity of bacterial species was also higher on the body surface of the honeybees than in their gut (Table 3). These bacterial species were observed to vary in the ecological zones during the seasonal periods. *Alcaligenes spp* was only found on the body surface of the honeybees from the guinea savannah zone during the wet season period and the dry season period. Similarly, *Aerobacter aerogenes* was also present only on the body surface of the honeybees from the derived savannah zone during the wet season and the dry season periods. *Bacillus spp* was not found on the body surface of all the honeybees collected during the wet season period. *Bacillus spp* was however present on the body surface of the honeybees from the rainforest and guinea savannah zones during the onset of rain and the dry season periods as well as on the body surface of honeybees from the derived savannah zone during the onset of rain period.

During the onset of rain and the dry season periods, only the gut of the honeybees from the guinea savannah zone contained *Bacillus spp*. However, *Bacillus spp* was present in the gut of the honeybees from the three ecological zones during the wet season period. Also, *Pseudomonas spp* was only present in the gut of honeybees from the rainforest zone during the onset of rain and the wet season periods. *Micrococcus spp* was only present in the gut of honeybees from the derived savannah zone during the dry season period.

**Fungal diversity (Yeast and mold)**

No yeast isolate was identified in the gut of honeybees from the rainforest zone during the wet season period (Table 4). However, yeast

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Table 2. Microbial load count of the body surface (x 10³ cfu/ml/g) and gut (x 10³ cfu/ml/honeybee) of honeybees from three ecological zones of Nigeria.

|                  | Bacterial load |               | Fungal load |               |
|------------------|---------------|---------------|-------------|---------------|
|                  | Body surface  | Gut           | Body surface| Gut           |
| Onset of rain    |               |               |             |               |
| RF               | 6.00 ± 0.80a  | 2.35 ± 0.10a  | 0.90 ± 0.70b| 0.15 ± 0.10b  |
| GS               | 4.90 ± 1.90b  | 1.175 ± 1.95b | 0.96 ± 0.36b| 2.20 ± 0.40a  |
| DS               | 4.15 ± 1.05b  | 0.55 ± 0.10b  | 4.40 ± 0.40b| 0.15 ± 0.10b  |
| Wet season       |               |               |             |               |
| RF               | 3.95 ± 0.95b  | 2.25 ± 1.70a  | 8.30 ± 1.50a| 0.00 ± 0.00b  |
| GS               | 6.80 ± 3.00a  | 1.73 ± 1.65b  | 5.86 ± 2.94b| 0.65 ± 0.50a  |
| DS               | 5.56 ± 0.24ₐ  | 0.63 ± 0.05ₜ  | 0.80 ± 0.40ₜ | 0.70 ± 0.60ₜ  |
| Dry season       |               |               |             |               |
| RF               | 3.00 ± 1.20ₐ  | 0.73 ± 0.15ₜ  | 1.78 ± 0.02ₜ | 0.39 ± 0.07ₜ  |
| GS               | 1.46 ± 0.24ₐ  | 0.30 ± 0.20ₜ  | 0.73 ± 0.30ₜ | 1.55 ± 0.10ₜ  |
| DS               | 1.96 ± 0.74ₐ  | 0.45 ± 0.10ₜ  | 0.64 ± 0.40ₜ | 0.55 ± 0.10ₜ  |
| Seasonal interactions | F value | 1.578 | 1.561 | 2.847 | 0.151 |
|                  | P value       | 0.26 | 0.26 | 0.11 | 0.86 |

a,bMeans (±Standard deviation) in the same column for each of the seasonal periods having similar superscripts are not significantly different at p < 0.05; RF = Rainforest; GS = Guinea savannah; DS = Derived savannah; *Interaction significant at p < 0.05
Table 3. Bacterial diversity of the body surface and gut of honeybees from the three ecological zones of Nigeria.

| Onset of rain | Body surface | Gut |
|---------------|--------------|-----|
| Wet season    | RS Streptococcus pyogenes, Bacillus spp, Proteus spp, Micrococcus spp, Enterobacter spp | Salmonellae spp, Pseudomonas spp |
|               | GS Bacillus spp, Enterobacter spp, Pseudomonas spp, Streptococcus Pyogenes, Micrococcus spp | Salmonellae spp, Bacillus spp |
|               | DS Salmonellae spp, Pseudomonas spp, Streptococcus pyogenes, Bacillus spp, Micrococcus spp | Salmonellae spp |

| Dry season    | RS Serratia spp, Micrococcus spp, Enterobacter spp, Pseudomonas spp, Serratia spp | Bacillus spp |
|               | GS Citrobacter spp, Micrococcus spp, Serratia spp, Alcaligenes spp, Proteus spp | Bacillus spp, Pseudomonas spp, Citrobacter spp |
|               | DS Citrobacter spp, Streptococcus Pyogenes, Pseudomonas spp, Proteus spp, Aerobacter aerogenes | Aerobacter aerogenes, Bacillus spp, Salmonellae spp |

| RF = Rainforest; GS = Guinea savannah; DS = Derived savannah |

Table 4. Fungal diversity (yeast and molds) of the body surface and gut of honeybees from the three ecological zones of Nigeria.

| Onset of rain | Yeast isolate | Mold isolate |
|---------------|---------------|--------------|
| Wet season    | RF Candida kruzei | Candida albicans, Candida tropicalis, Candida krusei | Rhizopus spp, Fusarium solani, Aspergillus fumigatus, Trichoderma viridae |
|               | GS Candida albicans, Candida krusei | Candida albicans | Nil |
|               | DS Candida albicans, Candida krusei | Candida albicans | Nil |

| Dry season    | RF Candida albicans, Candida krusei | Candida albicans, Candida tropicalis, Candida krusei | Rhizopus spp, Fusarium solani, Aspergillus fumigatus, Trichoderma viridae, Fusarium solani, Apophysomyces elegans |
|               | GS Candida albicans | Candida albicans | Nil |
|               | DS Candida albicans, Candida krusei | Candida albicans | Nil |

| RF = Rainforest; GS = Guinea savannah; DS = Derived savannah; Nil = Not detected |

isolate of the genus Candida was the only identified yeast on the body surface and in the gut of the honeybees from the three ecological zones regardless of the seasonal period. These isolates are Candida albicans, Candida krusei and Candida tropicalis.

On the other hand, there were no mold species identified in the gut of the honeybees from the three ecological zones during the onset of rain, wet season and dry season periods. Similarly, no mold isolate was identified on the body surface of the honeybees from the derived savannah during the onset of rain period and those from the rainforest during the dry season period. However, a total of eight mold species were identified on the body surface of the honeybees from the three ecological zones during the study period. These include Rhizopus spp, Scedosporium apiospermum, Paecilomyces spp, Fusarium solani, Aspergillus fumigatus,
Trichoderma viridae, Apophysomyces elegans, and Aspergillus nidulans.

Discussion

An array of microbial diversity was isolated from the body surface and gut of honeybees used in this study, regardless of the ecological zones or seasonal periods. Previous studies from other locations also showed that the honeybees could be associated with various microorganisms either on the body surface and alimentary canal/gut, bee bread, and the honey comb [13,17,23,33].

Although, 34, argued that honey production in honeybee colonies is not likely to increase as a result of probiotic bacteria intake by honeybee foragers, 35, reported that microbial species have been linked to digestive enzymes produced in the gut of honeybees. Some potential metabolic contributions of the gut microbial association in honeybees have also been identified in the expression of enzymes responsible for the degradation of complex polysaccharides in the diets of honeybees especially enzymes not produced by the honeybees [14,15]. 14, therefore classified the honeybee gut microbiome to contain glycoside hydrolases and polysaccharide lyases. This could be the reason why amylase and α-glucosidase had the highest concentrations in the gut of the honeybees from the three ecological zones in this study. According to 36, the role of amylase and saccharase is in the degradation of complex carbohydrates into monomers whereas glucose oxidase assists in the preservation of honey through the production of hydrogen peroxide in small amounts. Similarly, these enzymes were reported to be involved in disaccharide sucrose hydrolysis to glucose and fructose thereby supporting the breakdown of common sugars present in nectar [37].

Results of this study also showed that honeybee guts is made up of a specialized microhabitat of enriched activities of carefully selected microbes. For example, bacteria diversity was lower in the gut of the honeybees than the body surface. Also, despite the wide diversity of molds on the body surface of the honeybees, no species of mold was isolated in the gut of the honeybees from the three ecological zones during the study period. In a previous report, 13, also established differences in microbial load count and diversity of the gut and body surface of honeybees. Thus, it is possible that the honeybees could naturally pick up or exclude some microbial species from their gut when the need arise.

Among the microbial species identified in the gut and on the body surface of the honeybees from the three ecological zones are the Bacillus spp, Aspergillus sp and Rhizopus sp. These microbial species were earlier reported as common microbes of the honeybee comb, body surface and gut of honeybee [13]. In their report, 13, associated these microbial species with honeybee brood diseases and Colony Collapse Disease (CCD). According to 38, bacterial spores, especially of the genus Bacillus is often present in honey. The EU 3940, also pointed out that the Bacillus spp have not been linked to food poisoning, although it is a key organism responsible for the spoilage of foods and food products due to their production of heat-resistant spores and versatile metabolism. The principal sources of microorganisms in honey as reported by 40, are the nectar of the flowers visited by the honeybee. Hence, the honeybees could have picked up these microbes from the nectar during foraging activities.

Conclusion

This study has shown that honeybee workers, regardless of the ecological zones or seasonal periods harbor microorganisms on their body surface and in their gut. Findings of the study, however, suggests that the microflora of honeybees gut were cautiously selected to meet
a particular need which most likely could be to compensate for the digestion of some of its food substrate.

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The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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Declarations

Ethics approval and consent to participate: This research was done in compliance with the ethical standard set by the College of Biosciences of the Federal University of Agriculture, Abeokuta on the use of ‘Economic Insects’ for research study (Reference number PG-11/0216/004/2017).

Consent for publication: Not applicable

Availability of data and materials

The data set analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution

BJA and IAB conceived the idea. BJA, IAB and AKO collectively harmonized the research proposal. OAA together with BJA defined the field collection methods went to the field for honeybees sample collection.

AAO conducted microbial analysis in her laboratory. AKO assisted in the laboratory and collated literatures for the paper. BJA wrote the results and collated the manuscript. IAB gave technical advice all through the period. All the authors made financial contributions. All authors have read and approved the manuscript.

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