Isolation and Characterization of Bacteria from the Intestinal Tracts and Nests of Soil-Feeding and Fungus-Cultivating Termites

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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Peter Mochama Onkware

This thesis has been submitted for the examination with our approval as university supervisors.

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Signature…………………………....…….. Date…………………………..

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DEDICATION

To my wife, Sabina who tireless stood by me during the most trying times in life, my daughters Lucy and Alice, my late son Barack; may your soul rest in eternal peace; my dear parents, brothers; Dr. Kennedy Onkware, Tom, Mokua and Atogo and Sisters; Rose and Isabella. Thank you for all the support you offered me financially and morally when I needed you most.

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ABSTRACT

The intestinal tract of soil feeding and fungus cultivating termites harbour a dense community of prokaryotes. Majority of the termite gut microbiota have not been cultured. Termite nests and guts may provide novel bacteria with biotechnological potential. The study focused at isolating and characterizing bacteria from parent soils nests and guts of soil feeding and fungus cultivating termites, to assign the possible roles of the bacteria to nutritional physiology of termites and to assess the ability of the isolates to produce bioactive substances. *Cubitermes ugandensis* Fuller were collected from Kakamega rainforest in Western province Kenya and *Macrotermes michaelseni* Sjöstedt were collected from Juja in Central Province of Kenya. A total of two hundred and eighty six isolates were obtained. There was no significant effect of the pH (F=4.164) and different media (F=0.871) on the isolation of bacteria from termites. The isolates were screened for the ability to produce enzymes and antibiotics. Fifty isolates with ability to produce bioactive compounds were obtained and were characterized further. A combination of biochemical and morphological characteristics, distinguishing 16s rRNA gene sequences and phylogenetic analyses based on 16s rRNA genes provided strong evidence that isolates belong to the domain Bacteria and class Firmicutes and shared sequence similarity of 86-97% with known members of classes Clostridia, Enterococci, Bacilli and Carnobacteria. Based on results obtained from this study, it can be speculated that the isolates play digestive roles in termites especially protein and carbohydrate hydrolysis.

CHAPTER ONE

1.0 INTRODUCTION

Termites are terrestrial social insects that belong to the order Isoptera (Nutting, 1990). The order Isoptera contains five (5) families of lower termites and one (1) family of higher termites (Noirot, 1992) and contains over 26,000 described species (Ohkuma *et al*., 2001). Lower termites comprise of Mastotermitidae, Kalotermitidae, Hadotermitidae, Termopsidae, Rhinotermitidae and Serritermitidae. The higher termites consist of Termitidae (Krishna, 1989). The diet of lower termites is restricted to sound wood i.e.wood that is not decayed or nearly sound wood (wood that is decayed) and grass (Noirot, 1992)

The higher termites belong to the family Termitidae constitute approximately 75% of all termite species (Breznak, 1983). They harbour dense populations of gut bacteria but lack cellulolytic protozoa. Their digestive processes remain obscure although they rely partly on their own digestive enzymes including cellulases and symbiotic gut microorganisms (Breznak, 1983; Wood and Johnson, 1986). These termites colonize various biotypes due to their different dietary demands. Most of these thrive onrelatively refractory nitrogen poor resources i.e. lignocellulolytic plant material.

These include soil feeding, wood –feeding grass feeding and fungus - cultivating termites (Noirot, 1992).

Termites play vital roles as mediators of decomposition (Wood and, Johnson, 1986), humification, soil conditioning, and fragmentation of organic detritus, nitrogen fixation, aggregate binding and formation of clay-mineral complexes (Lee and wood, 1971; Collins, 1983; Tayasu *et al*., 1994).

Soil-feeding termites make up 62% of the genera of higher termites (Noirot, 1992) and 50% of all the termite species (Brauman *et al*., 2000). The digestive tract of soilfeeding termites harbours a dense and varied microbiota (Brune, 1998; Brauman *et al*., 2000), which are thought to be involved in the transformation processes inside the *t*ermite gut (Bignell *et al*., 1980). However the exact composition and role-played by the microbiota in the digestive tract of soil-feeding termites is poorly understood.

Most studies have focussed on wood feeders; analogous studies on other feeding guilds, especially soil feeders remain sparse owing to their habitat, delicate nature and the difficulty of establishing permanent laboratory cultures (Bignell *et al*., 1980;

Rouland *et al.*1993). Studies of soil-feeding termites, especially their functional role in digestion are still lagging behind (Brune; 1998).

Enumeration of microorganisms using 4,6-diaminoo-2-phenylindole (DAPI) staining techniques confirmed abundant colonisation of the guts *Cubitermes umbratus* and *C. ugandensis* with numerous filamentous organisms and bacteria (Miambi *et al*., 2006; Schmitt-Wagner *et al*., 2003). Numerous *Streptomyces* spp. from the gut, parent soil and mound materials were isolated (Bignell *et al*., 1991). Some cellulolltyic and lignin – solubilizing actinomycetes were isolated from several termites (Pasti and Belli, 1985; Pasti and Belli, 1990, Kuhnig and König, 1991). Watanabe *et al.* (2003) isolated and characterized actinomycetes from termite guts and established that actinomycetes isolated depended largely on geographical origin of the termite. For termites from the same area, taxonomic differences between the termites influenced the microbiota (Watanabe *et al*., 2003)

The symbiosis between the termite and fungi of the genus *Termitomyces* is crucial to the digestive process (Anklin-Mühlemann *et al*., 1995). Despite the symbiosis with the fungi,they are reported to harbour dense populations of bacteria and Archaea (Anklin-Mühlemann *et al*., 1995; Brauman *et al.,* 2001). They play an important role in the

degradation processes in the tropics (Buxton, 1981) but their symbiotic microbial community that plays a role in this process has hardly been studied. Earlier studies involved enumeration of bacterial cells and quantification of metabolites in the gut compartments of *Macrotermes subhyalinus* (Anklin-Mühlemann *et al*., 1995). In another study using group specific probes *Macrotermes subhyalinus* and *Macrotermes bellicosus* were also reported to harbour dense populations of bacteria and a smaller population of archaea (Brauman *et al*., 2001). Studies on microorganisms in *Macrotermes michaelseni*, a fungus – cultivating termite found in the savannah of tropical and sub-tropical Africa have only started. Groups of bacteria, which have been identified to be resident in the guts using molecular studies, include *Cytophaga - Flexibacter - Bacteriodes* (CFB), *Proteobacteria*, and gram-positive bacteria with low G + C content, clostridia. The observations were as result of two independent clone libraries by Mackenzie *et al*. (2006) Also consistently detected in the *Macrotermes* clone libraries are members of the *Anaerobaculum-Thermoanaerovibrio* i.e. synergistes and spirochetes, although only a few clones of the two groups were recovered.

The diversity of actinomycetes secondary metabolites is unrivalled and unmatched in medical significance (Magarvey *et al*., 2004). Structurally and functionally diverse bioactive compounds have also been isolated prokaryotes including the members of

myxobacteria and cyanobacteria as antibiotics with antimicrobial, antiviral, and antitumuor activities (Patterson *et al*., 1994: Reichenbuch 2001, Schawartz *et al.*1990: and Shimkets *et al.,* 2004). The rate of discovery of new compounds from the existing genera obtained from terrestrial sources has decreased, while the rate of reisolation has decreased (Magarvery *et al*., 2004). Moreover, the rise of number multidrug resistant pathogens and the limited success of strategies as such as combinational chemistry in providing new agents indicate uncertain forecast of future antimicrobial therapy (Projan, 2003 and Projan and Youngman, 2002). Thus it is critical that new groups of microorganisms unexplored be pursued as source of novel antibiotics and small – molecule therapeutical agents (Bull *et al*., 2000)

This study was designed to isolate and characterize novel bacteria from nests and guts of soil-feeding and fungus-cultivating termites. Isolates were characterized and identified and their ability to produce bioactive compounds (enzymes and antibiotics) investigated.

1.2 Statement of the problem

Termites harbour dense populations of gut bacteria yet as small percentage has been cultured. Little studies on the functional physiology of termite gut microbiota have been done. Their digestive processes remain obscure. Studies on the soil-feeding and

fungus cultivating termites, especially their functional role in digestion, are still lagging behind. Most studies have focused wood feeding termites Diversity of bacteria in termites has been investigated yet they hold a great potential that is still untapped .Investigations have focused on old and know producers of bioactive substances. Such should be diversified to include little or previously uninvestigated species of micro-organisms. With advent of multi-drug resistance pathogens there is need to search for novel bacteria with ability to produce antimicrobials from little or uninvestigated sources including termite nests and guts

1.3 Justification of the study

Most studies on termites gut microbiota have focused on wood feeders; analogous studies on the other feeding guilds especially soil feeders and fungus cultivating remain few. This is due to their typically remote habitat delicate in nature and the difficulty in establishing permanent cultures. Very little has been done to describe the bacteria of nests and guts of soil feeding and fungus – cultivating termites. To date only relatively few microorganisms have been isolated from termite guts. Very small numbers of microbiological studies have been performed on the subject, hence to perform more studies in the area. While characteristics of gut bacteria have been extensively studied by tracing the flow of carbon and nitrogen or characterizing isolated strains of bacteria using molecular techniques, the bacterial microbiota has remained a black box due to difficulties in cultivation of most of these bacteria. Very

little is known about the physiology of the larger proportion of microorganisms that have never been cultured cultivation.

There is pronounced difference among the microbiota not only between the gut and the ingested soil, but also among the different gut compartments. This study is aimed at isolating and characterizing bacteria from nests and guts of soil feeding and fungus cultivating termites. Soil as a source of bacteria with ability to produce bioactive compounds has been exploited thus search for novel bacteria with ability to produce novel bioactive substances has to be diversified to new and little or previously uninvestigated species in soil feeding and fungus cultivating termites. Termites could be a source of novel bacteria with biotechnological potential.

1.4 Objectives

1.4.1 General Objective

To isolate and characterize bacteria in parent soil, nests and intestinal tracts of soil feeding and fungus cultivating termites

1.4.2 Specific objectives

1. To enumerate, isolate and characterize bacteria in parent soil, nests and intestinal tracts of soil feeding and fungus cultivating termites

2. To assess the influence of media and incubation pH on numbers of microorganisms and diversity.

3. To assess the potential exploitation of isolates for production of bioactive metabolites and enzymes

4. To assign possible roles of the studied groups to the nutritional physiology of fungus-cultivating and soil feeding termites.

2.7 Hypotheses

1. Nests and guts of soil feeding and fungus cultivating termites are potential sources of bacteria for use in biotechnology.

2. There is no difference in the bacterial diversity in soils, nests and guts of soil feeding and fungus cultivating termites

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1Classification of termites

There are over 2700 described species that are remarkably diverse in the biology and nutritional ecology (Wood and Johnson, 1986; Myles, 2000). The order Isoptera contains five (5) families of lower termites and one (1) family of higher termites. The African continent is climatically and geographically diverse and contains the world's largest deserts and one of the greatest mountain peaks. Termite diversity also reflects this topological and climatological diversity. More than 1000 species of the over 2700 species recognized occur in Africa (United Nations Environment programme: UNEP, 2000). The most important genera include Macrotermes that belong to the family Termitidae and Hadotermes that belong to the family Hadotermitidae (UNEP, 2000). The fungus growers are abundant in the savannas and somewhat dry lands .The soil-feeders on the other hand are abundant in the tropical forests (Dari *et al*., 1995).

2.2 Termite feeding guilds

The diet of termites is diverse but is rich in cellulose, hemicellulose and lignin or derivatives of lignin. This trait places the termites in an important ecological position particularly in the tropical region where their activity can dominate the process of decomposition and nutrient cycling (Woods and Sands, 1978).

2.2.1 Soil feeding termites

Soil feeding termites ingest large amounts of soil (Wood, 1978; Okwakol, 1980) and due to their high biomass densities, their feeding activity is important for the biomass turnover in the tropical and sub-tropical ecosystems (Wood and Johnson, 1986; Wood,1989; Martius, 1994; Bignell *et* a*l.,* 1997). The food ingested by soil –feeding termitesis quite heterogeneous. The gut predominantly contains soil minerals and humus, but also plant tissue fragments, plant roots, fungal mycelia and macerated organic material.

The hindguts of soil-feeding termites are more elongated and compartmentalized than those of any other feeding guild and characterized by steep pH changes along the axis (Bignell and Eggleton1, 1995). Microscale pH measurements have shown that the pH increases sharply in the anterior hindgut, culminating in the proctodeal one (P1) region with the most alkaline values encountered in the biological systems (pH >12), and decrease towards the rectum (Brune, 1996).

Studies on the soil-feeding termites, especially their functional role in digestion, are still lagging behind (Brune, 1998).

2.2.2 Fungus cultivating termites

The fungus-cultivating termites are reported to harbour dense populations of bacteria and Archaea (Anklin-Mühlemann *et al*., 2001). The symbiotic fungi grow on a special culture within the nest maintained by the termites and called Fungus comb (Hyodo *et al*., 2000). The fungus comb is made from partly digested foraged plant litter, which passes rapidly through the termite‘s guts. The resulting faecal pellets are pressed together to make a comb-like matrix. As the comb matures, mycelium develops and produces conidial nodules, which together with older, senescent comb are consumed by workers. Because of their unique symbiotic relationship, many studies have been conducted on the termite –fungus association (Sands, 1969, Darlington, 1994). Several roles have been suggested for the fungal symbionts, such as the provision of heat and moisture (Lusher,1951), the provision of concentrated nitrogen source as conidia (Matsumoto, 1976) and the enrichment of nitrogen in foraged foodstuffs by the virtue of the fungal metabolism (Collins, 1983). It has been noted that by associating with the lignin decomposers, the fungus -cultivating termite make it possible to utilize lignocellulose nearly completely as reflected in the small volume of their final faeces (Darlington, 1994) and therefore to play a dominant role in the decomposition processes in many parts of the tropics (Abe, 1980; Buxton, 1981)

2.2.3 Wood feeding termites

These are termites that feed on wood and woody litter, including dead branches still attached on trees. Lower termites are wood-feeders and there are wood-feeding species in all the subfamilies of the Termitidae except the Apicotermitinae (Bignell and Eggleton, 1995). The main part of the digestion takes place in the hindgut especially in the pauch, under the action of symbiotic microorganisms. It is well established that lower termites digest cellulose using synergistic actions of the cellulolytic enzymes that originate from the termite guts and symbiotic protozoa in the hindgut.The protozoan fauna accounts for the most of the cellulolytic activities in the hindgut (Breznak, 1982). The role of bacteria in the digestion processes of the lower termites is controversial. However, Breznak (1982 recorded evidence of significant nitrogen fixation in both microbial isolates and intact termites.

2.3 The intestinal tracts of termites

The digestive tracts of termites consist of the foregut, the midgut and the hindgut. The foregut consists of oesophagus, the crop and the gizzard (Figure 1). The oesophagus is a simple, narrow tube, which extends as far as the thorax. It is followed by the crop and is not clearly separated, having practically the same structure (Figure 1). The gizzard is not generally separated from the crop, but its musculature is more powerful. The foregut terminates with an oesophageal valve which is always well developed and which penetrates deeply into the midgut (Figure 1). The midgut is a tube of uniform diameter and its histological structure is remarkably constant. The muscular

connective envelope is slightly developed. (Noirot and Noirot-Timothee, 1969; Bignell *et al*., 1979)

The hindgut is always well developed, and it exhibits variations depending on the different groups and their nutritive regime. There are five successive distinguishable segments (Hölmgren, 1969). The first segment may be short, tubular, long or it may even have a pronounced dilation. The 2nd segment is formed by a differentiation that characteristic of termites (Grasse and Noirot, 1954). The 3rd segment; whose entrance is controlled by the enteric valve, consists of voluminous dilation or pauch, containing an abundance of symbiotic microbes. This segment narrows progressively in its posterior part and drains into the following segment without any clear limits.
The 4th segment is a narrow and contorted tube, often called the colon, of variable but always considerable length (Grasse and Noirot, 1954)

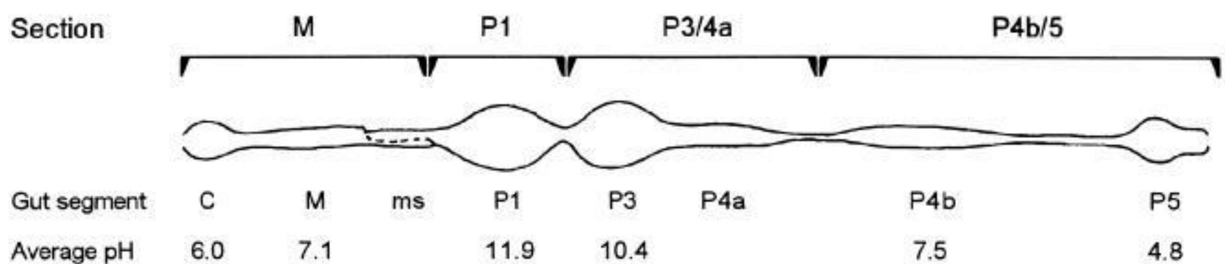

Figure 1: Gut morphology of a *Cubitermes* sp. worker termite, also representative of other soil feeding species of the subfamily *Termitinae*. The gut was drawn in its unraveled state to illustrate the various segments: C, crop; M, midgut; ms, mixed

segment; P1 to -5, proctodeal segments 1 to 5, respectively. The average luminal pH was determined for the indicated gut regions in C*ubitermes speciosus* by using intact guts and glass pH microelectrodes when gut sections were used; the guts were separated at the indicated positions. (Brune, 1998)

The rectum has a uniform structure and exhibits varying degrees of development from the 5th segment. It is elongated ampul; muscular, larger than the preceding sections and dilations, as in many insects, the rectum contains six more or less conspicuous longitudinal thickenings forming the rectal papillae that do not extend as far as the anus. The importance of this terminal chamber varies greatly among the species (Grasse and Noirot, 1954).

The arrangement of the digestive tube in the lower termite is not very variable, probably due to constant presence of symbiotic flagellates in the pauch, which indicates a rather uniform physiology (Grasse and Noirot, 1954). The crop is essentially symmetrical. The gizzard possesses a typical chitinized armature. The first segment of the hindgut forms a cone, more or less elongated and regular, whose tip corresponds to the enteric valve. The hind gut has been considered anoxic habitat where anaerobic micro-organisms ferment wood polysaccharides to short-chain fatty acids, which in turn serve as source of the sole carbon and energy source for the host (Noirot and Noirot-Timothee, 1969; Bignell, 1994)

In higher termites, the mensteron is considerably prolonged on one of the faces of the intestinal tube resulting in the mixed segment (Grasse and Noirot, 1954). The intestinal lumen is limited on one side by the mesenteron and on the other side by the proctoderm. When such a mixed segment occurs, it always contains abundance of bacteria, having the appearance of a pure culture. The mixed segment remains exterior to the peritrophic membrane and do not mix with the alimentary bolus. In Macrotermitinae, the structure of the digestive tube is very uniform and very close to that of the lower termites, notably Kalotermitidae and Rhinotermitidae that lack the mixed segment and the relatively unextended nature of the hindgut (Noirot and Noirot-Timothee, 1969). The gizzard is relatively long and there are four malpighian tubules that are inserted in a symmetrical manner at the junction of the mesenteron - proctodeum (Grasse and Noirot, 1954). It is unclear whether the relatively simple gut of Macrotermitinae reflects the absence of a symbiotic relationship with intestinal bacteria. It lacks the elongated and highly differentiated hindgut typical of other higher termites, (Anklin- Mühlemann *et al*., 1995).

2.4 Bacterial diversity guts of soil feeding and fungus cultivating termites

Earlier studies on the hindgut microbiota of soil feeders revealed that the hindgut is packed with morphologically diverse bacteria (Noirot and Noirot-Timothee, 1969;

Honig, 1970; Bignell *et al*., 1980; Noirot, 1992) whose metabolic products are thought to contribute to the nutrition of the termite (Noirot, 1992; Breznak and Brune, 1994).

Studies on the metabolic diversity of microorganisms present in the gut of soil feeders have revealed the presence of diverse metabolic groups of bacteria including homoacetogenic (Boga *et al*., 2003) and propiogenic bacteria (Boga *et al*., 2006) and methanogenic Archaea (Tholen and Brune, 1999)

Clone libraries obtained from a soil feeding termite *Cubitermes orthognathus* comprised a variety of phyla including the gram positive bacteria with low L+C content, the *Cytophaga-Flexibacter-Bacteroides* group, various groups of Proteobacteria and the spirochetes (Schmitt-Wagner *et al*., 2003)

Numerous *Streptomyces* species from the, parent soil and mound materials of *Procubitermes aburiensis* and *Cubitermes severus* have been isolated (Bignell *et al*.1980). Some cellulolytic and lignin solubilizing actinomycetes have also been isolated from several termites (Pasti and Belli, 1990; König, 1991).

Watanabe *et al*. (2003) isolated and characterised actinomycetes from termite guts and established that the actinomycetes isolated are largely dependent on the geographical origin of the termite. For the termites from the same area, taxonomic differences between the termites also influenced the microbiota in the gut (Watanabe *et al*., 2003).

These bacteria are primarily saprophytes and are best known from soils where they contribute significantly to the turnover of complex biopolymers such as hemicellulose, lignocellulose, pectin, keratin and chitin (Williams *et al*., 1984).

Spirochetes are a morphologically diverse group and may account for as much as 50% of all prokaryotes in some termites. The 12 to 15 spirochete morphotypes in the *Reticulitermes flavipes* were paralled by 21 different spirochete phenotypes, which were assigned to two major clusters of *Treponemes* within the Phylogenetic radiation of spirochetes (Lilburn *et al*., 1999). Leadbetter *et al*. (1999) were the first to isolate two spirochetes from *Zootermopsis angusticollis*

Lactic acid bacteria are typical and numerously significant carbohydrate utilizing microorganisms in the guts of many wood feeding and soil feeding termites (Tholen *et al*., 1997). Their presence in *R. Flavipes* was confirmed by 16s rRNA fingerprinting of the total hindgut community (Bauer *et al*., 2000). Isolates obtained from the hindguts of *Reticulitermes flavipes* and *Theracotermes macrothorax* show considerable diversity and comprise strains belonging to the genera *Enterococcus* and *Lactococcus* (Bauer *et al*., 2000). All isolates proved to be aerotolerant and exhibit high potential rates of oxygen reduction in the presence of fermentable substrates, which may explain that they are regularly encountered in the intestinal tracts of termites and other insects (Bauer *et al*., 2000).

Sulfate reducing bacteria seem to be common inhabitants of the intestinal tracts of many different termite species (Kuhnigk *et al*., 1996; Okhuma and Okudo, 1996; Fröhlich *et al*., 1999). All the isolates are related to free-living sulphate reducers of the genus *Desulfovibrio* and show high rates of oxygen reduction in the presence in hydrogen (Kuhnigk *et al*., 1996)

Only three species of methanogens have been isolated from termite gut. These isolates from *R. Flavipes* were identified as members of the genus *Methanobrevibacter* (Leadbetter and Breznak, 1996; Leadbetter *et al*.,1998). Methanogens are among the few groups of organisms where one can infer metabolic information from the 16s rRNA sequence (Brune and Friedrich, 2000).Isolation of *Bacteroides* from termites has been reported. Schultz and Breznak (1979), demonstrated cross feeding of lactate from a lactate producer to *Bacteroides*. A uric acid degrading bacterium was isolated from the termite gut and identified as *Bacteroides termitidis* (Protrikus and Breznak, 1980).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Termite collection

Cubitermes ugandensis Fuller were collected from Samia glade of Kakamega rainforest in Western Kenya. *Macrotermes michaelseni* Sjöstedt were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) when needed for experimental work. Termites were identified at the National Museums of Kenya and in addition, partial sequences (>650 bp) of the mitochondrial cytochrome oxidase II (COII) gene was determined by PCR of DNA extracts from termite heads with previously described primers (Thompson *et al.*, 2000). The nests of *Cubitermes* species were cut into blocks. The blocks were distributed in polypropylene containers. Containers were kept in the dark at room temperature. The containers were inspected regularly, and parts of the nest materials were removed and replaced with fresh soil; moisture was controlled by spraying the surface of the nest material covered with tissue paper with water (Schmitt-Wagner *et al.*, 2004).

Macrotermes michaelseni Sjöstedt were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) when needed for experimental work and were dissected within 12 hours of collection. For experiments only worker caste termites were used as they are metabolically diverse (Schmitt-Wagner *et al.*, 2003). Three parent soil samples were collected randomly at about 3 metres around the nests. They

were placed in sterile polythene bags and transported to the JKUAT GK Botany Laboratory and kept at 4 ^{o} C.

Figure 2: Photograph of major workers caste of *M. michaelseni* sampled at JKUAT Compound

3.2 Enrichment and isolation of bacteria from parent soil and nest materials

One gram of parent soil and nest materials was mixed with 0.1 g $CaCO_3$ and incubated at 25° C for 7-9 days in water-saturated atmosphere (Tsao *et al.*, 1960). Then the samples were air dried for 6 hours at room temperature. About 0.5 g were suspended in 49.5ml of sterile distilled water and shaken for one hour in a shaker incubator (200 rpm at 25°C). One hundred microlitres (100µl) was plated into glycerol casein potassium nitrate agar, starch casein potassium nitrate agar (Flaig and Kutzner, 1967) and glycerol arginine agar (El-Nakeeb and Lechevalier, 1963) whose pH was adjusted to 8, 10 and 12. The media effectively controlled eubacterial and fungal growth and aid in the isolation of more slowly growing bacteria (Reddi and Rao, 1970). The plates were incubated for 4-7 days at 25^{0} C. Colonies were counted under low power

microscope. The colonies were counted according to different colony morphologies and one colony from each different morphologies were streaked into freshly prepared plates of same media at same pH until pure colonies were obtained. The pure colonies were stocked in the ISP 2 broth (appendix I) supplemented with glycerol and were maintained at 4° C and sub cultured after every three months.

3.3 Enrichment and isolation of bacteria in gut homogenates

Termites were dissected with fine tipped forceps and homogenized in sterile buffered salt solution (BSS) (Breznak and Switzer, 1986) using glass tissue homogenizer. Ten serial dilutions were made up to 10^{-10}. Gut homogenates were prepared from different termites (Tholen & Brune, 1999) and were inoculated into glycerol- arginine agar, starch casein agar and glycerol casein agar as previously described in section 3.2.

3.4 Screening of the isolates for antibacterial, antifungal and enzyme activities

Bacterial isolates were screened for their ability to produce enzymes i.e. gelatinases, amylases, proteases, xylanases, lipases and cellulases and for their antagonistic activities against test fungi (*Candida albicans* and *Aspergillus niger* and test bacteria (gram negative: *Escherichia coli* and *Pseudomonas aeruginosa*, Gram positive Bacteria: *Staphylococcus aureus* and *Bacillus subtilis.* Isolates positive for the above attributes were selected and characterized further.

3.4.1 Determination of enzyme activities of the isolates

The ability of isolates to produce hydrolytic enzymes was determined by mixing cellulose, xylan, starch, egg yolk, gelatin or casein with agar/broth on individual assay plates and visualized by clear zone formation (Cappuccino and Sherman, 2002) using assays described by Shirling and Gottlieb (1966). These assays were used to detect the production of cellulases, proteases, lipases, xylanases, gelatinases and lipases.

3.4.1.1 Licithovellin reaction

The basal medium was autoclaved in batches of 95ml. Before pouring into the plates; 5ml of egg yolk emulsion were added to each batch. The plates were inoculated spot wise with 3 or 4 strains. After 3, 5 and 8 days, the plates were checked in transmitted light for the occurrence of opaque Zone around the colonies, that reached more than 10 mm in diameter. The zone of hydrolysis varied considerably in diameter. Lipase activity was indicated by the formation of a pearly layer detectable in oblique light; in licithovellin positive cultures, mostly at the edge of the opaque zone and in licithovellin negative culture around the colony (Korn-Wendisch and Kutzner, 1992).

3.4.1.2 Cellulose hydrolysis

Isolates were tested for their ability to degrade cellulose by two different methods. The first method involved inoculating a loopful well developed colonies on ISP 2 medium supplemented 3.0% NaCl) (appendix I) of the bacteria interest was spot inoculated on ISP 9 agar (appendix iv) supplemented with 1% carboxymethyl cellulose and

incubation of cultures was done for 14 to 21 days (Smibert and Krieg, 1981) colonies were there examined by dissecting microscope for the presence of zones of clearing (Rheims *et al*., 1998). The second method involved the use of ISP 9 broth (appendix ii) supplemented with sterile cellulose discs as a sole carbon source. A loopful of isolates of interest grown in ISP medium 2 broth (appendix i) was inoculated and incubated at 25°C for 14 to 21 days (Smibert and Krieg, 1981). The disappearance of filter paper discs from the medium showed a positive test.

3.4.1.3 Xylan hydrolysis

The ability of the isolates to degrade xylan was tested by inoculating each ISP 9 agar supplemented with 1% xylan as the only carbon source (4 isolates per plate). After incubation, clearing zones around the growth were recorded

3.4.1.4 Starch hydrolysis

Starch agar (1.5%) containing 0.2% soluble starch served as the polysaccharide substrate. The detection of hydrolytic activity following the growth period was made by examining for clearing zones around the colonies and by carrying out starch hydrolysis test. This was done by flooding the surface of agar with iodine. The presence of blue-black colour around indicated a negative test hence no starch hydrolyzed and absence of blue colour around the colonies and presence of a zone of hydrolysis indicated a positive test (Cappuccino and Sherman, 2002)

3.4.1.5 Gelatin liquefaction

Nutrient gelatin deep tubes were used to demonstrate the hydrolytic activity of gelatinase. The medium consisted of nutrients broth supplemented with 12% gelatin. Following inoculation and incubation for 4-6 days at 28° C, the cultures were placed in a refrigerator at 4° C for 30 minutes cultures that remained liquefied produced gelatinases and demonstrated rapid gelatin hydrolysis. Re-incubation was done for all solidified cultures for an additional 5 days and observed for liquefaction culture that remained liquefied were indicative of positive gelatin hydrolysis (Cappuccino and Sherman, 2002)

3.4.1.6 Casein hydrolysis

The isolates' ability to degrade casein was done using ISP 9 agar supplemented with 1% casein as sole carbon source. The casein gives the medium colloidal color and opacity because it deflects light rays rather than transmitting them. Following inoculation and incubation of agar plate culture, organism-secreting proteases exhibited a zone of proteolysis as demonstrated by clear zone surrounding the bacterial growth. In absence of protease activity the medium surrounding the growth of the organism remains opaque which a negative reaction (Cappuccino and Sherman, 2002).

3.4.2 Determination of antimicrobial activity of the isolates

The antimicrobial spectrum of the isolates was determined by making a single streak inoculation on nutrient agar supplemented with glucose, glycerol and calcium carbonate (Korn-Wendisch and Kutzner, 1992).The plates were incubated in an inverted position for 3 to 5 days at 25° C. Following incubation, on the bottom of each plate four perpendicular lines to the growth of antibiotic producing isolates were drawn. Aseptically a single line inoculation of each of the four-test organism on each plate was made .The test organism included, *Bacillus subtilis, Escherichia* coli*, Staphylococcus aureus*, *Pseudomonas aeruginosa* , *Aspergilus niger* and *Candida albicans*. After incubation the absence or presence of antibiotic activity was recorded for each isolate and the spectrum of antimicrobial activity also determined (Cappuccino and Sherman, 2002). Antibiotic producing isolates were characterized further.

3.5 Characterization of bacterial isolates

3.5.1 Morphological characterization

3.5.1.1 Gram stain

The Gram staining technique was used to divide isolates on the basis of reaction and morphology (Cappuccino and Sherman, 2002). Air dried smears of the isolates were heat fixed .the smears were then flooded with crystal violent for 60 seconds. Excess stain was washed in running water. The smears were then flooded with Gram's iodine

for 60 seconds and excess iodine was washed off in running water. The smears were then decolorized using acetone and counterstained with safranin for 60 seconds. The smears were the air dried and examined under 100 x objectives

3.5.1.2 Sporophore studies

The slide culture procedure (Hopwood, 1960) was adopted to study the morphological arrangement of the mycelia and spores. Mycelial and spore arrangement was determined by plating the organism on freshly prepared agar blocks aseptically placed at the Centre of the glass slide in a sterile glass Petri dish. Cover slips were gently used to cover the inoculated agar blocks on the glass slides to allow the spread of mycelia on both cover slips and glass slides. The Petri plates were then covered. The plates were incubated for 6 to 8 days to allow growth of the isolates after which both the cover slips and the glass slides were gently pulled out of the agar block. Cover slips and glass slides were heat fixed. The cover slips were air dried, gram stained and observed under inverted microscope at x100 objective (Keast *et al.,* 1984).

3.5.2 Cultural characterization of the isolates

Culture characterization of the isolates was done on 1SP 2 medium containing 3%NaCl with or without 2.0g/l of $CaCO_3$ (Magarvey *et al*., 2002 and Williams *et al*., 1992). One hundred microlitres (100 µl) of each isolate were plated into ISP 2 medium containing 3% NaCl with or without 2.0 g/l of $CaCO_3$. Incubation was done at 25° C for 7 days with regular inspection until characteristic aerial sporulation

appeared on colonies, pigmentation, optical characteristics, size of the colony, margin and elevation were determined as previously described by Cappuccino and Sherman (2002). The presence of characteristic colour of aerial mycelium and spores was examined.

3.5.3 Biochemical and physiological characterization

3.5.3.1 Utilization of sugars and similar compounds

The following compounds were tested for use as sole carbon source: glucose, arabinose, sucrose, xylose, mannitol, lactose, rhamnose, D-mannitol, D-meliobiose, D-galactose, D-mannose and lactose. One percent (1%) of these compounds was supplemented to ISP 9 broth (appendix ii). The medium was dispensed in tubes,autoclaved and inoculated with the bactaterial isolates. Growth was recorded after 7 and 14 days in terms of turbidity and optical densities. An uninoculated tube with complete medium for each sugar /carbohydrate served as negative control (Williams *et al*., 1989).

3.5.3.2 Formation of organic acids

Ability of the bacterial isolates to produce organic acids was determined by medium containing solution A, B, and C. Test tubes were charged with 0.2 ml of solution C and autoclaved. Solutions A and B were autoclaved separately and combined. One point eight (1.8ml) was added to the tubes acid formation was indicated by disappearance of $CaCO_3$ and colour change to yellow within 5-15 days (Williams *et*

al., 1992). Solution A contained 20.0g of glucose extract, 1.2 g of yeast extract, 0.25 g of $MgSO_47H_20$, 12.0 mg of bromocresol purple, 4.0 g of agar and 400 ml of water. Solution B contained 534 mg of $Na_2HPO_4.2H_2O$, 272 mg of KH_2PO_4 and 500ml of distilled water. Solution C contained 1.0 g of $CaCO_3$ and 100 ml of distilled water. Uninoculated tube with complete medium served as a control (Korn-Wendisch and Kutzner, 1992).

3.5.3.3 Resistance to sodium chloride

The ISP medium 2 broth without NaCl was prepared in six batches of tubes that were supplemented with NaCl: 0, 20, 40, 70,100 and 130 g/l. Tubes were divided 6 sections, each being inoculated with isolates. Observations were made after 10 days. The highest concentration of salt that allows growth was recorded (Korn-Wendisch and Kutzner, 1992)

3.5.3.4 Reduction of nitrate to nitrites

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out in ISP 8. Following incubation of the cultures isolates ability to reduce nitrates to nitrites was determined by addition of two reagents sulfanilic acid followed by alpha – Naphthylamine.

3.5.3.5 pH requirement

ISP medium 2 broth was prepared in five batches whose pH was adjusted to 3, 6,8,10 and 12 by using 1M HCl and 1M NaOH .The medium was then dispensed in tubes and autoclaved. Each batch was inoculated with the bacterial isolates in duplicates. Observation was made after six days incubation at 25° C. The presence of growth was indicated by turbidity and was recorded by sign (+) and the absence of growth was indicated by the absence of turbidity and denoted by negative sign (-). Optical densities were taken by spectrophotometer (Cappuccino &Sherman, 2002)

3.5.3.6 Citrate utilization

The capability of the isolates to use citrate as carbon source for their energy was carried out Simmons's citrate agar slants as previously described (Harold, 2002 and Williams *et al*., 1989). Utilization of citrate was indicated by alkalinization of medium after incubation

3.5.3.7 Catalase test

Catalase production was determined by addition of 3% hydrogen peroxide to tryptic soy agar culture of each isolates as previously described by Cappuccino and Sherman (2002).

3.5.3.8 Hydrogen sulfide production

Sulfur- indole motility (SIM) agar media was used to tests for the ability to produce hydrogen sulfide from substrate such as sulfur containing amino acids. Blackening of the medium indicated that the organisms produced hydrogen sulphide and was motile. Absence of blackening indicated absence of hydrogen sulfide (cappuccino and Sherman, 2002)

3.5.3.9 Hydrolysis of urea

The ability of the isolates to produce urease was determined using urea broth containing phenol red as previously described (Cappuccino and Sherman, 2002). Urease activity was indicated by alkalinization of the medium (Williams *et al*., 1992)

3.5.4 Molecular characterization

3.5.4.1 DNA extraction from bacterial cells

Protocol was devised for efficient bacterial DNA extraction. Isolate were cultured in 20ml of Lauri Bartani broth in universal bottles for 7 days and then 1.8ml of culture was added to eppendorf tube and centrifuged at 4000 rpm for 5 minutes, the supernatant was discarded. The pellet was resuspendsed in 200 ml of solution 1 containing 50 mm Tris pH 8.5, 5M EDTA pH 8.0 and 25% sucrose solution. Five microlitres (5µl) of lysozyme (20 mg /ml) were added and contents gently mixed. Ten microlitres (10µl) RNase A (20 mg/ml) were added and gently mixed and incubates at 37 ^{o}C for 1hour. Following incubation, 600 ml of solution 2 (10 mm Tris pH 8.5,

5mM EDTA pH 8.0 and 1% SDS) was added and contents were mixed by inverting several times. Ten microlitres (10µl) of proteinase K (20 mg/ml) was added and mixed gently. Incubation was done at 50 ^{o} C for 30 minutes.

DNA was extracted by adding equal volumes of phenol chloroform and centrifuged for 5 minutes at 13000 rpm and carefully pipetted out the aqueous phase, which contained the crude DNA. Proteins and lipids were removed by addition of 0.3 volumes of Diethyl ether and phases were mixed by inversion and centrifuged at 12000 rpm for five minutes. The aqueous phase was transferred to a new tube and DNA was stabilized by addition of 0.1 volumes of 3M NaCl and precipitated with an equal volume of absolutes ethanol. After the genomic DNA was centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt, dried and redissolved in 200 ml of TE for immediate use or storage at – 20° C. The DNA was semi quantified on a 1% agarose 1X TAE buffer and visualized under ultraviolet by staining with ethidium bromide (Magarvey *et al.,* 1989).

3.5.4.2 16s rRNA gene amplification

Nearly full length 16s rDNA gene sequences were amplified using bacterial primers pair 27F forward 5'-GAG TTT G (AC) T CCT GGC TCA G-3' and 1492R reverse primer 5'-TAC GG (CT) TAC CTT GTT ACG ACT T-3' (Eurofins MWG GmbH). A model 9800 fast cycler from applied Biosystems was used. The PCR reaction volume was 30µl. Reagents used included four (4µl)of 10xPCR buffer, 4ul of dNTPs

(2.5mM), 2.5µl of reverse primer (5pmol), 2.5µl of forward primer (5 pmol), 16.6 µl of PCR grade water 0.4 µl of Taq polymerase (50u/µl) and 1.5µl of the template. Temperature cycling profiles were 35 cycles; initial denaturation temperature of 94°C for 5minutes; denaturation at 94°C for 45 seconds, primer annealing at 55 ^{o} C for 30 seconds and, chain extension at 72 ^{o} C for 60 seconds and a final 8-minute extension 72 ^{o} C. Five microlitres (5µl) of each DNA sample was loaded on an ethidium bromide containing agarose gel (1%) in TAE buffer and run at 80 volts for one hour. Gel documentation was done using the Gel Logic 200 imaging system.

3.5.4.3 Purification of PCR products

QuickClean 5M Gel extraction kit was used to purify PCR products. A clean sharp razor blade or scalpel was used to excise the DNA band from the agarose. The gel slice was placed in a colourless pre-weighed tube. Three volumes of binding solution II were added to one volume of the gel slice (100 mg = 100µl). Incubation was done at 50 ^{0}C for 10 minutes with occasional vortexing or until the gel slice was completely dissolved. Usually, the color of the mixture is yellow. When the color of the mixture was violet, 5 to 19µl of 3M sodium acetate (pH5.0) was added and contents mixed. The colour changed to yellow. One volume of isopropanol was added (with respect to the original volume) and mixed. The mixture was the transferred, when less than 600µl to QuickClean column and centrifuged at 12,000 rpm for 30 seconds. For the mixtures with volumes more than 600µl, loading and centrifugation were done using

the same column. The flow-through was discarded from the tube. Five hundred microlitres (500µl) of the wash solution was added to the column and centrifuged at 12,000 rpm for one minute. The wash procedure step was repeated once more. The flow – through was removed and discarded and the mixture was centrifuged at 12,000rpm for additional 60 seconds to remove residual wash solution. The column was transferred to a clean 1.5µl elution buffer to the centre of the column membrane and incubated at 25^0 C for one minute and centrifuged at 12,00rpm for one minute to elution and collection of DNA.

3.5.4.5 Phylogenetic data analysis

Sequence data were edited and aligned by Bioedit (Hall,199).The 16S rRNA gene sequences were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Centre For Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov) to determine the similarity to sequences in the genebank database. The alignments were corrected manually where necessary based on conserved regions. The 16S rRNA gene sequences with high similarity to those determined in the study were retrieved and added to the alignment. The new sequences were added to the ARB database and analyzed with the Mega 4 (Tamura *et al.*, 2007). Phylogenetic tree was constructed by Maximum composite Likelihood method using Mega 4 (Tamura *et al.*, 2007). The evolutionary distances

were computed using the Maximum Composite Likelihood method (Tamura *et a*l., 2004]. The percentage of replicate trees in which the five isolates clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Sequence data was analyzed with ARB software package (Version 2.5; Strunk and Ludwig, Technishe Universitae Munchem (http://www.arb.home.de).

3.6 Statistical data analysis

Colony forming units obtained on different media at different pH and from different materials were compared by F ratio as described in Statistical package for social sciences (SPSS). The mean numbers of isolates obtained on different media at different pH from the samples were computed and compared by SPSS

CHAPTER FOUR

4.0 RESULTS

4.1 Enumeration of bacteria in parent soils, nest materials and gut homogenates of *Cubitermes ugandensis* Fuller and *Macrotermes michaelseni* Sjöstedt

The gut homogenates of the two termite species yielded the highest numbers of colony forming units, followed by the nest materials and the least was parent soils (Table 1).

Table 1. Summary of colony forming units × 10^6 ±SD/ml of parent soils and nest materials and colony forming units × 10^6 ± SD/gut of *Macrotermes michaelseni* and *Cubitermes ugandensis* using different media

Media	**pH**	**Termites**					
		Macrotermes michaelseni			***Cubitermes ugandensis***		
		gut	Nest	Soil	Gut	Nest	Soil
Starch casein agar	8	206±36.06	8.064±11.3	10.7±36.8	360±69.3	17.1±38.9	16.7±50.9
	10	96.±6,36	4.03±21.2	5.61±21.2	201±62.9	11.0±31.8	11.6±19.1
	12	61.25±7.8	3.072±4.1	3.062±2.8	106±26.9	3.39±13.4	4.4±28.99
Glycerol arginine agar	8	258±27.6	11.23±28	19.2±84.8	282±28.3	18.1±13.4	17.9±34.7
	10	203.8±13.4	4.32±14.14	10.4±33.9	180±28.5	11.90±19.8	9,1±22.6
	12	165±18.9	2.21±5.66	2.5±6.36	64.1±11.3	4,5±4.14	3.52±24.8
Glycerol casein agar	8	222.5±26.9	10.56±20.4	13.67±48	27±23.3	19.5±62.9	13.9±39.6
	10	120±25.5	7.296±20	8.83±23.3	22.7±28.9	11.6±56	10.9±52.3
	12	97±12.73	2.4±13.43	5.10±14.1	49±20.51	4.8±38.9	3.16±5.7

4.2 Isolation of bacteria from guts, soils and nests

A total of two hundred and eighty six isolates were obtained from parent, soils, nest materials and gut homogenates the termites

Table 2. Summary of isolates obtained from parent soil, nests and intestinal tracts of *M. michaelseni* and *C. ugandensis* using three different media

Medium		Starch casein agar			Glycerol arginine agar			Glycerol casein agar			Total
Ph		8	10	12	8	10	12	8	10	12	
Macrotermes michaelseni	gut	7	4	6	6	7	6	6	6	5	53
	nest	6	6	4	5	4	5	5	5	4	42
	soil	6	6	4	4	6	5	4	6	4	47
Total		**19**	**16**	**14**	**15**	**17**	**16**	**15**	**17**	**13**	**142**
Cubitermes ugandensis	Gut	4	4	4	6	5	5	6	6	4	44
	Nest	7	6	6	6	4	4	7	4	5	49
	Soil	7	6	7	6	4	4	6	6	5	51
Total		**18**	**16**	**17**	**18**	**13**	**13**	**19**	**10**	**14**	**144**

A total of hundred and forty-four isolates were obtained from parent soils, nests and guts of *Cubitermes ugandensis* fuller as shown in the Table 2 while a total of 142 isolates were obtained from *Macrotermes michaelseni* (Table 2).

4.3 Screening of the isolates for enzyme and antibiotic activities

A total of 286 isolates obtained from the soils, nest materials and gut homogenates were tested for their ability to produce antibacterial and antifungal activities. The isolates were also screened for their ability to produce six groups of enzymes namely, amylases, proteases, gelatinases, lipases, xylanases and cellulases (Table 4)

4.3. 1 Enzyme activities of the isolates

The 286 isolates obtained from two termite species were tested for their ability to produce six enzymes namely, amylases, proteases, gelatinases, lipases, xylanases, and cellulases. These enzymes have a biotechnological potential for exploitation in food industry, leather industry and fermentation industry. Emphasis was placed on alkiliphilic extracellular enzymes that are widely used in the detergent, food processing and tanning industries especially proteases and amylases. Fifty isolates were found to produce at least four groups of enzymes. The fifty isolates were selected based on this attribute for further characterization using biochemical and morphological characteristics. All the isolates were amylolytic, proteolytic, lipolytic and xylanolytic. All the isolates were non-cellulolytic perhaps indicating the absence of one or more of cellulosomes. Isolates KSC8S21 and KSC12S21 produced gelatinase (Table 3).

Table 3. Summary of isolates from parent soils, nest materials and gut homogenates showing positive activity for enzymatic activities

Sample	Number of isolates	Isolates positive for enzymes					
		Xylanase	lipase	gelatinase	amylase	Cellulase	Protease
Macrotermes ugandensis							
Gut	53	3	3	3	3	0	3
Nest	42	10	10	10	10	0	10
Soil	47	6	6	6	6	0	6
Cubitermes ugandensis							
Gut	44	8	8	8	8	0	8
Nest	49	14	14	14	14	0	14
Soil	51	9	9	9	9	9	9

4.3.2 Antibiotic activities of isolates

A total of 286 isolates obtained from the soils, nest materials and gut homogenates were tested for their ability to produce antibacterials and antifungals. A total of fifty isolates were found to have significant antibacterial and antifungal activities against test organisms. The fifty isolate were also found to produce at least four extracellular enzymes of biotechnological potential. The fifty (50) isolates were characterized further using morphological and biochemical characteristics. (Table 4)

Table 4. Summary of isolates from parent soils, nest materials and gut homogenates showing positive activity for enzymatic activities

Sample	No of isolates	Isolates positive for antibacterial and antifungal activities Against					
M. michaelseni		SA	EC	PA	BS	CA	AN
Gut	53	3	3	3	3	3	3
Nest	42	10	10	10	10	10	10
Soil	47	6	6	6	6	6	6
C. ugandensis							
Gut	44	8	8	8	8	8	
Nest	49	14	14	14	14	14	14
Soil	51	9	9	9	9	9	9

KEY SA =*Stapylococcus aureus*, BS=*bacillus subtilis*, EC =*Escherichia coli*
C =*candiada albicans*, PA =*Pseudomonas aeruginosa*, AN =*Aspergillus niger*

These isolate might be producing novel antibiotics that can be used against multi-drug resistant microorganisms (Table 3). They were antagonistic against test bacteria and fungi

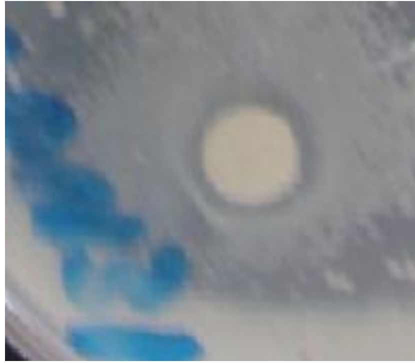

Figure 3a: Antagonistic activity of isolate KGA8N31 against *Aspergillus niger*

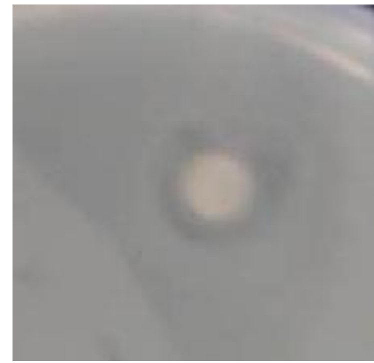

Figure 3b: Antagonistic activity of JGA10N22 against *Bacillus subtilis*

Figure 3c: Antagonistic activity of KSC12S21 against *Pseudomonas aeruginosa*

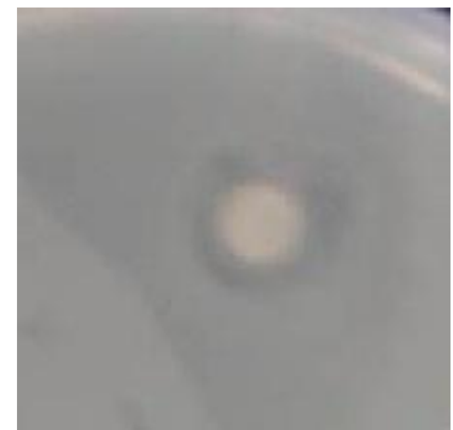

Figure 3d: Antagonistic activity of KGA831 against *Candida albicans*

The isolates produced varied sizes of zones of inhibition against test bacteria and fungi (Table 5.)

Table 5. Inhibition zone diameters (cm) of isolates against test organism

Isolate	*S. aureus*	*P. aer*	*E.coli*	*B.subtilis*	*C. albicans*	*A. niger*
JGA10N22	0.8	0.65	0.7	0.8	0.95	0.6
JGC10G21	0.6	0.6	0.9	0.7	1.15	0.6
KSC8S23	0.6	0.8	1.05	1.3	0.75	0.6
KSC12S21	0.65	0.7	0.9	1.25	1.1	0.9
KGA8N31	0.7	0.9	1.05	1.0	1.05	0.7

4.4 Characterization of the isolates

Fifty isolates were found to produce at least four groups of enzymes and antibiotics. A polyphasic approach was used to characterize isolates with biotechnological potential i.e. those with ability to produce enzymes and antifungal and antibacterial agents. A combination of biochemical and morphological characteristics provide strong evidence for the isolates that produced bioactive compound belong the domain bacteria.

4.4.1 Morphological characterization

Colonies were observed under dissecting microscope after 3-7 days .The colonies not only had characteristic morphologies but some were arranged in concentric rings .The isolates did not produced a variety of coloured pigments.

Figure 4a: KSC12S21 colonies examined Under dissecting microscope

Figure 4b: KSC12S21gram smear(x1600)

Figure 5a: JGA10N22 colonies examined Under dissecting microscope

Figure 5b: JGA10N22 gram smear(x1600)

Figure 6a: JGC10G21 colonies as examined dissecting microscope

Figure 6b: JGC10G21 gram smear(x1600)

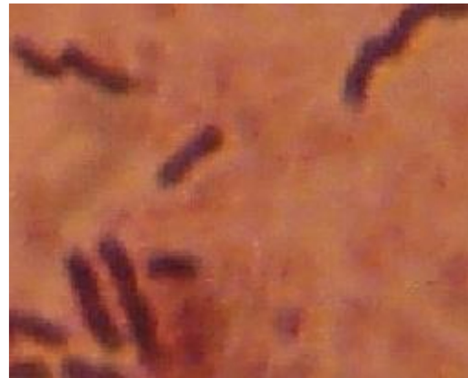

Figure 7a: KGA8N31colonies examined under dissecting microscope

Figure 7b: KGA8N31gram smear (x1600)

Figure 8a: KSC8S23 examined under dissecting microscope

Figure 8b: KSC8S23 gram smear(x1600)

4.4.2 Physiological and biochemical characterization of the isolates

4.4.2.1 Utilization of sugars

All the isolates utilized glucose except JGC10G21 that did not. All the isolates were manninitol and galactose positive except KSC8S23. Isolate JGA10N22 utilized all the sugars except cellulose indicating high metabolic diversity. JGC10G21 utilized only two sugars namely meliobiose and mannose (Table 6)

4.4.2.2 pH requirement of the isolates

The isolates grew well at pH 6 to 10. There was poor growth at pH 12. At pH 3 there was hardly any growth with exception of KSC8S23 and KGA8S23 (Figure 9)

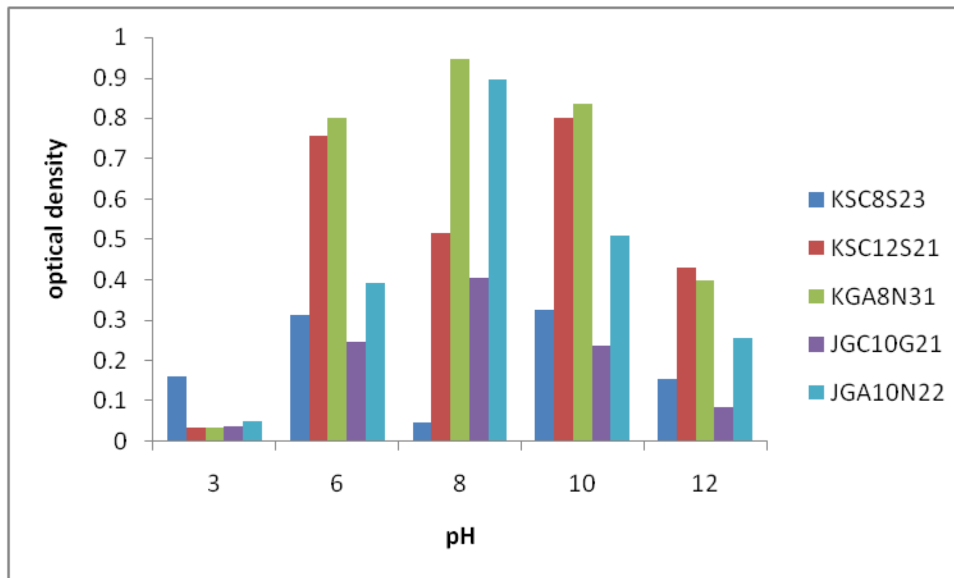

Figure 9: A bar graph showing pH requirement by the isolate

4.4.2.3 Resistance to NaCl

All the 5 isolates did not require NaCl for growth. Highest optical densities were recorded at 0 and 20g/l 0f NaCl. Isolates JGC10G21 and JGA10N22 resisted salt concentration up to 40 g/l and were unable to grow at 70g/l. Isolates KSC12S21, KGA8N31 and KSCS23 resisted concentrations of NaCl up to 70g/ l (Figure 10)

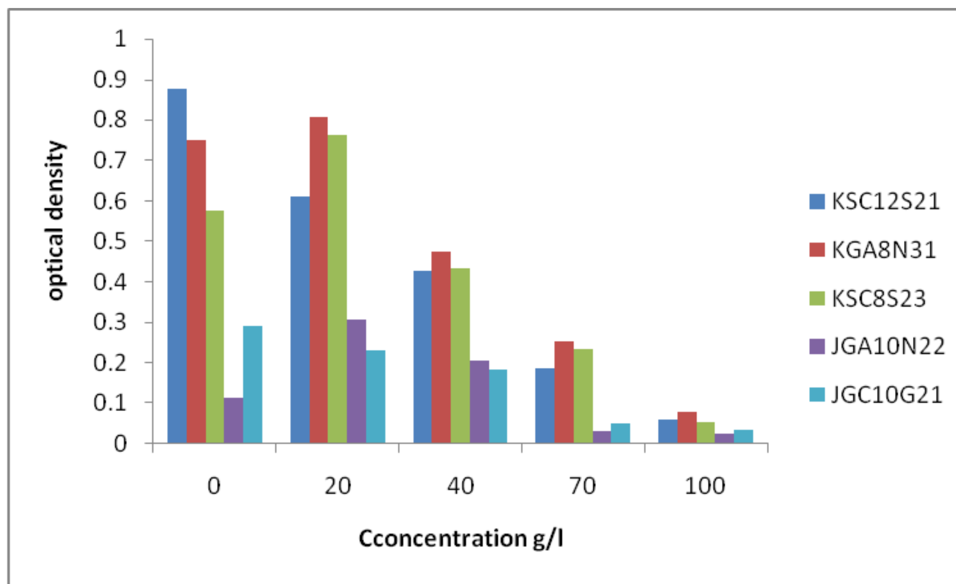

Figure 10: Bar graph showing resistance to NaCl by the isolates

4.4.2.4 Biochemical characteristics of the isolates

The five isolates exhibited variations in biochemical characteristics. All the isolates were non –cellulolytic, xylanolytic, proteolytic and amylolytic (Table 6). All the isolates produced organic compounds and were citrate positive and hydrogen sulfide negative.

Table 6. Biochemical characterization of isolates

ISOLATE	JGA10N22	JGC10G21	KSC12S21	KGA8N31	KSC8S23
Amylase	+	+	+	+	+
Lipase	+	+	+	+	+
Cellulose	–	–	–	–	–
Xylanase	+	+	+	+	+
Protease	+	+	+	+	+
Gelatinase	–	–	+	–	–
Catalase	+	–	+	–	–
Sulfide	–	–	–	–	–
Nitrate	–	+	–	–	–
Organic acid	+	+	+	+	+
Urease	+	+	+	–	–
Citrate	+	+	+	+	+
Arabinose	+	–	+	+	+
Glucose	+	–	+	+	+
Sucrose	+	–	–	+	+
Galactose	+	–	+	+	–
Lactose	+	–	+	–	+
Rhamnose	+	–	+	–	–
Melibiose	+	+	–	–	+
Mannitol	+	–	+	–	+
Mannose	+	+	+	–	+

Key (+) positive (-) negative

4.5 PCR amplification of 16s rDNA gene from isolates

Nearly full length 16S rRNA genes sequences of the fifty (50) isolates were PCR amplified using bacterial primer pair 27F (forward) primer and 1492R, (reverse) primer. Out of the 50 isolates' 16S rRNA genes only 33 were amplified by PCR. The remaining seventeen 16s rRNA genes were not amplified by protocol conditions probably due to inhibitory effect of impurities in samples to amplification. The isolates visualized after ethidium bromide staining. The amplification products were approximately 950bp from all the isolates (Figure11)

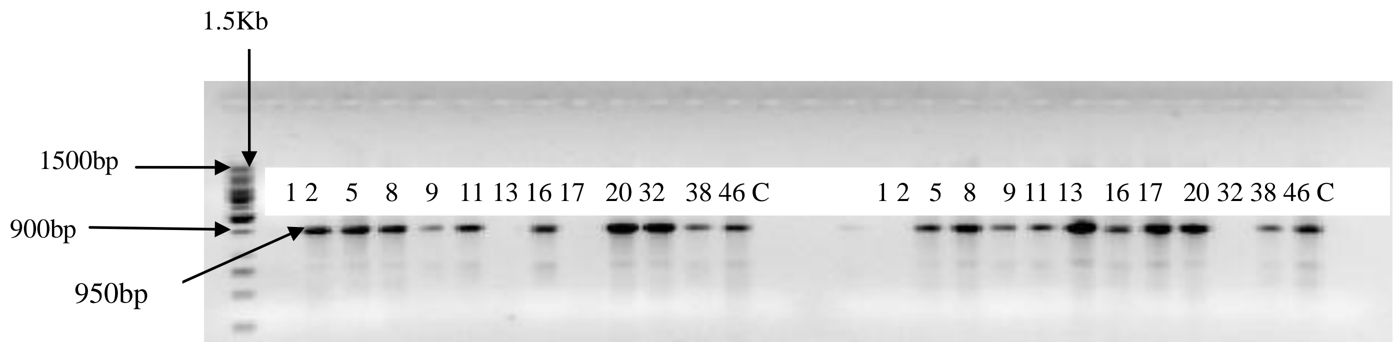

Figure 11: A 1% agarose gel showing PCR amplification of 16S rDNA of the isolates as visualized by ethidium bromide staining

The 5 isolates whose 16S rDNA was sequenced positions are 8,13,17,20 and 32 representing isolates KSC8S23, KSC12S21, JGA10N22, KGA8N31 and JGC10G21. Marker used was 1.5 Kb plus DNA ladder (fermentas). The 16S rRNA sequences of the fifty isolates amplified by PCR were digested by HhaI enzyme to determine the restriction fragment length polymorphisms (RFLP).

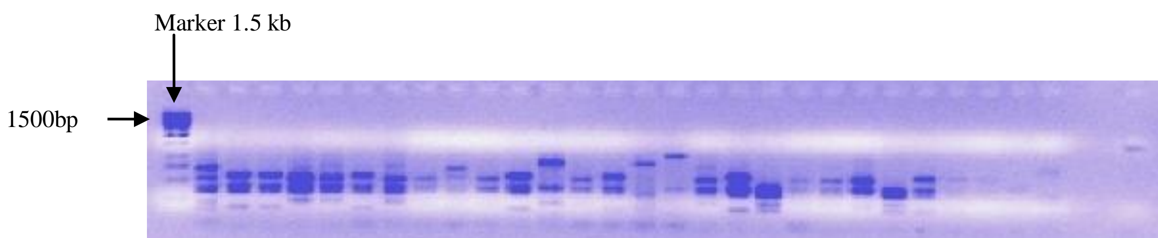

Figure 12: Restriction fragment polymorphisms some isolates (RFLP) profiles

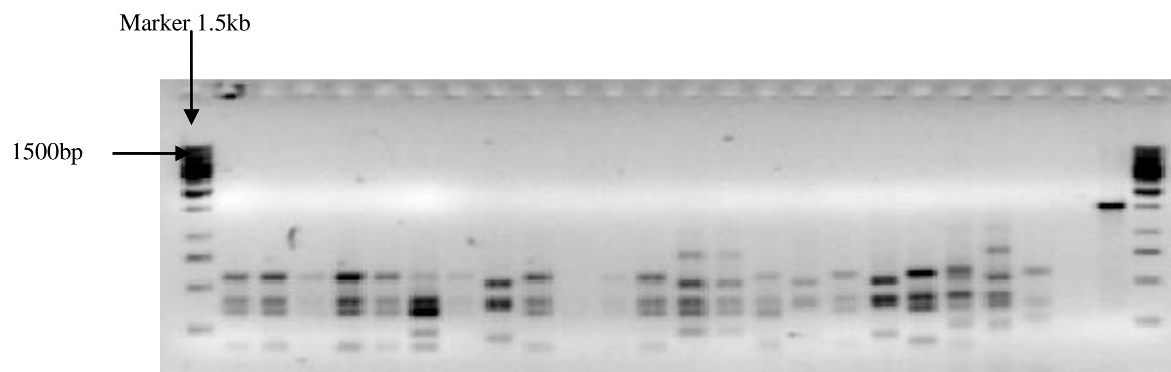

Figure 13: Restriction fragment polymorphisms the remaining isolates (RFLP) profiles

The RFLPs indicated that thirty three (33) out of the fifty (50) isolates with ability to produce bioactive compounds fall into eight profile (Table 7). Ten representative(s) were selected from the eight profiles, amplified further by PCR and sent for sequencing sequencing.

Table 7. RFLPs of bacterial isolates with their representative sample identity, and next relative

RFLP	Sample Identity	Representative sent sequencing	Next relative	% Sequence similarity
RFLP 1	KGA8S23	KGAS23	Variable from gamma to Delta proteobacteria	Not applicable
RFLP2	KSC8G21,KSC8S23, KSC12S21,JGC8S21, KGA8N31,JGA12S31, JGA12S22	KSC12S21	*Bacillus subtlis*	96
		KGA8N31	*Bacillus luciferensis*	96
		KSC8S23	*Vagoococcus Carniphilus*	97
RFLP 3	JGC10G21 and JGC8N22	JGC10G21	*Enterococcus faecalis*	96
RFLP 4	KGA8N24,JGC10S22 JGC10N22,JGA12S22	KGA8N24	None. Sequence was bad	Not applicable
RFLP 5	JGC10N22, JGA12S21 JGC8S21	JGC10N22	None. Sequence was bad.	Not applicable
RFLP 6	JGA10G22,KSC10N22 , JGC10N23	JGC10N23	None. Sequence was bad	Not applicable
RFLP7	KSC12N32	KSC12N32	None ,sequence was bad	Not applicable
RFLP 8	JGA10N22	JGA10N22	*Clostridium* sp (Accession DQ 479417)	86

4.6 Phylogenetic cluster analysis of sequences

The PCR amplified products from the10 isolates were sequenced. Only five sequences were good and were used to construct a Phylogenetic tree. The rest of the sequences gave hits that had no significant similarities from BLAST search results due to low percentage similarity <86%). In addition the BLAST search results showed that the isolates belong to the domain bacteria (Figure 14). The five isolates clustered within phylum Firmicutes, These isolates shared sequence similarity of 86-97% with known members of classes Clostridia, Bacilli and Carnobacteria.

The phylogenetic tree constructed showed the Phylogenetic position of each isolate (Figure 14). Isolate JGA10N22 clustered with Clostridium sp (Accession number DQ 479417) with a sequence identity of 86 %. This was supported by a bootstrap value of 98% indicating that isolate JGA10N22 could be related to the Clostridium sp. KSC8S23 clustered with *Vagococcus carniphilus* (Accession number AY 179329) with sequence identities of 97%. This was supported by bootstrap value of 100%. KGA8N31 clustered with *Bacillus luciferensis* (Accession number AJ419629) with sequence identity of 96%, which was supported by Bootstrap value. KSC12S21 clustered with Bacillus subtilis with sequence identity of 96% as supported by Bootstrap value of 100%. This was JGC10G21 clustered with *Enterococcus faecalis* (Accession number AB012212) with sequence identity value of 96% that is supported by Bootstrap value of 100%

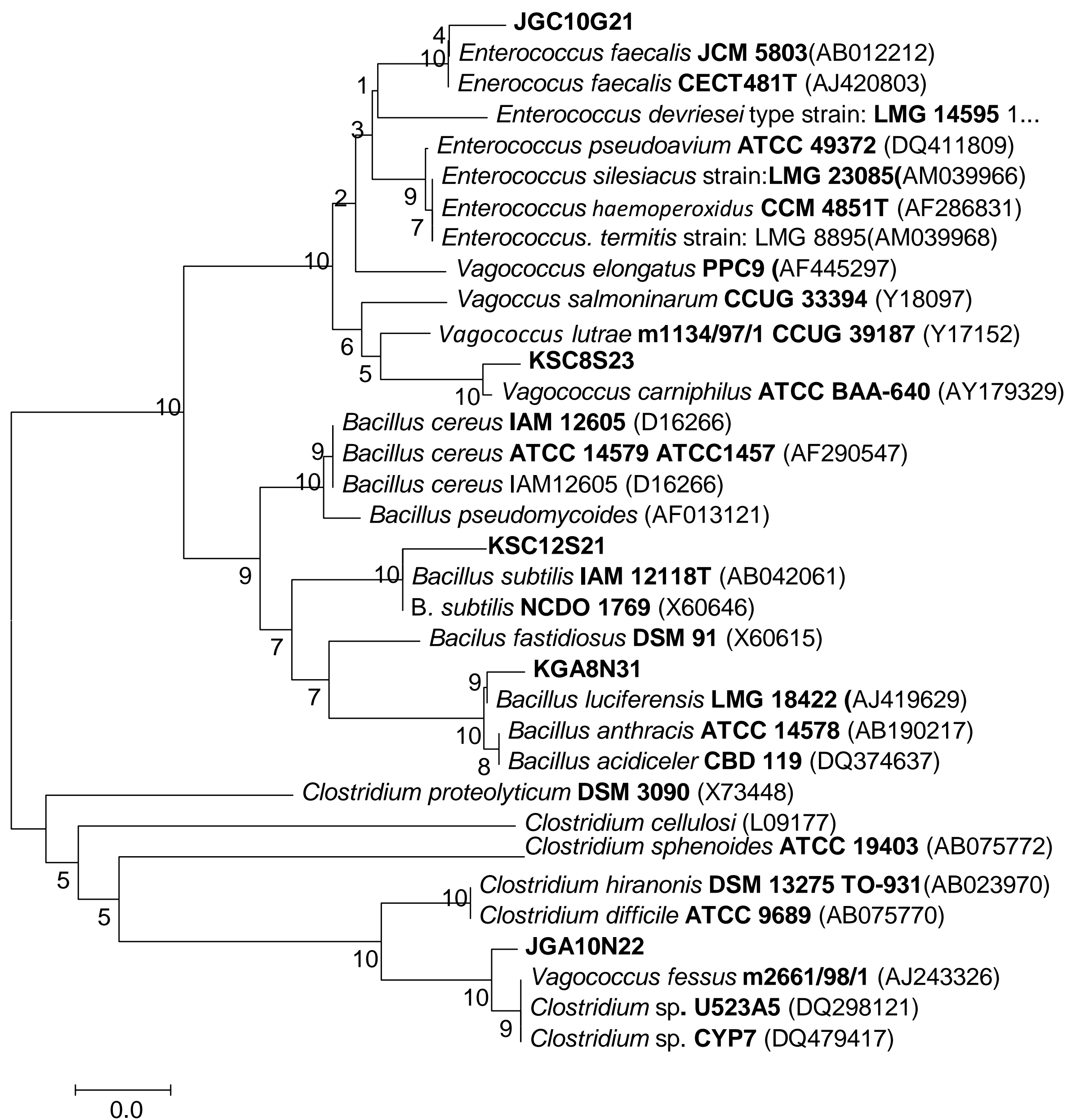

Figure 14: phylogenetic positions of the 5 isolates with the ability to produce bioactive compounds

CHAPTER FIVE

5.0 DISCUSSION

The gut homogenates of the two termite species yielded the highest numbers of colony forming unit followed by the nest materials and the least colony forming units observed was from parent soils (Table 1). The gut of *Cubitermes ugandensis* contained of about 10^8 colony-forming units per gut. This is consistent with earlier studies by Schmitt- Wagner *et al*. (2003) that have shown that enumeration of bacteria in gut sections *C. ugandensis* by DAPI staining contained of about 10^8 microorganisms in all major gut sections. The gut of the two termite species had the highest mean of colony forming units($1.795 \times 10^8 \pm 2.136$ colony forming units /gut), followed by nest ($1.015 \times 10^7 \pm 1.756$ colony forming units /ml) and the least was from parent soil ($9.4 \times 10^6 \pm 0.6$/ml) .This is consistent with earlier findings that show that the intestinal tract of soil feeding termites contains a high density of microbial cells (Bignell *et al*., 1980) and high concentrations of microbial products in the individual gut compartments indicating the presence of an active gut microbiota (Tholen, 1999).The environment for microorganisms derived from soil and faeces is modified not just by an increase in available organic compounds but also by change of their qualities (C/N, humic/sugar content (Fall *et al*., 2001; Sall *et al*., 2002). This richness in organic matter appears to be the reason for the increase in microbial density in the termite mounds (3 to 24 times) (Fall *et al*., 2004). This increase in density is not accompanied by a significant increase in bacterial activity (mineralization) with the neighboring soil. Termite

mounds therefore provide a site where organic matter is protected from strong mineralization that is characteristic of the tropical ecosystem. Previous studies have shown that the bacterial genetic structure of the mounds of soil feeding termites differs from that of their surrounding soils (Fall *et al*., 2004). It has been well established that termites maintain endosymbiotic relationships (digestive bacteria) and exosymbiotic relationships (Fungus-growing termites) with microbial communities (Bignell and Eggleton, 2000).

There was no significance on the effect of the different media on colony forming units ($F=0.023$, $P=0.977$). The cell densities were not depended on the type of medium used. This could be attributed to there being no preference for starch to glycerol and vice versa as carbon source and also on no preference to arginine to casein as nitrogen source. The effect of pH on the colony forming units was not significant ($F=2.175$, $P=0.124$) with the highest cell densities recorded at pH 8 followed by pH 10 and the least cell densities were recorded at pH 12 (Table 1).

Soil had the highest diversity with mean number of isolates of 5.44±SE 0.232, followed by the gut with a mean of 5.39±SE 0.244 and the nest had the least with a mean of 5.06±SE 0.262.The bacterial diversity was low with an average of 3 isolates being obtained per plate. This is confirmed by earlier studies that have shown that using cultivation techniques, only a small number of isolates are obtained (0.1%) as

supported by earlier findings by Miambi *et al*. (2006) The bacterial diversity in this study was low consistent with earlier findings (Mackenzie *et al*., 2007) This could be attributed to the fact that cultivation is known to capture a small insignificant fraction of microbial diversity in a given sample.

The morphological characteristics of isolates KSC8S23 are consistent with those of carnobacteria. The general description of carnobacteria corresponds to that of the lactobacilli in as far as the former are gram positive, catalase negative non-spore forming rods that are usually non-motile do not reduce nitrate and have a fermentative metabolism (Collins *et al*., 1987; Holzapfel and Gerber, 1983; Show and Harding, 1985). The genus contains seven (7) validly described species with *Carnobacterium* being the type species. These organisms share some habitats with lactobacilli but differ in certain physiological properties (Hammes and Hertel, 2006). The carnobacteria may be described as short to medium length, straight slender rods, with rounded ends occurring singly in pairs or sometimes in short chains (Hammes *et al*., 1992). Other properties that are shared with most lactobacilli include the inability to produce gelatinase, indole or H2S. No growth occurs at 45o C or in the presence of 8% NaCl or the pH 3.9 (Collins *et al*., 1987; Holzapfel and Gerber, 1983). Isolates KSC8S23 had sugar utilization patterns consistent with those of family Carnobacteriaceae. This is confirmed by earlier studies that show that carnobacteria strains seem to have the following common properties (Borch and Molin, 1988;

Collins *et al*., 1987): acid production from celliobose, D-fructose, D-glucose, maltose, D-mannose, ribose, salicin and sucrose. Acid is not produced from arabinose, fucose, arabinose, arabitol, erythriol, glycogen, inositol, raffinose, L-Rahamnose, L-sorbose, xylitol and xylose. All strains by Collins *et al*. (1987) produce arginine deaminase. Isolates KSC8S23 grew well at pH 6 to 10. This is consistent with earlier studies that have shown that a neutral to high pH ranging from 6.8 to 9.0, favours growth and that range 8.0 to 9.0 may serve as selectively inhibit lactobacilli that are often found in association with carnobacteria (De Man *et al*.,1960). The five isolates formed organic acids as indicated by the disappearance of CaCO3 and colour change to yellow with 5-15 days in the basal medium (Table 6).

JGC10G21 was obtained from gut of *M. michaelseni* and it produced organic acids as shown by the disappearance of $CaCO_3$ and colour change in the medium to yellow. This is consistent with earlier studies that have shown that there is accumulation of butyrate and succinate in various gut sections of *Cubitermes orthognathus* and other soil feeding termites (Tholen, 1999) that is in general agreement with the presence of these groups.

The five isolates did not require salt for growth but were able to tolerate concentrations up to 7% (Figure 10). Adaption to high salt concentration has been reproduced in vitro (Okazaki and Okami 1975; Okami and Okazaki, 1978), salt

sensitive streptomycetes subcultured in increasing concentrations of NaCl yielded salt tolerant variants. However there is no isolate that proved to be obligate halophile unable to grow without salt. This is consistent with earlier studies (Okazaki, and Okami, 1976) who found only few isolates that were obligate halophiles.

Isolate JGC10G21 was able to grow in glycerol-based medium, which is consistent with characteristics of genus *Enterococcus.* Earlier studies in this genus indicate that most species of ferment glycerol under anaerobic or microaerophilic conditions (Gunsalus and Sherman, 1943; harrier *et al*., 1997; Huycke, 2002). Among the enterococci only *Enterococcus faecalis* has been shown to utilize glycerol as a source of energy under anaerobic conditions (Gunsalus and Sherman, 1943). Only *E. faecalis* among the enterococci has been shown to utilize agmantine and L-serine as sources of energy (Deibel, 1964). Isolate JGC10G21 was catalase negative that is in agreement with characteristics of genus *Enterococcus* (Leblanc, 2006).

The isolate JGA10N22 had characteristics consistent with those of the genus *Clostridium*. The genus *Clostridium* exhibits a great diversity with respect to the morphological and metabolic, nutritional requirements and G+C content (Andreesen, 1990). Clostridia produce interesting fermentation products, carry out bioconversions and secrete useful enzymes and proteins. Clostridial fermentation products of importance are n-Butanol, acetone, propanediol-1, 2, propanediol -1, 3, ethanol and

butyrate (Hippe *et al*., 1992). Isolate JGA10N22 did not produce pigments. This is consistent with characteristics of clostridia. Most cells of this group as well as of most anaerobes don't contain bright pigments such as caretenoid derivatives. Surface colonies can be translucent to dull white and buff coloured to dark, dull or shiny gray (Wiegel *et al*., 2006). Isolate JGA10N22 grew well at pH 6 to 10 (figure 9). This is consistent with earlier studies that have shown that several clostridia grow at pH values above 8.0 although their optimum pH is at neutral pH values (Alkalitolerant species). True alkaliphilic species are the thermophilic *Clostridium paradoxum* and *Clostridium thermoalkaliphilum* (Peptostreptococcaceae) with pH around 10 (Wiegel *et al*., 2006). The isolate JGA10N22 was oxygen tolerant .The oxygen tolerance of clostridia species ranges from relative insensitivity such as *Clostridium aerotolerans* (Van Gylswyk and Van de Toon, 1987) to extreme sensitivity such as for some of the hydrogen oxidizing acetogens (Drake,1994).

Results from molecular characterization of the isolate indicate that all the isolates belongs to the domain Bacteria, phylum Fermicutes classes; Carnobacteria, enterococcaceae, bacilli and *Clostridium*. Similarity searching using BLAST shows that the isolates belong to the domain Bacteria. A phylogenetic tree showing the Phylogenetic position of each isolate is shown in Figure 14. Since the isolates did not cluster 100% with respective species, it can be concluded that they are novel isolates or they have never been cultured. The findings of the study are consistent with earlier

studies that have shown that the largest number of clone libraries represent gram-positive with low G+C content affiliated with clostridia and Gram negative bacteria of *Cytophaga – Flexibacter -Bacteroides* phylum (Schmitt-Wagner *et al.*, 2003). Representatives of these phyla are typical components of the intestinal microflora of mammals including human (Franks *et al*., 1998; Hold *et al*., 2002; Leser *et al.*, 2002; Suau *et al*., 1999) and have also been recovered from wood feeding and soil feeding termites either by cultivation (Breznak and Brune, 1994) or as 16S rRNA gene.

Isolates KGA8N31 and KSC12S21 clustered members of genus *Bacillus* and were capable of producing antibiotics as secondary metabolites in the late logarithmic or early stationary phase of growth. Various strains of *Bavillus subtilis* have been shown to produce 68 antibiotics while *Bacillus brevis* can produce 23 (Katz and Demain, 1977).These isolates showed antagonistic activities against test organisms. This is consistent with earlier studies that have shown that most of antibiotics are active against Gram-positive organisms, although there are exceptions. The majority is peptide antibiotics but some belong to other chemical classes such as aminoglycosides.

Isolates KSC8S23, JGA10N22 and JGC10G21 (Table 5) also had antagonistic properties against test organisms, although sequence identities indicate that they belong non-antibiotic producing genera; *Vagococcus*, *Clostridium* and *Enterococcus*

respectively (Table 4). Isolate JGC10G21 was antagonistic to test bacteria probably due lytic activity of cytolysins produced by the isolate which is consistent with earlier findings that indicate that the cytolysins produced by *Enterococcus faecalis* are able to lyse a variety of eukaryote cell types, including erythrocytes, as well as several species of Gram positive bacteria (Haas and Gilmore, 1999).

There has been considerable interest in producing large quantities of enzymes for industrial purposes- proteases for detergent supplementation, the brewing industry, various uses in the food industry and in leather manufacturing; and different amylases for brewery use, in bread making, and in the paper industry (Debabov, 1982). Isolates KGA8N31 and KSC12S21 were proteolytic which is in agreement with the characteristics of the genus bacillus. Studies have shown that during vegetative growth and subsequent sporulation, a variety of proteases are produced (Priest, 1977). There are six extracellular proteases and at least three major intracellular proteases—ISP, esterase A, and esterase B. They may be involved with turnover of intracellular proteins, the processing of protein precursors for spore coats, or inactivation of later sporulation enzymes, as well as other functions.

Isolate JGA10N22 was non – cellulolytic (Table 5) however a special configuration of extracellular enzymes has been found in the anaerobic, thermophilic and mesophilic clostridia and related bacteria which are able to hydrolyze crystalline cellulose (Table

5). The cellulolytic enzymes are excreted as large enzyme complexes called cellulosomes (Bayer and Lamed, 1986) containing various cellulases, xylanases and esterases. Cellulolytic clostridia digest cellulose through extracellular multienzyme complexes (Béguin & Lemaire, 1996; Bayer *et al.*, 1998). The cellulosomes are very efficient degradative activity against crystalline cellulose due to a high synergism of the different cellulase components (Boisset *et al.*, 1999). Isolate JGA10N22 was xylanolytic consistent with earlier findings in which xylosomes associated with anaerobes had been proposed but not yet unequivocally demonstrated for any lo G+C gram-positive bacteria strains (Table 4). However for several of the anaerobic Fermicutes (such as *Thermoanaerobacter* and *Thermoanaerobacterium*), protrusions of cell wall compounds have been observed to contain extracellular starch hydrolyzing enzymes (Antranikian *et al*., 1987).

The five (5) isolates produced a variety of extracellular enzymes including amylases, lipases, gelatinases, proteases and xylanases (Table 5). It can be postulated that these microbial enzymes are involved in the nutritional physiology of the soil feeding and Fungus- cultivating termites. This is in agreement with earlier studies in which it was noted that physiological functions of symbiotic prokaryotes in termites were extremely diverse including cellulose digestion (Hether *et al*., 1992), hemicelluose digestion (Taguchi *et al*., 1996), acetogenesis (Taguchi *et a*l., 1996; Taguchi *et al*., 1993), methanogenesis (Brauman *et al*., 1992), sulfate reduction (Kuhgink *et al*., 1996), and

nitrogen fixation (Ohkuma *et al*., 1996; Tayasu *et al*., 1994). Intestinal bacteria also contribute to creating suitable conditions for symbiotic flagellates through production of nutrients and the maintenance of the pH and anaerobic conditions in lower termites. It has also been reported that termites belonging to the Termitidae which do not have symbiotic protozoa and are known as 'higher termites 'possess bacteria not only in the hindgut but also in mixed segment (Bignell *et al.,* 1983; Czolij *et al*., 1983). Clostridia are usually known as soil bacteria and are also found in the rumens and intestines of animals and humans (Chen 1995; Dehority *et al*., 1991 and Bignell *et al*., 1983).

Intestinal bacteria are important to the health of humans (Bignell *et a*l., 1983) and some predicted roles of intestinal bacteria are similar to those in humans such as producing short chain fatty acids from carbohydrates or synthesizing amino acids (Cummings and Mcfarlane, 1997). Because many clostridia degrade polysaccharides to produce acetone alcohol, acetate, lactate, CO2 and H2 (Chen, 1995; Hazlewood and Gilbert, 1993; Rainey and Stackbrandt, 1993; Mitchell, 1992 and Johnson and Goldfine, 1985) and other can ferment nitrogenous or lipidic compounds ((Johnson *et al.,* 1985), it is expected that the isolate JGA10N22 obtained from nest materials of *Macrotermes Michaelseni* play roles in the nutritional physiology of the fungus cultivating termites. Some clostridial species are source good stable enzymes especially amylolytic and cellulolytic enzymes (Saha *et al*., 1989). Since several *Clostridium* and *Desulfovibrio* species are known to be N_2-Fixing bacteria, the isolate

JGA10N22 that cluster with clostridium represent possible N_2-fixing candidate in the termite hindgut (Ohkuma and Kudo, 1996).

All the five isolates were proteolytic indicating their role in decomposition of organic matter in nature (Table 5). This is consistent with findings with other researchers in which Peptides are have been shown to be by far the most abundant nitrogenous compounds in soil organic matter (Sowden *et al.*, 1976; Schnitzer, 1985). Feeding experiments with ^{14}C-labeled humic model compounds have provided evidence the peptidic component of humic substances is preferentially solubilized and mineralized during gut passage (Li and Brune, 2005).

CHAPTER SIX

6.0 CONCLUSIONS

The study demonstrated that parent soils, nest materials and intestinal tracts of *Cubitermes ugandensis* fuller and *Macrotermes michaelseni* are rich in bacteria with antagonistic properties against bacteria and fungi.

The study shows that there was no significant effect of the incubation pH and media on the isolation of bacteria from *M. michaelseni* and *Cubitermes ugandensis*. The study also indicates that the isolates grew well at pH 6 to 10

The effect incubation pH on the colony forming was not significant (F=2.175=0.124) with the highest cell densities recorded at pH 8 followed by pH 10 and the least cell densities were recorded at pH 12

The study shows that the isolate have ability to produce bioactive compounds (Enzymes and antibiotics).These compounds could be novel with biotechnological potentials.

Based on observations from the study it can be speculated that isolates play role in the nutritional physiology of soil feeding and fungus-cultivating termites as indicated by production of amylases, xylanases, proteases, and lipases.

6.1 RECOMMENDATIONS

There is need to investigate the variations of bacteria in relation growth parameters such as temperature, pH requirements and culture media composition for isolation to correspond with those of natural habitats of different ecological zones. Changes could result in differences in the abundance and diversity of isolates obtained from each area as some isolates may grow under some conditions and not others.

Further studies should be carried to determine how bacteria diversity influences environmental functions as nutrient cycling, degradation of xenobiotics and ecosystem stability

More research should be done on cultivation techniques and new approaches and Media developed that can be used to isolate uncultured bacteria obtained from Gut and nests

Further studies using molecular characterization of community DNA should be carried out to enhancing the understanding and knowledge of the structural composition and dynamics of the microbial population.

Further molecular characterization should be done to the remaining isolates that did not produce bioactive compounds,

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Appendices

Appendix I: International *Streptomyces* project (ISP) medium 2

Agar 20.0g

Yeast extract 10.0g

Malt extract 4.0g

Glucose 4.0g

Trace salts solution 1.0ml

Trace salt solutions

Composition per litre

$FeSO_4 . 7H_2O$ 0.1g

$MnCl_2.4H_2O$ 0.1g

$ZnSO_4.7H_2O$ 0.1g

Appendix II: International *Streptomyces* project medium 9

Composition per litre

K_2HPO_4 5.65g

$(NH_4)_2SO_4$ 2.64g

$MgSO_4.7H_2O$ 1.0g

KH_2PO_4 2.38g

Carbohydrate solution 100ml

Pridham and Gottlieb trace salts 1.0ml