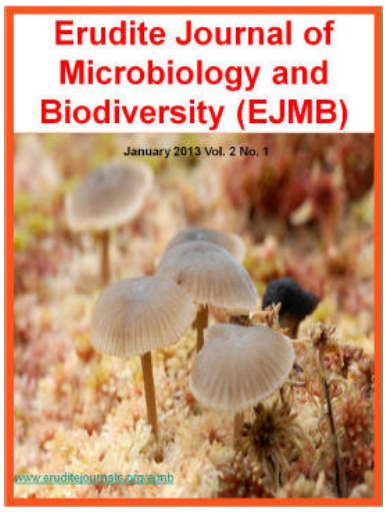
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Isolation and Identification of microorganisms in comb and body parts of wild and domesticated honey bees of two ecozones of Nigeria

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Abstract

The survival of the honey bee depends on the successful defense against microbial invaders, parasites and predators. This research was carried out to isolate and identify microorganisms associated with the honeybees of two ecological zones of Nigeria. Thirty adult worker honeybees were collected from the wild, modern and traditional beekeeping methods and analyzed for microbial presence. The body and bee comb were swabbed using sterile normal saline moistened swab stick. The swabs were diluted, homogenized and cultured for microbial growth. The contents of the fore, mid and hind guts were emptied, homogenized and cultured. 1ml of homogenized sample was diluted, from this into four sterile dilutions of $^1/_{20}$ $^1/_{40}$ $^1/_{80}$ and $^1/_{160}$. 1.0 ml each of dilutions which was inoculated into molten Sabourand dextrose Agar (SDA) containing tetracycline for fungi growth, while nutrient agar and De Ma Rogossa Sharbroth (MRS) agar were inoculated for the bacteria. SDA plates were incubated at 30° C for 3 days while SDA and MRS were incubated at 37°C overnight. The total number of colonies forming units (CFU) per plate was counted and was correlated to the dilution factor. Each different colony was identified using morphological and biochemical tests. The microbial examination of the comb, body and gut of the bees showed the presence of six moulds and fungi: *Mucor hiemalis, Penincillium frequentans, Aspergilus repens, Rhizopus stolonifer, Geotrichums* sp. and *Botryotricum* sp. and three bacteria species in the three beekeeping methods in both ecological zones. The species of the bacteria encountered were: *Staphylococcus aureus*, *Streptobaccillus* sp. and *Baccillus pulyifacien.* Some of the isolated microbes such as *Mucor hiemalis, Aspergilus* species and *Rhizopus* species have been implicated as causative organisms of chalkbrood disease of bee. Further research is been suggested for the roles of the isolates in much dreaded CCD.

Key words: Honeybee, Isolates, Microorganisms, Bee diseases, Ecozones

INTRODUCTION

There are many threats to bee survival, including the risk of disease caused by microorganisms. The vast majority of our knowledge of bee diseases focus on the honey bee, *Apis mellifera,* although there are actually over 20,000 other species, both stingless and stinging, from those with solitary lifestyles to complex societies such as honey bee hives.

Viruses, fungi, protozoa and bacteria are all known to cause infections in bees, sometimes leading to collapse of colonies, and causing serious threats to the bee-keeping industry (Glinski, and Buczek, 2003). Bees have two distinct life forms, brood (egg, larva and pupal stages which develop within the hive) and adult. Most diseases are specific to just one of these life stages. While the list of diseases is quite long, only a few are of serious concern to apiculturists (Chrles, 1995; Glinski and Jarosz, 1992).

Chalkbrood is usually considered a minor disease of bees, as is stonebrood, caused by the fungus *Aspergillus* (Glinski and Buczek, 2003). The high density populations and other conditions within the bee colony (such as enclosed, moist, dark, poorly ventilated) are ideal for the outbreak and spread of diseases (Jacob, 1993). Although bees are constantly exposed to pathogenic microorganisms, they have evolved strategies to resist infection (Nasr, et al., 2001).

The cuticle of bees acts as a barrier to penetration, and immune system based defense can prevent infection of many minor pathogens. However, the recent completion of the honey bee genome sequence has shown that they have only about a third of the number of known immunologically related genes when compared to houseflies or mosquitoes, suggesting that bees rely less on individual immunity than most insects (Jarosz, 1997; Jarosz, 1993). Bees, in common with a number of other social insects, have well developed behavioural responses to combat disease. These behavioural responses are collectively known as hygienic behaviour and include recognition and removal of diseased brood by worker bees. Bee species, and even different hives of the same species, differ in their ability to perform hygienic behaviours, with some colonies far superior to others (Rilter et al., 1987).

The disease caused by the spore-forming bacterium *Paenibacillus larvae,* was first described in 1769. Acid Fast Bacillus (AFB) is probably the most virulent disease of honey bee brood and is capable of causing the collapse of bee colonies. The name describes the symptoms of diseased brood; infected cells become discoloured, sunken and there is a characteristic smell. Adult bees are not affected. This disease has traditionally been treated with the antibiotic such as oxytetracycline, but some bacterial strains have developed resistance to this and the disease is now increasing in prevalence around the world (Taber, 1993). Although the disease was first described over 200 years ago, much is still unknown about this disease (Taber, 1993).

A German research team has only recently discovered how the bacteria kill larvae, by building up to very high numbers in the gut before bursting into the haemocoel, causing death (Chen and Siede, 2007). European foulbrood (EFB) is caused by the non-spore-forming bacterium *Melissococcus* (*Streptococcus*) *plutonius*. Unlike AFB, EFB usually affects unsealed brood, and the recently dead larvae present as watery and yellowish brown cadavers twisted inside the cell (Rilter et al, 1978). Despite the importance of EFB, the disease is poorly understood, but like AFB, has increased in prevalence in recent years (Wilkins et al., 2007). *Ascosphaera apis* is the causative agent of the well known chalkbrood disease in honey bees, so called because of the chalky appearance of infected brood. Chalkbrood is usually considered a minor disease of bees, as is also stonebrood, caused by the fungus *Aspergillus (*Snowdown and Cliver, 1996). Viruses can also cause devastation in bee colonies. At least 18 types of viruses have been found infecting honey bees alone. Going by some delightfully descriptive names (e.g. deformed wing virus, chronic paralysis virus, acute bee paralysis, sacbrood virus and black queen cell virus), these viruses range from non-lethal to causing significant mortality in nests. One of the more interesting aspects of viral disease is that many infections have no obvious symptoms. Of the fungi known to infect bees, species of the fungus *Ascosphaera* are the most common.

Microbes are constantly evolving, leading to the emergence of new strains with novel pathogenic abilities. For example, some honey bee diseases appear to have widened their host range in recent years (Morse, 1990). Protozoa of the genus *Nosema* infect many invertebrates, and individual species are typically quite limited in their host range. *Nosema apis* has long been recognized as causing one of the most important diseases in adult honey bees, infecting the guts of adult bees. However, *Nosema ceranae*, thought to infect only the Asiatic or Eastern honey bee, *Apis cerana*, has recently been shown to infect the European honey bee, *A. mellifera*. Evidence is emerging of recent on the spread of *N. ceranae* in honey bee populations around the world since around 1998 (Wilkins et al., 2007). There is ongoing risk that other highly virulent diseases of honey bees will emerge.

There is still so much we don't know about how combinations of microbial diseases, parasites, pollution and urbanization are affecting bees. Colony Collapse Disorder (CCD) is the name given to the recent widespread mortality of worker (adult) honey bees on several continents, especially North America. However,

startlingly high mortality rates have not been attributed to a particular cause. Several recent studies suggest that some colony collapse is caused by a combination of disease and the parasitic infections of *Varroa* mites (Chen, and Siede 2007). Israeli Acute Paralysis Virus (IAPV) was recently found to be the most consistent indicator of CCD, as well as Kashmir bee virus and *Nosema* spp. However, no causal link has been made between IAPV and CCD (Glinski and Jarosz, 1998; Omoya and Akharaiyi 2010).

As with all living things, stress increases the susceptibility of the host to a pathogen, and if bees are under stress, disease can be more debilitating. Whether CCD is only caused by the interaction between a specific stress such as *Varroa* and some other diseases is unclear. Combinations of stresses could include multiple diseases. Using molecular techniques, several studies have shown that multiple diseases infecting single bees. So diseases, some of which may not normally cause death, could act together to kill. Additionally, nutritional stress can exacerbate the incidence of pathogens (Omoya and Akharaiyi 2010). Very few works have been done in this geoecozone on organisms associated with diseases of bees. Information on features and pattern of disease causing organisms in this zone would help the bee keepers on protective measures. This study is aimed at isolating and identification of potential pathogens in body, hives and anatomical parts of bees of geoecozone of Kwara State, Nigeria.

METHOD

Thirty honeybees were collected (10 each from the wild, modern and traditional beekeeping sites) using sweep nets and the bees were killed with chloroform. The body and the bee comb were swabbed using sterile normal saline moistened swab stick. The swabs were diluted and homogenized bees from each of the habitats and passed through microbial studies. The body of each bee was disinfected by swabbing with iodine followed by 70% ethanol to avoid contamination of the gut with external microbes the dissection of the bee gut was carried out with sterile scissors following the method previously described by Youdeowei (1974). Like all other invertebrate, insects are always dissected from the dorsal side, this is because the nerve cord lies ventrally and this exposes the required internal parts. For the dissection to expose the gut, the parts were dismembered to make observation and subsequent culturing easier. The contents of the fore, mid and hind guts were emptied using flame sterilized forceps. The contents were homogenized separately and culture on appropriate media.

One ml of homogenized sample was diluted in 9 ml of sterile normal saline. From this, four sterile dilutions were further made to give. $^1/_{20}$ $^1/_{40}$ $^1/_{80}$ and $^1/_{160}$ 1.0 ml each of dilutions which was inoculated into molten sabourand dextrose agar (SDA) containing tetracyline for bacteria growth, while nutrient agar and De Ma Ro (MRS) agar were inoculated for the bacteria growth. SDA plates were incubated at 30^{o}C for 3 day while NA and MRA were incubated at 37^{o}C for 3 days. The plates were prepared in duplicates. The plates were examined De Ma Rogossa Sharbroth (MRS) day for growth. These media were prepared following the manufacturer instructions.

The total number of colonies forming units (CFU) per plate was counted using colony counter and was correlated to dilution factor. Each different colony after counting were subcultured to obtain pure culture and were identified using morphological and biochemical methods as previously described (Cowen and Steel, 1998). Colony forming unit (CFU) growth on SDA were counted, sub-cultured on fresh new SDA and identified using staining morphology and bio chemical tests methods (Cheesbough, 1987; Mahon and Manuselis 1995)

RESULT

Distribution of the microorganisms on the comb, body and gut of honey bees of geo-ecozones of Kwara State.

The microbial examination of the comb, body and gut of the bees showed the presence of six fungi and three bacteria species in the three habitats in both ecological zones. The species of the mould encountered were *Mucor hiemalis, Penincillium frequentans, Aspergilus repens, Rhizopus stolonifer, Geotrichums sp.* and *Botryotricum sp.* while *Staphylococcus aureus, Streptobaccillus sp.* and *Baccillus pulyifacien* were the bacteria encountered (Table1.).

Microbial counts of the moulds

The colony forming units of the mould in each ecological zone were presented in Figures 1 to 4. In the Guinea savannah, the colony forming units (CFU) of the mould in the combs of the wild habitat, modern and the traditional beekeeping were 4.79 x10^4, 3.70 x10^4 and 3.89 x104 CFU/ml respectively while the CFU of the body of the bees from the wild habitat, modern and the traditional beekeeping were 3.61 x10^4, 3.17 x10^4 and 3.55 x10^4 CFU/ml respectively. The CFU of the gut from the wild habitat, modern and the traditional beekeeping were 5.19x10^4, 4.55x10^4 and 4.56x10^4 CFU/ml respectively. The CFU of the mould was significantly higher ($p<0.05$) in the comb, body and the gut from the wild habitat relative to modern and the traditional beekeeping (Figure 1).

Table 1: Microflora isolated from bee comb, body and gut of honeybee from three habitats at the study area

Moulds	Bacteria
Mucor sp.	Staphylococcus sp .
Penicillium sp	Streptobaccillus sp.
Aspergillus sp.	Baccillus sp
Rhizopus sp	
Geotrichums sp.	
Botryotricum sp	

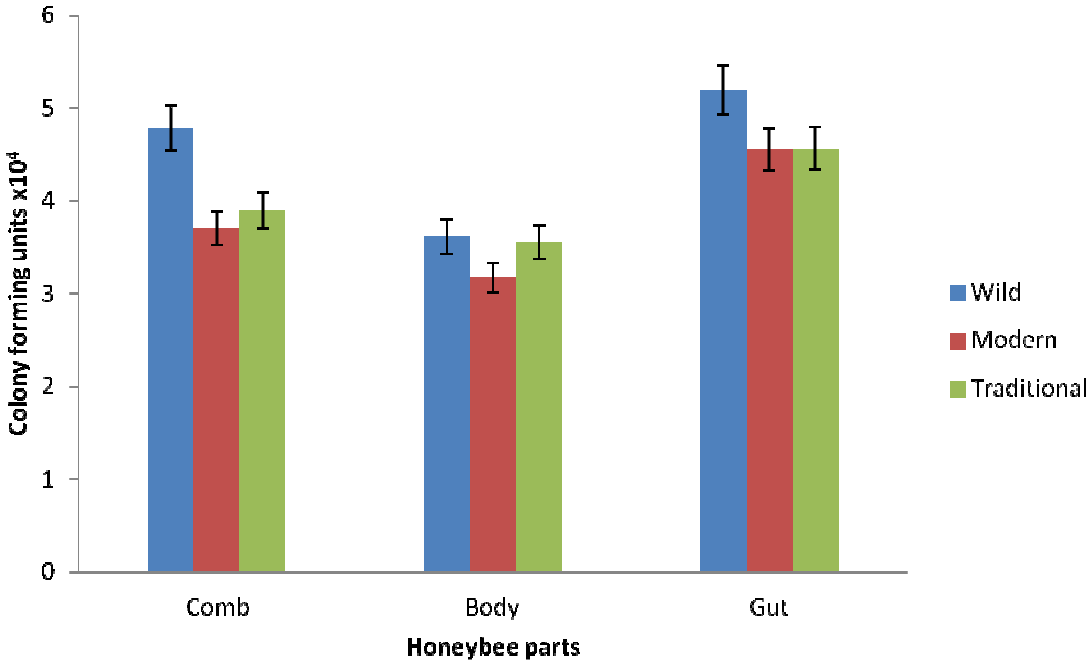


Figure 1. The microbial loads of the moulds isolated from the bees in the Guinea Savannah.

However, there was no significant variation in CFU of the comb and the gut of bees from the modern and the traditional beekeeping except the CFU of the bee body which differed significantly ($p<0.05$) in both habitats (Figures 2 and 3).

The CFU of the comb and the bee body and the gut varied significantly in the Derived Savannah and the highest CFU was found in the traditional beekeeping (6.37 $x10^4$ and 7.20 $x10^4$ CFU/ml) for comb and gut respectively) with the exception of the bee body from the wild which had the highest CFU (5.11 $x10^4$ CFU/ml) as compared with the two other habitats (4.78 $x10^4$ and 4.50 $x10^4$ CFU/ml for the modern and the traditional beekeeping respectively) (Figure 2). Though, the CFU of the comb, bee body and gut were higher in the three habitats in the Derived Savannah than the Guinea Savannah, the difference was not statistically significant ($p>0.05$) (Table 1).

Microbial counts of the bacteria

The results of the colony forming units (CFU) of the bacteria showed that bee body had the highest CFU of bacteria followed by bee gut; while comb had the least in both ecological zone (Figures.3 and 4). The CFU of the bacteria in the comb from the wild habitat, modern and the traditional beekeeping in the Guinea Savannah were 5.79 $x10^4$, 5.09$x10^4$ and 5.33 $x10^4$ respectively. The CFU of the bee body were 24.0 $x10^4$, 23.73$x10^4$ and 23.84 $x10^4$ for the wild habitat, modern and traditional beekeeping respectively while the gut had CFU of

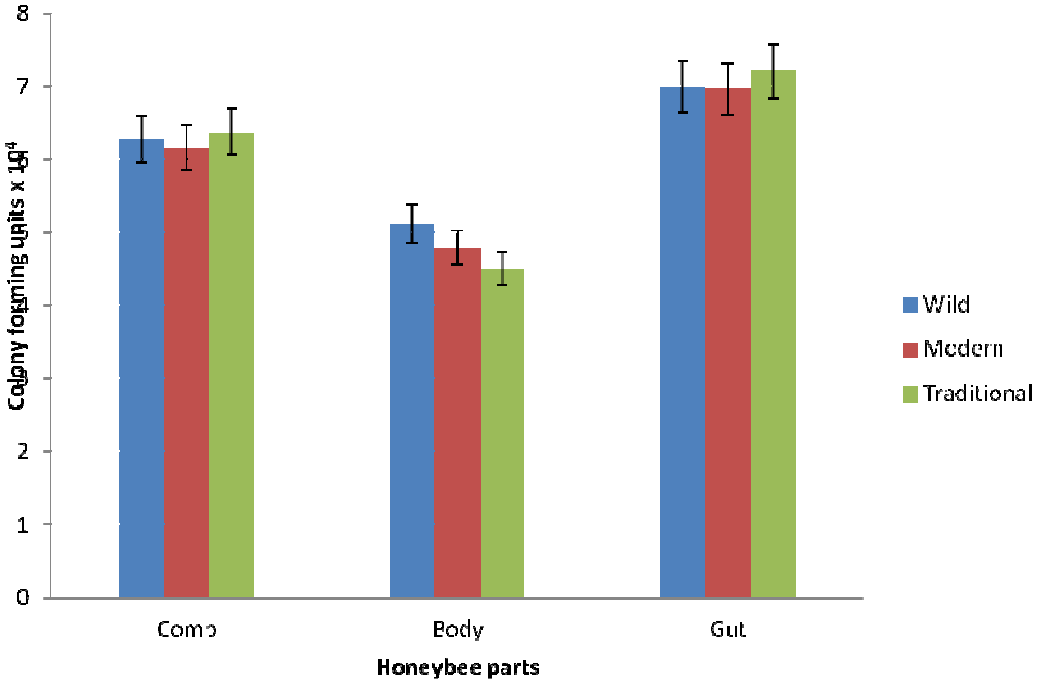


Figure 2. The microbial loads of the mould isolated from the bees in the Derived Savannah.

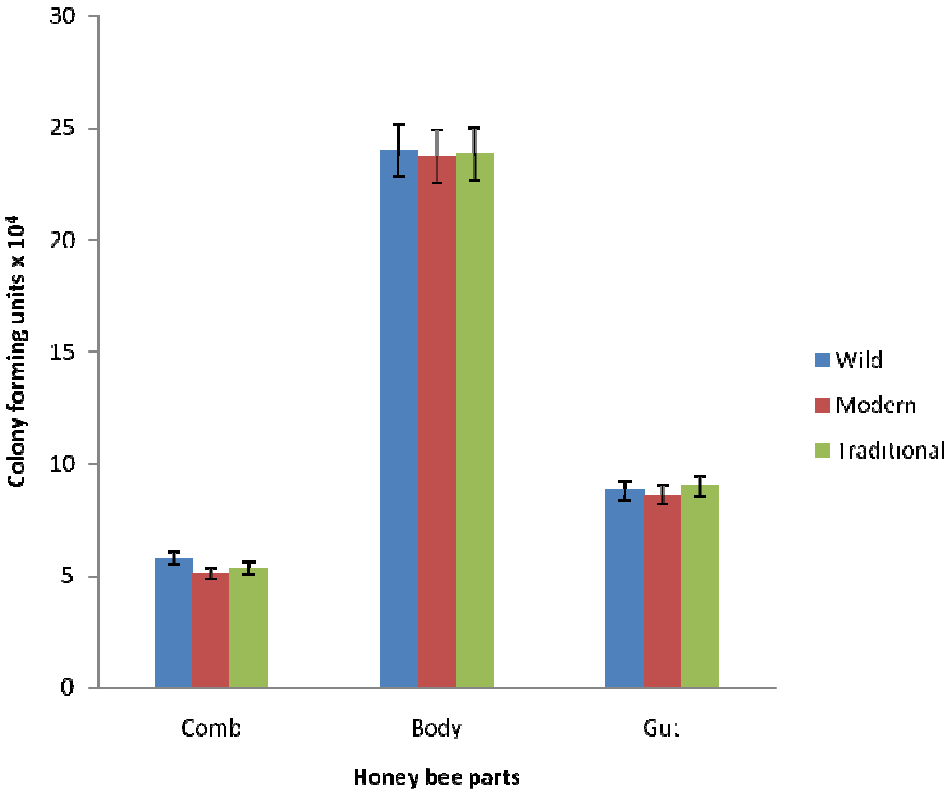


Figure 3. The microbial loads of the bacterial isolated in the bees in the Guinea Savannah

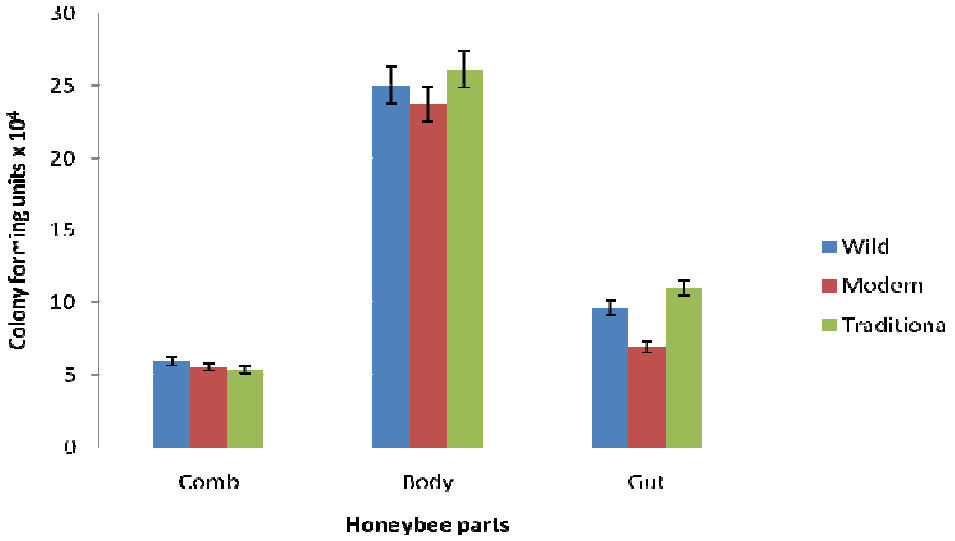


Figure 4. The microbial loads of the bacterial isolated in the bees in the Derived Savannah

Table 2. Distributions of moulds isolated from honeybees from Guinea and Derived Savannah.

Locations	Isolates	Wild bees		Modern reared		Tradition reared	
		GS	DS	GS	DS	GS	DS
Comb	MI,MII	2.90±1.9	4.90±	2.50±1.3	4.98±1.2	2.40±1.5	4.70±1.7
Body	MI,MII,MIII,MIV	2.3±1.3	3.31±1.8	2.01±1.2	3.39±1.4	2.00±1.6	3.51±1.0
Gut	MI,MII,MIII,MIV,MV,MVI.	3.30±1.9	5.30±1.7	3.10±1.5	5.37±1.6	3.11±1.5	5.80± 1.4

NB- MOULDS: MI = Mucor hiemalis, MII = Penincillium frequentans, MIII = Aspergillus repens, MIV = Rhizopus stolonifer, MV = Geotrichumus sp, MVI = Botryotricum sp. GS = Guinea Savannah, DS = Derived Savannah

Table 3. The P- values of the comparison of the microbial loads of the moulds between Guinea Savannah and the Derived Savannah for the bee habitats.

Honey bee parts	Wild habitat	Modern beehive	Traditional beehive
Comb	0.08*	0.15*	0.15*
Bee body	0.10*	0.12*	0.07*
Bee gut	0.09*	0.131*	0.14*

*Indicates significant difference at $p<0.05$

$8.83x10^4$, $8.61x10^4$ and $9.00x10^4$ in the wild habitat, modern and traditional beekeeping respectively (Figure 4.). Apart from CFU of the comb which showed significant difference in the three habitats, the bee body and the gut did not exhibit significant difference in the CFU at the three habitats. (Table 2, 3 and 5).

The CFU of bacteria in the comb, bee body and the gut differed significantly in the three habitats in the Derived savannah. The bee body and the gut recorded the highest CFU in the traditional beekeeping (bee body, $26.10\ x10^4$; gut, $11.00\ x\ 10^4$) followed by modern beekeeping (bee body, $23.72x10^4$; gut, $6.90x10^4$) and the wild habitat (bee body, $25.0\ x10^4$; gut, $9.59x10^4$) while the comb recorded the highest CFU in the wild habitat ($5.94x10^4$) (Figure 4). Table 4 presents the comparison of bacteria CFU in the two ecogeological zones. There were significant differences in the CFU of the comb and bee body in the two ecological zones. However, the CFU of the gut did not differ in the two ecological zones except in the wild habitat where significant variation was detected.

Table 4. Distributions of bacteria isolated from honeybees from Guinea and Derived Savannah.

Locations	Isolates	Wild bees		Modern reared		Tradition reared	
		GS	DS	GS	DS	GS	DS
Comb	BII	3.90±1.9	4.25± 1.7	3.60±1.5	4.15±1.4	3.84±1.5	5.25±1.7
Body	BI, BII	2.80±21.3	3.80±21.3	2.55±21.2	3.40±21.4	2.65±21.2	4.80±21.1
Gut	BI, BII,BIII,	6.30±2.6	7.30±2.3	6.22±2.4	7.50±2.5	6.20±2.8	8.30± 2.7

NB- BACTERIA: BI = Staphylococcus aureus, BII = Streptobaccillus sp, BIII = Baccillus pulvifacien. GS = Guinea Savannah, DS = Derived Savannah

Table 5. The comparison of the microbial loads of the bacterial isolated from the bees in three habitats between Guinea Savannah and the Derived Savannah.

Honey bee parts	Wild habitat	Modern beehive	Traditional beehive
Comb	0.008*	0.027*	0.001*
Bee body	0.013*	0.001*	0.029*
Bee gut	0.026*	0.070*	0.063*

* Indicates significance difference at $p<0.05$

DISCUSSION

The similarity in the species composition of the microorganisms in the three habitats in the two geo-ecological zones could be a reflection of the microorganisms found around the environment of the bees of the study areas. Hunt and Charnley (1981) and Idowu and Edema (2002) reported that the microorganisms in the guts of the insect usually indicate the types and numbers of microorganisms in the environment as microbes normally get into the gut of the insect along with the food. The microorganisms would have been picked from the field and transferred onto the combs in the hives through body contact and regurgitation. The variation in microbial load in the three habitats and between the two ecological zones could also be attributed to the numbers of the microorganisms in the diets of the bees. The microorganisms encountered in this study may be of both beneficial and detrimental importance. Microbiota had been known to be of high nutritive potentials and is rich sources of macronutrients such as lipid, protein and carbohydrate (Martin and Kukor, 1984; Idowu and Edema, 2002). Apart from nutritive values, some of the microorganisms encountered have been linked with the production of digestive enzymes which aids food digestion (Idowu and Edema, 2002; Idowu et al., 2009). Honeybees are phytophagus insects and utilize different plant hosts during foraging (Omoloye and Akinsola, 2006; Akinwale and Badejo, 2009). Most of these plants have secondary compounds which need to be digested by extracellular enzymes. Similar observation had also been reported in *Zonocerus variagatus*, cockroaches, locusts and termites that also use the microorganisms in their guts to digest cellulose and cyanogenic glucosides (Idowu and Edema, 2002; Ohkuma, 2003; Delalibera et al., 2005; Idowu et al., 2009).

Previous studies have implicated some isolates in some honeybee diseases that are of concern to apiculturalists. Diseases of brood of bees are caused by several different fungi, the most common of these species are *Aspergillus sp* which affect the brood of all ages (US. Agric. Bulletin No 313). *Mucor hiemalis and Rhizopus sp* isolated from the bees in this study have been implicated in fungi diseases of honeybees (Burnside, 1935; Glinski and Buczek, 2003). *Baccillus sp* has also been implicated in bacterial bee diseases (Glinski and Buczek, 2003).

The result of this study revealed the presence of six fungi of which three *Mucor hiemalis*, *Aspergillus* and *Rhizopus sp* are implicated in both brood and adult bee diseases such as chalk brood while *Baccillus sp* has been implicated in chalkbrood a bacterial disease of bee. Further research is recommended to understudy the possible implication of the isolated and identified species of microorganisms in bee stress and challenges posed by Colony Collapse Disease (CCD).

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