ASSESSMENT OF BACTERIAL PATHOGENS DIVERSITY AND BEEKEEPING CONSTRAINTS IN *Apis mellifera* COLONIES OF EMBU COUNTY, KENYA

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

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DEDICATION

I dedicate this work to my family.

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LIST OF ABBREVIATIONS/ACRONYMS

AFB	American Foul Brood
BLAST	Basic Local Alignment Search Tool
EDTA	Ethylene Diamine Tetra-Acetic Acid
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
OTU	Operational taxonomic unit
TSA	Tryptone soy agar
SDS	Sodium dodecyl sulphate
QIIME	Quantitative insight into microbial ecology
RPM	Revolutions per minute
SSU	Small subunit
FLAB	Fructo lactic acid bacteria
TAE	Tris Acetate EDTA

ABSTRACT

Wild and managed honeybees (*Apis mellifera*) contribute towards economic gain as a source of income and food. Additionally, honeybees provide pollination services to numerous plants thus contributing greatly to food chains, ecosystem functions and world economy. Bee health is thus crucial in the attainment of these diverse roles. Over time persistent bee population decline has been reported globally due to biotic and abiotic stresses. Among the biotic factors are the honeybee diseases caused by bacteria, fungi and viruses. Among the bacterial pathogens, the most pernicious and with a world wide distribution is the American foulbrood (AFB) caused by *Paenibacillus larvae*. Additionally, the honeybee microbiome and its immediate hive environment is composed of various microbial symbionts that are involved in mutualistic, commensalistic or parasitic interactions, and which have significant impact on bee health. The objective of this study was to determine the diversity of bacteria and constrains associated with *Apis mellifera* keeping in Embu County, Kenya. Specifically, the study established constraints associated with *Apis mellifera* production, determined bacteria diversity within the honey and honeycombs using culture-dependent approach and metagenomic approach. Questionnaires were used to establish constraints associated with beekeeping. Samples of adult bees, honey, honeycombs, frame scrappings and bee larvae were collected randomly from bee farmers, and total DNA was extracted and genotyped using the 16S rRNA marker gene. Results showed that colony decline was associated with pests (42.3%), swarming (20.6) and hive abscondment (37.1%) across the traditional, transitional and frame hives. A total of 17 bacteria isolates were obtained using the culture-dependent approach. Based on comparative analysis of their sequences with closest relatives from National Center for Biotechnology Information (NCBI) GenBank database, results revealed that these isolates were affliated with the phylum Firmicutes, Proteobacteria and Actinobacteria. They were represented by the genera *Bacillus, Microbacterium, Micrococcus, Serratia, Staphylococcus, Klebsiella, Erwinia* and *Devosia*. Key observation was the utilization of multiple carbon sources exhibited by bacterial isolates. This indicates the significance of bacteria degrading diverse polysaccharides in honeybee nutrition.Using metagenomics approach, 499,300 nucleotide sequence reads were obtained. These were represented by the phyla Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria and Cyanobacteria. The most abundant genera comprised of *Fructobacillus*, *Lactobacillus*, *Gilliamela*, *Bombella*, *Frischella*, *Enterobacter*, *Lactococcus*, *Serratia*, *Leuconostoc* and *Bacillus*. These were similar to the core honeybee gut bacteria signifying stability in honeybee metabolism and nutrition. The findings of this study showed that the bacterial community associated with honeybees derived from the host and the bee-hive environment play a crucial role in host nutrition and health modulation. The study recommends the prioritization of management strategies to reduce potential stressors that result in a hive absconding. Data generated from this study can be used in conservation and preservation of this important insect and ecosystem service provider.

CHAPTER ONE
INTRODUCTION

1.1 Background of the study

Bees are winged insects that mainly depend on floral nectar and pollen for food and nutrients. They belong to phylum Arthropoda, class Insecta and order Hymenoptera. Of particular interest are the honeybees, *Apis mellifera* L. (Family: Apidae) which have significant economic value due to their role in honey production and crop pollination. Honeybees are known for their sociality and have a haplodiploid sex determination system in which the queen's unfertilised eggs develop into males whose role is to mate with the queen (Moškrič *et al*., 2020). Fertilised eggs develop into either queens or worker bees. Worker bees are non-reproductive and are involved in foraging pollen, nectar and water outside the hive and the inhive cleaning (Remolina & Hughes, 2008).

Honeybees, both managed and the wild are important for pollinating a variety of plants and food crops (Usta *et al*., 2025). Pollination is critical in ecosystem balance maintainance and crop production. Pollinators are thus essential in crop yield process and these include water, wind and animals. Animal pollinators are key in production of healthy crops for fibres, food, medicines, edible oils and other derivatives (Samanta *et al*., 2024). They have been estimated to account for pollination of about 75% of over 1,300 varieties of plants that are cultivated for food, spices, beverages, medicine and fabric (Das *et al*., 2018). Bees have been reported as the primary insect pollinators of plants with high preference due to increased crop yield in terms of both quality and quantity (Stein *et al*., 2017;Sáez *et al*., 2020).

The global annual economic value of pollination is estimated at between USD$ 195 billion and $387 billion (Porto *et al*., 2020). This guarantees food security in the light of the expanding human population and contributes to the economy at large through trade (Human, 2016). Globally, honeybee domestication throughout most of the world is associated with their long history of interaction with humans and their global distribution in addition to pollination services and production of various hive products. Honeybees provide food, nutritional supplements, and traditional medicinal treatment (Chantawannakul *et al*., 2018).

In Kenya, there are efforts to develop beekeeping as a way of income generation for which people in the Arid and semi-arid (ASAL) can earn income in the wake of climate change (Gakenia *et al*., 2024). Further, two broad systems of beekeeping have been described; the intensive and extensive systems. The intensive system is characterized by use of modern hives or topbar hives on small to medium scale farms mostly on agriculturally productive lands near human settlements (Zocchi *et al*., 2020). Extensive beekeeping system on the other hand is characterized by use of traditional hives which are usually hung on trees and are located away from human settlements. Mainly practiced in arid and semi arid lands (Carroll & Kinsella, 2013). Locally, beekeeping is an important emerging venture owing to the required inputs and space. Increasingly, it has also become a more popular way for small-scale farmers to suppliment their incomes and meet dietary demands (Ongus *et al.*, 2018).

Concerns of the honeybee population decline have been raised since they reflect on stability in food production and eventual loss of livelihood. Numerous stresses have been linked to the decline, including pesticides, agricultural intensification, habitat fragmentation and emerging diseases and pests (Regan *et al*., 2018). Human associated activities such as bush clearing and overgrazing affect the ecosystem's overall health by reducing nesting sites and food resources. This eventually damages the bee population (Kasina *et al.*, 2009). Pesticides, migratory beekeeping practices, contaminated water and poor nutrition have contributed to bee decline (vanEngelsdorp & Meixner, 2010). Pathogens and parasites affecting bee health which include bacteria, viruses, fungi, mites and microsporidia have also been described to influence the beekeeping operations (Awino *et al*., 2018).

Honeybee bacterial microbiome studies have been carried out and major focus has been on the gut associated communities, honeybee pathogenic bacteria and the hive associated bacteria (Zheng *et al*., 2018). The honeybee gut is consistently composed of specific bacterial species occupying distinct regions tasked with different functions. The honeybee gut bacteria functions include formation of biofilms, breakdown of pollen walls, carbohydrate metabolism and fermentation (Zheng *et al*., 2019). These bacteria species include the *Bifidobacterium* and *Lactobacillus* (firm 4 and 5) occupying the rectum, *Frischella perrara*, *Gilliamella apicola* and

Snodgrassella alvi occupying the ileum, while the midgut region is dominated by *Bombella apis* (Kwong & Moran, 2016).

Among the bacterial pathogens, the most damaging honeybee disease is *Paenibacillus larvae* causing American foulbrood (Giménez *et al*., 2020). American foulbrood is distributed throughout Africa except in the Central African region (Mumoki *et al*., 2014). As a result of the occurrences, temporal monitoring studies aimed at understanding the factors that influence honeybee colony health, disease prevalence and abundance over the geographical regions and seasons are crucial (Glenny *et al.*, 2017).

The hive environment harbors a diverse bacterial community introduced from the foraging environment into the foodstores, wax comb, pollen and the propolis. These bacteria have been implicated in the maintainance of healthy microbiomes within the hive and perhaps involved in processing, storage and preservation of pollen (Rokop *et al*., 2015). This study was aimed at elucidating the bee microbiome and bacterial pathogens diversity and constraints associated with bee health and beekeeping industry at large.

1.2 Statement of the problem

Over the years, concerns have been raised on the alarming bee population decline, given their important role in the ecosystem and economy at large (Human, 2016). The decline, however, has been variable in terms of regions such as in the USA, which recorded 59% colonies loss between 1947 and 2005, whereas a 25% colony loss was recorded in Central Europe between 1985 and 2005 (Potts *et al.,* 2010). In Kenya, reports on colony losses characterised by a declining number of colonized hives, infrequent migrating swarms which are also reduced in size, and empty hives that are not easily colonized have been documented (Muli *et al.,* 2014). At the hive level, stressors such as pests and predators have been linked to the weakened colonies either by killing the bees, comb destruction and eventual abscondment of the hives. At the individual level, honey bees are also attacked by various pathogens ranging from fungi, viruses and bacteria that threaten the entire bee's health.

1.3 Justification of the study

Locally, honeybees play a significant role in nutrition and income for farmers since beekeeping is mostly practiced for the honey and other hive products production. Additionally, honeybees play an integral part in providing essential pollination services. Wild plants and major food crops are dependent on pollination. Frequent absconding of hives have been reported despite being a common occurrence and usually associated with swarming, which gives rise to new colonies. Factors such as loss of habitat, poor management practices, toxin exposure through unwise application of agrochemicals, malnutrition, pathogens, parasites and climate change have also been linked to the declining bee population trend.

In Kenya, there have been no reports on the occurrence of honeybee bacterial pathogens, a case which may be associated with the near absent routine screening and surveillance of pathogens (Ongus *et al*., 2018). Information on beekeeping constraints will help in regular monitoring and routine surveillance towards maximization of beekeeping.Using modern sequencing technologies such as the 16S rRNA gene sequencing will help generate high throughput data on the composition of microorganisms associated with hive environment (Dash *et al*., 2025). The study findings will bring out an improved understanding of the indigenous bacteria diversity within the hive materials and also help appreciate the interaction, function, spread and communication of microorganisms both in stressed and healthy bees. In summary, the generated data will be used in the management of honeybee production constraints and in the conservation of honey bees population.

1.4 Research questions

1. What are the constraints associated with *Apis mellifera* keeping in Embu County, Kenya?
2. What is the culturable bacterial pathogens diversity within the honey and honeycombs from Embu County, Kenya?
3. What is the total bacterial pathogens diversity associated with honey and honeycombs in Embu County, Kenya?

1.5 Objectives

1.5.1 General Objective

To assess the diversity of bacterial pathogens and associated constraints in *Apis mellifera* keeping in Embu County, Kenya.

1.5.2 Specific Objectives

The specific objectives of the study were:

1. To establish the constraints associated with *Apis mellifera* production in Embu County, Kenya.
2. To determine the culturable bacterial pathogens within the honey and honeycombs from Embu County, Kenya.
3. To establish the total bacterial pathogens diversity and distribution associated with the honey and honeycombs in Embu County, Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bees

Bees are winged insects that depend on plant derivatives: nectar and pollen for food. They are usually classified taxonomically as belonging to the phylum Arthropoda, class Insecta and order Hymenoptera. Seven families are recognized and classified based on the morphology of the labial palp: families *Apidae* and *Megachilidae* making up the long-tongued bees while the families of short-tongued bees consist of the *Strenotritidae*, *Adrenidae*, *Colletidae*, *Melittidae,* and *Halictidae* families (Michez *et al,*. 2007). Family *Apidae* forms the most iconic group characterized by the possession of corbicula; a specialisation of the hind tibia in females of pollen collecting species (Starr, 2021). This lineage of corbiculate bees comprise of the Euglossini (orchid bees), Meliponini (stingless bees), Apini (honeybees) and Bombini (bumble bees) (Engel & Rasmussen, 2020).

Honeybees (*Apis mellifera* L) are members of the *Apidae* family in the Apini linage (Cédric *et al*., 2015). Ten species in the genus *Apis* have been described and are grouped into three categories namely the enclosed-nesting bees which include *A. mellifera*, *A. koschevnikovi* Enderlein, *A. cerana* Fabricius, and *A. nulensis* Lin (Nowak *et al*., 2021). Dwarf bees include, *A. florea* Fabricius and *A. andreniformis* Smith while the Giant bees include *A. dorsata* Fabricius, *A. laboriosa* Smith, *A. binghami* and *A. nigrocincta* Smith (Awino *et al,*. 2018). Globally, many *Apis mellifera* L. subspecies have been described. Classification is based on molecular techniques and morphological characteristics (Ilyasov *et al*., 2020). Africa is home to 11 honeybee subspecies namely: *Apis mellifera intermissa*, *Apis mellifera sahariensis*, *Apis mellifera lamarckii*, *Apis mellifera jemenitica*, *Apis mellifera sudanensis*, *Apis mellifera monticola*, *Apis mellifera litorea*, *Apis mellifera adansonii*, *Apis mellifera scutellata*, *Apis mellifera capensis* and *Apis mellifera unicolor* (Human, 2016).

2.2 History of beekeeping

In Kenya, beekeeping is mainly practiced in environments where the natural vegetation is composed of many species of plants suitable for bees to forage on and agricultural fields where croppings are limited. This has been embraced by the communities living in arid and semi arid areas as well as those living in forested areas owing to the reliance on traditional and indigenous knowledge and skills (Zocchi *et al*., 2020). Two broad sytems have been put into practice that is; extensive beekeeping practice where traditional log hives are hung in trees and scattered over large areas away from human settlements. The practice is common in the dry and semi-arid regions in Kenya (Gakenia *et al*., 2024). The other practice is the intensive system where modern hives are mostly used and bees are kept primarily on small to medium scale agriculturally productive land in the country (Carroll & Kinsella, 2013).

2.3 Importance of bees

In agricultural and non-agricultural settings, different bees species dominate insect pollination. When managed pollination is not available, crops rely on solitary bees as well as honeybees for pollination (Kasina *et al*., 2009). Honeybees are domesticated for their pollination services, conserving biodiversity, apitherapy, revenues and production of various hive products, including bee waxes, honey, propolis, royal jelly and venom (Belsky & Joshi, 2019). Bee waxes from the colony's foundation have wide application in candle making, pharmaceuticals, cosmetic industry, art, varnishes and polishes. Bee pollen is known to have antioxidant properties that are used in apitherapeutic treatment and is also used as a nutritional supplement (Komosinska-vassev *et al*., 2015). Royal jelly produced by young nurse bees is a popular product for the cosmetic industry and functional food industry (Collazo *et al*., 2021). Propolis is entirely composed of plant exudates with antibacterial properties, and is used in food, therapeutic and pharmaceutical industries (Aryal *et al*., 2020). Bee venom has been used for treatment of chronic inflammatory disorders (Khalil et al., 2021).

2.4 Honeybee keeping constraints

Honeybees pollinate plants for a huge percentage of the world's crops growing. Locally, beekeeping is vital for food security, poverty reduction and health in cases such as where honey is used as medicine. Several factors have been linked with the

weakening of the beekeeping practice. Biotic and abiotic factors all come into play for the successful practice (Wakgari & Yigezu, 2021). Natural bee enemies, pests and predators ranging from birds, honey badgers and rodents cause significant harm to the beehive, honey and other hive products. Skunks prey on beehives at night and eat a large number of bees. All of these disruptions are known to trigger migration and disappearance. As a result, number of colonized hives reduce as well as the honey produced since few bees take much time to make sufficient wax and thus energy that would have been directed towards pollen collection and honey production is lost (Bekele *et al*., 2017). Vulnerability to numerous pathogen and parasites such as bacteria, fungi and viruses serves as a blow to the honeybee colonies and products. Their presence translates to reduced longevity and reduced yields (Genersch, 2010).

Pesticides ranging from insecticides, fungicides and herbicides pose a great risk to the pollinators in terms of associated toxicity and level of exposure. In indirect cases, herbicide application reduces flowering plants diversity that offers pollen and nectar to the pollinators (Yang *et al*., 2024). In direct cases given the nature of honeybees visiting different flower sites while foraging, consumption of the residues is inevitable. Foraging adult honeybees are directly exposed to pesticides by flying over sprayed surfaces and the consumption of contaminated pollen and nectar. This causes a change in the hive environment as all the lifecycles are affected either by weakening or death, which further renders them vulnerable to other hive associated enemies (Potts *et al*., 2016).

2.5 Symbionts associated with honeybee

The bee microbiome comprises a variety of microbial symbionts, which interact in mutualistic, commensalistic or parasitic ways all of which have major effects on bee health (Gakenia *et al*., 2024). Nutritional mutualism is essential for adult bees which rely on the gut microbiota to meet their nutritional needs from different compounds in their food. However, in order to achieve their nutritional needs, larval bees depend on external pollen-borne microbial symbionts (Dharampal *et al*., 2019). The phylotypes for honeybee microbiota have mostly been identified and classified into two major ecological niches of the adult gut; the core gut (midgut through hindgut) and the crop (foregut), where each has a unique composition (Schwarz *et al*., 2015).

Past studies have been directed towards identifying the core microbial symbionts existing in honeybee guts and their diversity, function and activity such as anaerobic breakdown, pollen predigestion, and the effects of the dysmbiosis (Disayathanoowat *et al*., 2020). Honeybees are social insects; thus, the host–symbiont relationship is seen in the perspective of the colony niche (Anderson *et al*., 2011). Commensal fungi and bacteria adapted to one niche are likely to occur in another hive microenvironmentfor a short time or regularly. The microbiota of honeybees resides on two levels: within the holding hive that contains the developing young and the food stores and within the relatively basic alimentary tract (Anderson *et al.,* 2011).

Symbiotic relationship between hymenoptera and fungi have been reported, where fungi act as the food source (Yun *et al.,* 2018). Environmental fungi also represent bee pathogens and yeasts tht can thrive in honey (Wirta *et al.,* 2021). On the other hand, bacteria can be of many origins, such as the gut and the pollination environment (Wirta *et al*., 2021). The dominant microbiota of the honeybees that have been described include families *Acetobacteriaceae, Bifidobacteriaceae, Enterobacteriaceae, Lactobacillaceae*, *Neisseriaceae*, *Orbaceae* and *Rhizobiaceae* (Bleau *et al.,* 2020). In Kenya, core gut microbiota of the honeybee described include: the genus *Gilliamella, Lactobacillus, Snodgrassella, Bifidobacterium, Frischella*, *Commensalibacter*, *Bombella*, *Apibacter*, and *Bartonella* (Tola *et al.*, 2020).

2.6 Bee pathogens

Like most living species, bees are vulnerable to a range of diseases caused by pathogens, which might be fungal, viral or bacterial in origin. These negatively impact productivity and control host bee population within their native habitat (Belsky & Joshi, 2019). Honeybees exhibit a combination of social and individual traits. At the individual level, honeybees have three types of caste in the colony namely workers, drones and queens where each has own specialization within the honeybee society. Food exchange among colony members accompanied by the high density of individuals within the hive presents a suitable environment for pathogens, a condition that translates to an increase in infection rates and diversity of pathogens in failing bee colonies (Tozkar *et al*., 2015).

2.6.1 Fungal pathogens

When enough moisture is available, the beeswax habitat, supplies in the hive, brood and excreta provide a favorable condition for fungal development. Yeast and other fungi associated with bees can be mutualistic, commensal, or parasitic. Crucially, the bee brood especially larvae and pupae, are the most vulnerable members of the colony. This is accelerated by their inability to groom spores from their bodies. Despite the hygienic grooming behavior of the honeybee nurses, establishment of opportunistic fungal infections is inevitable (Miller *et al*., 2021). Common fungal pathogens include: chalkbrood caused by *Ascosphaera apis*, which mostly affects honeybee brood thus affecting the productivity of overall colony (Svečnjak *et al.*, 2019), Nosema disease particularly caused by *Nosema ceranae* and *Nosema apis* that impairs the midgut of the honeybee digestive tract (Mumoki *et al.*, 2014); and Stonebrood caused by a number of *Aspergillus* species, mainly, *Aspergillus fumigatus* and *Aspergllus flavus* (Foley *et al*., 2014).

2.6.2 Bacterial diseases

Two bacterial pathogens that directly affect bee health are *Paenibacillus larvae*, which causes American foulbrood and *Melissococcus plutonius* which causes European foulbrood (Engel *et al.*, 2016). American foulbrood (AFB) is associated with death of infected larvae and is also potentially lethal to infected colonies. The disease spread is facilitated by usual exchange of bee and hive materials between colonies, trading of queens, colonies and honey and managing numerous hives in a confined area (Genersch, 2010).

2.6.3 Viruses

Viral diseases are associated with physiological abnormalities, physical deformities, behavioral disturbances, and decreased longevity of bees. Individual hosts may also have varying degrees of the disease, and the virus can persist indefinitely and asymptomatically. The acute bee paralysis virus, Kashmir bee virus disease, deformed wing virus, chronic bee paralysis and black queen cell virus are some of the recognized bee viral diseases (Rana *et al.*, 2011). Sac brood virus affects the brood resulting in perforation of sealed brood, pre-pupal death due to pupation failure and accumulation of fluid around the body and integument.

Black queen virus is characterized by death of the pupae, larvae and the queen. Death of the larvae and pupae occurs after the cells have been sealed which eventually results in black coloration of the larvae and cell walls. Worker and drone brood show no clear symptoms. This ensures maintenance of black queen virus throughout the year in the colony. This results in pathogen spill over to uninfected bees in the pollination environment (Belsky & Joshi, 2019).

Chronic bee paralysis virus results in greasy or shiny bodies with bloated abdomen, crawling on the surface, and demonstrated body trembling caused by an inability to control flight muscles. Bee-to-bee transmission of the virus occurs through the unusually extended body contact or rubbing. This may cause bristles and hair breakage eventually exposing live tissues and bees die after a few days of the disease onset. Both the adult honeybees and brood are impacted by acute bee paralysis (Mumoki *et al.*, 2014).

2.7 Metagenomics as a microbial community assessment method

Culture-dependent methods involve growing the microorganism on appropriate media prior to identification. Biochemical testing is frequently required at the species level. Eventual bias to cultures as a result of the limitations of media used for growth is inevitable. As a result, culture methods can only verify existence of microorganisms that grow on that specific medium (Hilton *et al*., 2016). Despite the activities and time required, the cultivation of microorganisms is considered fundamental for understanding microbial physiology and morphology. However, these approaches of conventional culture cultivation only access a tiny subset of the varied diverse microorganisms which are presumed to be present in any given environment. Furthermore, the microorganisms that exhibit rapid growth and can thrive in nutrient rich media (Tyson & Banfield, 2005).

In culture-independent methods, there are no intermediary laboratory culture steps that are necessary for the culture-dependent procedures. In this case however, microbial DNA or RNA is directly analysed from the samples, thereby removing the bias toward culturable species. Metagenomics, which involves the sequencing of entire nucleic acids from a sample, yields extensive information including insights

into the metabolic and functional characteristics of the microbiota (Romero *et al.*, 2019).

The two main methods of microbial community study are the marker gene and the whole genome shortgun metagenomics. Marker gene studies are based on specific gene-region sequencing which reveals the composition and diversity of specific taxonomic groups present in an environmental sample (Pérez-Cobas *et al*., 2020). Several marker genes are used in microbial ecology and they include the internal transcribed spacer (ITS) used for characterization of fungal community (Schoch *et al*., 2012), the 16S rRNA gene used to target and analyse bacteria and archaea communities (Case *et al*., 2007) and the 18S rRNA gene used to target the eukaryotes (Banos *et al*., 2018). Whole genome shortgun metagenomics entail sequencing all the existing genomes in a microbial community targeting the functional ability and their biodiversity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site description and sampling design

This study was conducted in Embu County, Kenya. Embu county lies at an elevation of 1350 m above sea level and is located approximately between latitude 0° 8 and 0° 50 S and longitude 37° 3 and 37° 9 E. It borders Machakos County to the south, Murang'a County to the south west, Kirinyaga county to the west, Meru County to the north west, Tharaka Nithi County to the north and Kitui County to the east. Administratively, Embu county is divided into four constituencies; Manyatta, Runyenjes, Mbeere north and Mbeere south (**Figure 3.1**). The county is characterized by typical agro-ecological profile ranging from cold and wet upper highland zones bordering Mt Kenya to hot and dry low midland zones bordering Kitui and Machakos counties.

These representative agro-ecological zones are; the upper highlands characterised by annual rainfall ranging from 1750-2000 mm and temperatures 15.8 to 17.7°C, upper midlands with annual rainfall ranging from 980-1800 mm and temperatures 17.5 to 20.9°C. The lower midlands characterized by annual rainfall ranging from 700-1100 mm and temperatures 21-23.9°C and the inland lowlands with annual rainfall ranging from 590-710°C and temperatures 24-25.4°C (Jaetzold, 2007). Purposive sampling targeting bee farmers in the different agroecological zones across the five sub-counties in Embu county was used.

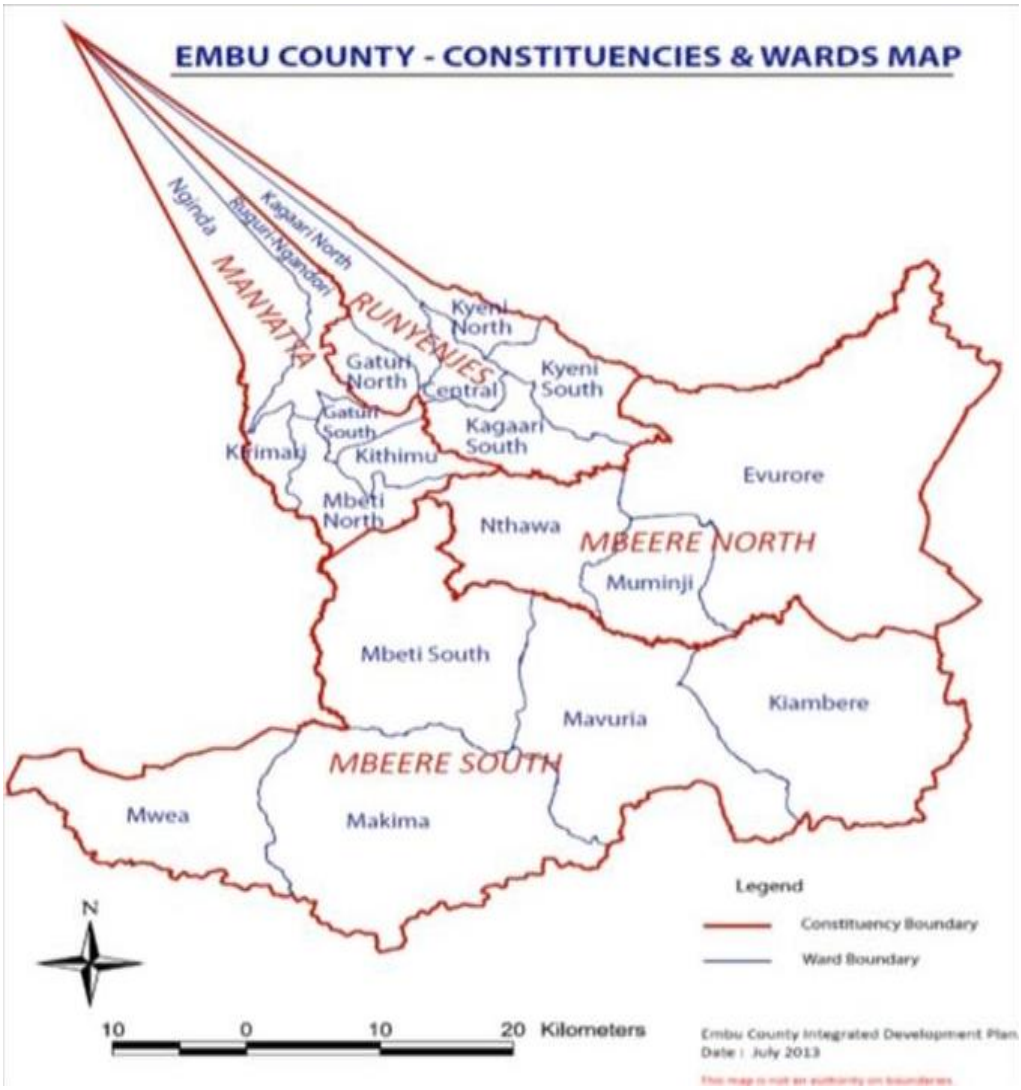

Figure 3.1: A map showing administrative wards in Embu County (Government, 2017).

3.2 Objective one: Evaluation of constraints associated with *Apis mellifera* production in Embu, Kenya

3.2.1 Determination of sample size

The following formula was applied to calculate the sample size for questionnaire application:

$$n = \frac{(z^2 \times p \times q)}{e^2}$$

Where **n** denotes the sample size, $\mathbf{Z^2}$ represents area under the acceptance zone in a normal distribution, **p** is the estimated proportion of an attribute which is present in the population (colony decline) and **q** is **1-p** while **e** is the level of precision that has

been preferred (Nanjundeswaraswamy & Divakar, 2021). Number of farmers interviewed was thus determined using the formula; 95% confidence interval, 0.5 standard deviation and a level of precision of +/-10%:

$$\frac{((1.96)^2 \times 0.5 \times 0.5)}{(0.1)^2} = 96.04$$

Therefore 96 respondents were involved in the study. These were distributed according to the administrative subdivisions; Manyatta and Runyenjes constituency made up of Embu east (14), Embu west (20) and Embu north (21) sub-counties. Mbeere south and Mbeere north constituencies made up of Mbeere south (21) and Mbeere north (20) sub-counties.

3.2.2 Questionnaires

Questionnaires were used for a guided interview with the farmers to survey on the constraints affecting honeybee production (**Appendix 2**). Core focus of the questionnaires was on the colony decline. Other focus areas included beekeeping situation in terms of source of starter colony and duration of the beekeeping practice. Level of education, number of bee colonies owned, hive placement, hive abscondment, major pests and predators threatening beekeeping and number of harvests per year (**Table 3.1**). To assess for accuracy, a sample of 10 questionnaires were first pretested and reframed to enable collection of reliable data and ease of interpretation by the farmers.

Table 3.1: Study variables

Variable	Description	Measurements
Demographics	Age	Number of years
Education	Level of education acquired	1=Primary, 2=Secondary,3=Tertiary
Beekeeping	Number of years practicing beekeeping	1=< 1 year, 2= 1-5 years, 3= 5-10 years, 4= 10-15 years
Bee source	Source of starter colony	1= Buying, 2=catching swarms, 3= caves and forest
Hive type	Type of hive used	1=Traditional, 2=frame(langstroth) , 3=transitional (Kenyan topbar)
Hive used	Traditional, frame, transitional hive is used	1=Yes, 0=No and the frequency Farmers preference for hive used
Hive placement	Backyard, apiary site, tree	Number of hives
Colony decline	Colony decline reported	1=Yes, 0=No
Decline causes	Pests, swarming, hive absconded	1=Yes, 0 =No
Hive abscond	Hive frequently absconded	0= None, 1=traditional, 2=frame, 3 =transitional
Harvest	Number of harvests per year	1=Once, 2=twice,3=thrice,4=quarterly

3.3 Sample collection

Samples for the study included honeycombs, honey, adult worker bees, frame scrappings and brood (larvae) (**Appendix 1**). With the assistance of honey harvesters, 30 samples were purposively collected within the sampling sites from strong healthy colonies and absconded hives resulting from non-reproductive swarming. Brood combs, honey, honeycombs and frame scrappings were cut using sterile blade and put in zip-lock bags and sticker labels were mounted indicating the collection sites. Adult worker bees samples were collected using cap tubes and preserved in 70% ethanol. The collected samples were then put in cooler box and transported to the University of Embu research laboratory for analysis.

3.4 Objective two: to determine the culturable bacterial pathogens with the honey and honeycombs from Embu, county Kenya

3.4.1 Bacteria isolation

A pooled sample (10g) of honey, combs, adult bees, larvae and frame scrapings samples mixture were crushed using mortar and pestle and the material mixture suspended in 100 ml phosphate buffer. The extract (100 µl) was transferred to 900 µl of phosphate buffer solution to constitute a tenfold serial dilution. Inoculation mixture from the serial dilution 10^8, 10^9 and 10^{10} series were then spread plated on Tryptone soy agar (TSA) cyclohexamide (50 µg/ml) supplemented media plates. The plates were inverted and incubated at at 30° C for 48 hours to enable bacterial growth. Individual colonies were then transferred into fresh plates and re-streaked to get pure colonies for further analysis.

3.4.2 Morphological characterization

Physical examination of the colony form, elevation, margin and pigmentation was done. To identify the cell shape, Gram staining was carried out where crystal violet was added to colony smear on the glass slide and and allowed to stand for 1 minute. The slide was passed through a gentle and indirect stream of water. Fixing of the crystal violet dye followed where Gram's iodine was flooded on the smear and allowed to stand for 1 minute followed by a washing step. Slides were then decolorized with 95% ethyl alcohol dropwise until the discolorizing agent ran clear. To counterstain, safranin was added onto cells and let to settle for 45 seconds and then rinsed with distilled water. The slides were subsequently dry blotted with an absorbent paper and viewed under a microscope using oil immersion.

3.4.3 Biochemical characterization

Bacterial isolates were tested for ability to utilize different carbon sources including starch, cellulose, xylan, chitin, pectin and xanthan. Tryptic soy agar basal media was prepared by supplementing TSA with respective substrate separately and autoclaved for 15 minutes at 121°C. Individual respective pure isolates were spotted then incubated for a period of 24 hours at 30°C to allow for growth. Formation of a zone of clearance around colony growth after flooding the plates with iodine solution for

starch plates, Congo red for cellulose, xanthan and xylan plates while Cetyltrimethylammonium bromide (CTAB) for pectin plates indicated utilization of the substrates.

3.4.4 Molecular characterization

3.4.4.1 DNA extraction

Pure isolates were grown in trypticase soy broth (TSB) media for 24 hours. The broth containing the cultures was centrifuged to harvest the bacteria cells. The extract (1 ml) was transfered into sterile 2 ml tubes, spun at 13200 rpm and the supernatant carefully removed. The cell pellet obtained was redispersed in 100 µl buffer solution {100 mM EDTA (pH 8.0), 400 mM Tris-HCl (pH 8.0)}, 10 µl of lysozyme (20 mg/ml solution) and then incubated for 30 minutes at 37°C in a water bath. Lysis buffer (400 µl) {60 Mm EDTA (pH 8.0),400 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% sodium dodecyl sulfate}(Arenas *et al*., 2022) were added and the tubes were inverted multiple times to gently mix. After adding 10 µl of 20 mg/ml proteinase K, the mixture was incubated in a waterbath at 65°C for 1 hour. Equivalent amount of chloroform was added to the mixture and centrifuged at 13,200 rpm for 10 minutes at 4°C. The supernatant was transferred into a new tube and the volume was noted. Thereafter, 150 µl of Sodium acetate (pH 5.2) was added to the supernatant. An equal volume of isopropyl alcohol was added to the mixture. The tubes were inverted gently and then centrifuged at 13,200 rpm for 10 minutes. The DNA pellet were washed in 300 µl of chilled 70% ethanol by spinning at 13,200 rpm for 1 minute and the supernatant discarded. The tubes were allowed to airdry and thereafter DNA was resuspended in 30 µl of nuclease free water. The DNA product was then run in 1% agarose gel and viewed under ultraviolet (UV) light.

3.4.4.2 PCR amplification and sequencing

DNA from individual isolates was used for amplification of the 16S rRNA gene. Universal bacteria primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) were used (Kakumanu *et al.*, 2016). The Amplification program involved initial denaturation step at 94 °C for 5 minutes, then 35 cycles of alternate temperatures;(94°C denaturation for 30 seconds, 1 minute of annealing at 51°C, 30 seconds extension at 68°C) and a final extension step at 72 °C for 5 minutes. The PCR products were separated in a 1% agarose gel electrophoresis

and viewed under UV light transilluminator. Negative control contained nuclease free water instead of DNA. Sequencing was done at Inqaba Biotech, South Africa.

3.5 Objective three: total bacterial pathogens diversity and distribution associated with the honey and honeycombs using molecular approaches

3.5.1 Total DNA extraction from hive materials (frame scrappings, adult worker bees, larvae, honeycombs and honey) and sequencing

Using mortar and pestle, 2g of each sample type (honeycombs, honey, adult worker bees, frame scrapping and larvae samples) was minced and the resulting mixture was suspended in 20 ml phosphate buffer in falcon tubes. The extract (1 ml) was added into sterile 2 ml microcentrifuge tubes and then centrifuged at 13,200 rpm. The resultant supernatant was discarded and the pellet used for DNA extraction. The resulting cells were resuspended in 100 µl buffer solution which contained {100 mM EDTA (pH 8.0), 400 mM Tris-HCl (pH 8.0), 10 µl of lysozyme (20 mg/ml solution)} and incubated for 30 minutes at 37° C in a water bath. Lysis buffer (400 µl){150 mM NaCl, 1% SDS , 400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH8.0)} (Arenas *et al*., 2022) were added and the tubes left to stand at room temperature for 10 minutes. After adding 10 µl of 20 mg/ml proteinase K, the mixture was incubated in a water bath at 65°C for 1 hour. Equivalent amount of chloroform was added to the mixture and spun at 13,200 rpm for 10 minutes at a temperature of 4°C. The supernatant was transferred into a new tube and the volume was noted. Sodium acetate (pH 5.2) was added in a volume of 150 µl to the supernatant. Equal volume of isopropyl alcohol was added to the mixture. The tubes were inverted gently, spun at 13,200 rpm for 10 minutes and the resulting supernatant was discarded. The DNA pellet was washed in 300 µl 70% ethanol, centrifuged at 13,200 rpm for 1 minute and the supernatant discarded. The tubes were allowed to airdry and thereafter 30 µl of nuclease free molecular water was added to the tubes to suspend the DNA. The DNA product was then run in 1% agarose gel and viewed under ultraviolet (UV) light transilluminator. The total DNA was shipped to Inqaba Biotech, South Africa for amplicon generation on a PacBio following the manufacturer's instructions.

3.6 Data analysis

Objective 1: Constraints associated with *Apis mellifera* production in Embu county

Coded data was entered in MS Excel worksheet. Data on responses was analyzed using SPSS version 25 with a significance level set at $p \leq 0.05$. Descriptive statistics comprising the arithmetic means, percentages, frequencies and standard deviation were performed.

Objective 2: to determine the culturable bacterial pathogens with the honey and honeycombs from Embu, county Kenya

All bacteria isolates were subjected to partial sequencing of the 16S rRNA region. Sequences generated were edited using Chromas Lite software. The Basic Local Alignment Search Tool (BLASTn) on the National Center for Biotechnology Information (NCBI) website was then used to query and compare the edited sequences with the reference type strain sequences in the GenBank database.

The partial sequences phylogenetic relationships were determined in MEGA X using maximum likelihood analyses. The Neighbor-Joining method was used to infer the evolutionary history as described by Saitou & Nei (1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992) and were in the units of the number of base substitutions per site. Fifty one nucleotide sequences were involved in this analysis. All positions with less than 95% site coverage were eliminated, that is; fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 355 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018) and rooted using *Escherichia coli* (HQ012019.1) as an outgroup.

Objective 3: total bacterial pathogens diversity and distribution associated with the honey and honeycombs using molecular approaches

All sequence reads were processed by amplicon analysis pipeline of the silva project, SILVAngs 1.4 (Quast *et al.*, 2013). Each read was aligned using SILVA Incremental Aligner (Pruesse *et al.*, 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast *et al*., 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2% of homopolymers, were omitted from further processing. Following first stages of quality control which included the identification and exclusion of, putative contaminations and artefacts, reads with low alignment quality were identified and excluded from the downstream analysis.

These identical reads were identified and the unique reads clustered into operational taxonomic units (OTU) on a per sample basis and reference read of each OTU classified (Leite *et al.*, 2021). Dereplication and clustering was done using VSEARCH version 2.15.1 (Rognes *et al.*, 2016) applying identity criteria of 1.00 and 0.98 respectively. The classification was performed by BLASTn (Camacho *et al.*, 2009). Taxonomy assignment was done against the Silva database v138. Reads without any or weak classification remained unclassified and were assigned to the metagroup 'no relative' in the SILVAngs fingerprint and krona charts (Camacho *et al*., 2009). Eukaryotic OTUs were removed using filter_otu_table.py in QIIME and statistical analysis were performed using the R packages; Phyloseq for taxonomic classification (McMurdie & Holmes, 2013) , Ampvis2 for plotting and visualization (Andersen *et al*., 2018) and Vegan for diversity analysis (Oksanen *et al*., 2020).

An OTU network was generated using QIIME, filtered using an edge cut-off of 0.001 and then visualized in Cytoscape version 3.9.1 with an "edge-weighted spring-embedded layout" (Otasek *et al*., 2019). Association value was used to weigh the network edges (Shannon *et al*., 2003, Cline *et al*., 2007). Sample types were used as source nodes and the bacteria genera as target nodes. A non-metric multidimensional scaling (NMDS) was performed using Bray-Curtis dissimilarities employing the "vegan" package (Oksanen *et al*., 2015) to visualize thr multivariate dispersion of the community composition.

Alpha diversity metrics for each sample was estimated using Chao1, Ace and fisher's alpha. Good's coverage to indicate the sequencing effort while Simpson and

Shannon as richness and diversity estimators using vegan package of R software. Sequences from this study were deposited into the Sequence read archive (SRA) database of the NCBI under the accession SRP383332.

CHAPTER FOUR
RESULTS

4.1 Questionnaire data

4.1.1 Characteristics of sampled farmers

4.1.1.1 Social demographics of sampled bee farmers

In terms of age, majority (39.6 %) of the bee farmers were between the ages of 36-50 years while 33.3% of the farmers were between 51-65 years of age, the youthful age bracket of 20-35 years made up of 14.6 % of the farmers and 12.5% of the farmers were above 66 years of age. According to the highest level of education attained, tertiary education training accounted for (45.8%) of the farmers, (44.8%) bee farmers with secondary education, while (9.4%) bee farmers had attained primary education as the highest form of education training. In terms of beekeeping experience, majority (41.7%) of the farmers sampled had kept bees for 5-10 years, (35.4%) farmers in range between 1-5 years, while (11.5%) farmers in the range of 10-15 years and (11.5%) less than 1 year. The primary source of starter colony among the beeekeepers was predominantly through catching swarms (69.8%), buying starter colonies (19.8%) while (10.4%) sourced their starter colonies from forests and caves (**Table 4.1**).

Hive type preference was based on ownership of the three hive types where some farmers owned more than one hive type. Frame hives were preferred by (66.7%) of the farmers, while (53.1%) farmers preferred transitional hives and (49%) preferred traditional hives for beekeeping (**Table 4.2**). In terms of hive placement and location preference, number of hives per location were recorded where (47.3%) hives were placed or hung on trees, (45.2%) placed in an apiary site, while (7.5%) hives were placed in the backyard. Colony decline cases were reported by 88 (91.7%) farmers while 8 (8.3%) did not report such cases. Major causes of colony decline were associated with absconding of hives, colony swarming and pests. Traditional hives were frequently absconded (32.3 %) followed by frame hives (31.3%), transitional hives (28.1%) while (8.3%) had no recorded cases of hive abscond. Number of honey harvest done in a year were reported as 6 (6.3%) once, 40 (41.7%) twice while 50 (52.1%) reported to harvest honey thrice a year which were the

majority (**Table 4.1**). Among the major causes of colony decline where multiple responses were allowed, pests accounted for (83.3%), absconding (72.9%) while colony swarming (40.6%) of the colony decline causes. Further, major pest reported were ants (67.7%), wax moths (61.5%), wasps (17.7%) and small hive beetles (16.7%) where multiple responses were allowed (**Table 4.2**).

Table 4.1: Characteristics of sampled bee farmers in Embu county

Variable	No. of farmers	Percentage
Age		
20-35	14	14.6
36-50	38	39.6
51-65	32	33.3
66-80	12	12.5
Mean= 49.64	Std dev=13.28	Max=78, min=25
Education level		
Primary	9	9.4
Secondary	43	44.8
Tertiary	44	45.8
Beekeeping		
Less than 1 year	11	11.5
1-5 years	34	35.4
5-10 years	40	41.7
10-15 years	11	11.5
Sources of starter colony		
Buying	19	19.8
Catching swarms	67	69.8
Caves and forests	10	10.4
Colony decline		
Yes	88	91.7
No	8	8.3
Hive frequently absconded		
None	8	8.3
Traditional	31	32.3
Frame	30	31.3
Transitional	27	28.1
Harvests		
Once	6	6.3
Twice	40	41.7
Thrice	50	52.1

Table 4.2: Response on hive placement, hive type preference, colony decline causes and common honeybee pests where multiple responses were allowed

Variable	Frequency (N=96)	Percentage
Hive placement		
Backyard	188	7.5
Apiary site	1130	45.2
Trees	1182	47.3
Hive type preference		
Traditional	47	49
Frame	64	66.7
Transitional	51	53.1
Colony decline causes		
Absconding	70	72.9
Swarming	39	40.6
Pests	80	83.3
Common pests		
Ants	65	67.7
Bettles	16	16.7
Wasp	17	17.7
Wax moth	59	61.5

4.1.2 Beekeeping situation in Embu county

To evaluate the beekeeping situation in Embu county, cross tabulations were used to establish association of selected variables.

4.1.2.1 Association between the education background and beekeeping experience

In the study findings, education status did predict the honey beekeeping experience. Farmers across the education levels were actively involved in beekeeping and the majority had 5-10 years experience in the practice. Primary education as the highest training had fewer farmers and were distributed across the different years of experience **(Table 4.3).**

Table 4.3: Association between education status and beekeeping experience

		Beekeeping				Total	
		< 1 year	1-5 years	5-10 years	10-15 years		Fishers exact test value=8.113
Education	Primary	3	1	3	2	9	P value=0.192
	Secondary	4	14	19	6	43	
	Tertiary	4	19	18	3	44	
Total		11	34	40	11	96	

4.1.2.2 Association between education background and bee colony source

Catching swarms was the most common source of starter colony and accounted for 69.8% (67) respondents across all the education levels. Further, preference for colony source differed where beekeepers with tertiary education majorly obtained their starter colony by catching swarm and also accounted for the largest group to buy swarms as a source of starter colony. Secondary education as the highest level training similar to tertiary level had the majority preference on catching swarms and also recorded the most farmers preferring caves and forest as a source of starter colony across the three education levels. Beekeepers with the highest level of training as primary on the other hand prefered catching swarms as the source of colony. Caves and forest as a source of starter colony was the least preferred method across the education levels (**Table 4.4**).

Table 4.4: Association between education status and source of starter colony

		Starter colony source			Total	
		Buying	Catching swarms	Caves and forest		Fishers exact test value=10.927
Education	Primary	0	9	0	9	
	Secondary	8	26	9	43	P value=0.019
	Tertiary	11	32	1	44	
Total		19	67	10	96	

4.1.2.3 Association between beekeeping experience and honey harvest

Cross tabulation between the beekeeping experience and number of honey harvests indicated that honey harvest was influenced by beekeeping experience where most farmers harvested twice (41.7%) and thrice (52.1%) in a year. There was an increase

in frequency of honey harvest across the experience ranges evident by the number of farmers reporting to harvest thrice except in the less than 1 year category. Most farmers with few harvests per year had less than 1 year beekeeping experience (**Table 4.5**).

Table 4.5: Association between beekeeping experience and number of honey harvests

		Honey harvest			Total	
		1	2	3		Fishers exact test value=18.803
Beekeeping	Less than 1 year	4	6	1	11	
	1-5 years	1	13	20	34	P value=0.002
	5-10 years	1	19	20	40	
	10-15 years	0	2	9	11	
Total		6	40	50	96	

4.1.2.4 Association between beekeeping experience and colony decline

Colony decline was recorded independent of the farmers experience. This was indicated by widespread colony decline across the honeybee keeping experiences. Farmers with <1 year experience had the highest percentage of colony loss compared to other experience ranges (**Table 4.6**).

Table 4.6: Association between beekeeping experience and colony decline

		Colony decline		Total	
		Yes	No		Fishers exact test value=14.479
Beekeeping	< 1 year	6	5	11	
	1-5 years	33	1	34	P value <0.001
	5-10 years	39	1	40	
	10-15 years	10	1	11	
Total		88	8	96	

4.1.2.5 Association between colony decline and hive abscondment

Hive abscondment was recorded cross all the hive types with all cases reporting colony decline. In 8 cases, neither hive abscond nor colony decline was reported indicating a strong association between absconding and colony loss (**Table 4.7**).

Table 4.7: Association between colony decline and hive abscondment

		Hive Absconded				Total	
		None	Traditional	Frame	Transitional		Fishers exact test value=45.777
Colony decline	Yes	0	31	30	27	88	
	No	8	0	0	0	8	P value<0.001
Total		8	31	30	27	96	

4.1.2.6 Association between hive abscondment and honey harvest

Majority of the farmers harvested honey thrice and twice a year. Traditional hives had multiple harvests indicating reliability in terms of honey production and colony retention. Hives without abscond had the lowest number of harvests with the possibility of having weak colonies or less intensively managed (**Table 4.8**).

Table 4.8: Association between hive absconded and number of honey harvests

		Harvest			Total	
		1	2	3		Fishers exact test value =18.686
Hive Absconded	None	3	4	1	8	P value=0.002
	Traditional	3	14	14	31	
	Frame	0	8	22	30	
	Transitional	0	14	13	27	
Total		6	40	50	96	

4.2 Isolation of bacteria from hive material samples

The samples of hive materials yielded a total of 17 bacterial isolates (**Table 4.9**).

4.2.1 Cellular and morphological characterization

The isolates had varying colony characteristic, that is; cell morphology and gram staining. Bacteria isolates were gram positive cocci and rods and gram negative rods. Colony color observed were cream, yellow and white while elevations observed were flat, raised and convex (**Table 4.9**).

Table 4.9: Cellular and morphological characterization of 17 isolates from hive materials.

Isolate Code	Gram stain	Cell shape	Color	Form	Elevation	Margin
J1	Positive	Rod	White	circular	flat	entire
J2	Positive	Rod	Cream	circular	flat	entire
J3	Negative	rod	Red	circular	umbonate	entire
J4	Positive	cocci	Yellow	circular	flat	entire
J5	Negative	rod	White	circular	umbonate	entire
J6	Positive	cocci	Yellow	circular	flat	entire
J7	Positive	cocci	White	circular	convex	entire
J8	Negative	rod	White	circular	umbonate	entire
J9	negative	rod	White	circular	convex	entire
J10	negative	rod	White	circular	raised	entire
J11	negative	rod	Cream	circular	raised	entire
J12	positive	cocci	White	circular	raised	entire
J13	positive	rod	White	circular	flat	entire
J14	positive	cocci	Yellow	circular	convex	entire
J15	Positive	rod	White	circular	flat	entire
J16	Positive	rod	White	circular	convex	entire
J17	positive	cocci	Yellow	circular	convex	entire

4.2.2 Biochemical characterization of bacteria isolates

From the results enzymatic activity recorded, all isolates were positive for amylase production and negative for pectinolytic activities except isolate J 11 (**Table 4.10**).

Table 4.10: Biochemical characterization of bacteria isolates in hive materials from Embu county.

Isolate	Xanthan	xylan	starch	cellulose	Chitin	pectin
J1	+	-	+	+	+	-
J2	-	+	+	+	+	-
J3	+	-	+	+	+	-

J4	-	-	+	+	+	-
J5	+	+	+	+	+	-
J6	-	-	+	+	+	-
J7	-	-	+	+	+	-
J8	-	-	+	+	+	-
J9	-	-	+	+	-	-
J10	-	-	+	+	+	-
J11	-	-	+	+	-	+
J12	-	-	+	+	+	-
J13	+	-	+	+	+	-
J14	+	+	+	+	+	-
J15	+	+	+	+	+	-
J16	-	-	+	+	+	-
J17	+	+	+	+	+	-

Key:(+) indicates activity while (-) indicates no enzyme activity

4.2.3 Molecular characterization

DNA was extracted for all the bacteria isolates obtained from the samples using the phenol-chloroform method (**Figure 4.1**).

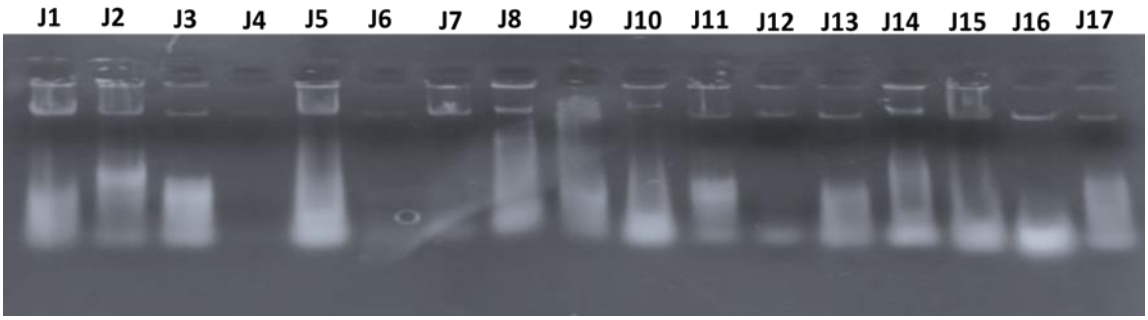

Figure 4.1: A gel electrophoresis image showing the DNA of the isolates. Lane 1-17 denoting isolates J1- J17.

In J4 and J6 wells there was no substansive DNA however they were amplified following the PCR as indicated by **Figure 4.2.**

4.2.4 PCR amplification of the 16S rRNA gene

Partial sequencing for 16S rRNA gene was done using universal bacterial primer pair 27F and 1492R which produced approximately 1400 base pairs of the amplification product.

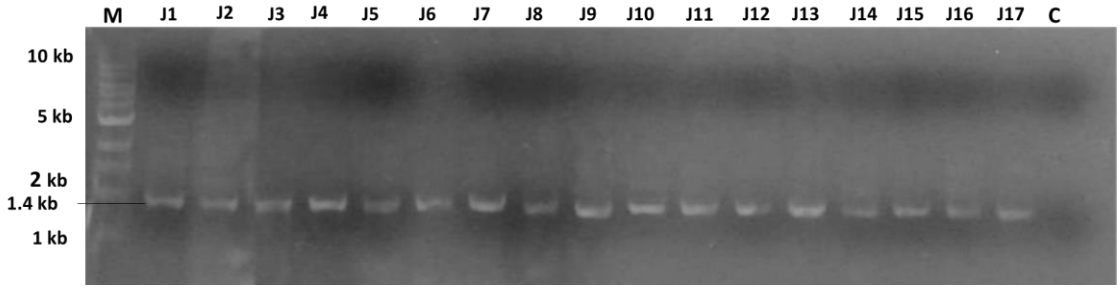

Figure 4.2: Agarose gel electrophoresis (1% agarose w/v) of PCR amplified products.

Lane M, 1kb ladder II DNA size marker, lanes J1- J17 indicates samples and lane C negative control (nuclease free water).

4.2.5 Phylogenetic analysis of the obtained bacteria sequences

From the total of 17 isolates only 1 isolate was ambiguous after editing using Chromas Lite software (http://www.technelysium.com.au) and thereafter only 16 isolates sequences were matched against the public databases of the NCBI using BLAST program. MEGA X software was used for pairwise alignment to determine the afflitiation of the isolates and their closest reference strains. From the results eight bacterial genera namely; *Bacillus*, *Microbacterium*, *Micrococcus*, *Serratia*, *Staphylococcus*, *Klebsiella*, *Erwinia* and *Devosia* were present **(Table 4.11)**.

Table 4.11:Taxonomic affiliation of partial sequences of bacterial isolated from hive materials

Isolate Code	Genus	Closest strain	Accession number#	% Identity
J1	*Bacillus*	*Bacillus drentensis* NA-14	KU254658.1	99.77%

J2	*Microbacterium*	*Microbacterium oleivorans* CS15KG0LA534	OM971582.1	99.76%
J3	*Serratia*	*Serratia nematodiphila* BAB-6746	MF319832.1	97.02%
J4	*Staphylococcus*	*Staphylococcus arlettae* NR_113349.1	MN851081.1	99.66%
J5	*Serratia*	*Serratia marcescens* strain LJYL2	MT131169.1	99.09%
J6	*Staphylococccus*	*Staphylococcus arlettae* strain NR_113349.1	MN851081.1	99.21%
J7	*Staphylococcus*	*Staphylococcus edaphicus* strain Uyi_20	MT507214.1	99.77%
J8	*Serratia*	*Serratia marcescens* strain LJYL2	MT131169.1	95.07%
J9	*Devosia*	*Devosia riboflavina* strain SR 131	MW036915.1	99.49%
J10	*Klebsiella*	*Klebsiella oxytoca* strain B892.21	OM220627.1	99.42%
J11	*Erwinia*	*Erwinia tasmaniensis* strain SRRNI51	KF724025.1	99.31%
J12	*Staphylococcus*	*Staphylococcus saprophyticus* strain 95B (BP2)	KF254616.1	99.44%
J13	*Bacillus*	*Bacillus cereus* strain CGNE-67	OL697787.1	99.44%
J14	*Micrococcus*	*Micrococcus luteus* strain BH001218	OL851613.1	87.71%
J15	*Bacillus*	*Bacillus cereus* Sihong_833_1	MN309966.1	99.77%
J17	*Micrococcus*	*Micrococcus yunnanensis* strain SR3-17-1	MN421481.1	99.53%

Accession number#: GenBank accession number of reference strains. % identity represents similarity between the isolate and closest relative sequences in the GenBank as indicated in **Table 4.11**.

The partial sequences phylogenetic relationships were determined in MEGA X using maximum likelihood analyses. The bacterial isolates were identified based on query and sequence comparison against the GenBank, NCBI and reference type strain. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Fifty one nucleotide sequences were involved in this

analysis. There were a total of 355 positions in the final dataset. Bacteria were clustered into distict groups along with their reference strains (**Figure 4.3**).

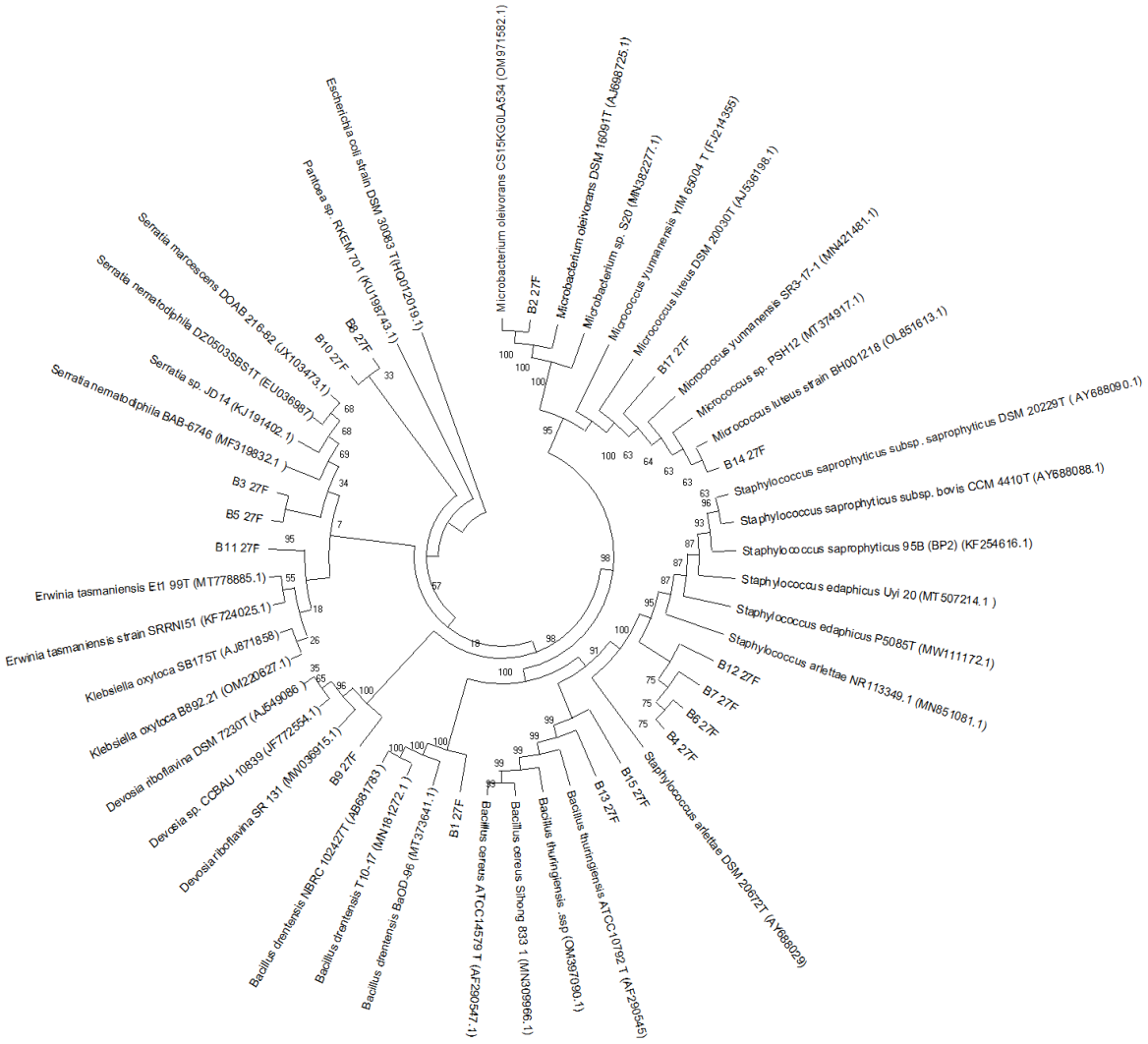

Figure 4.3: Phylogenetic tree showing the evolutionary relationship between the isolates.

4.3 Bacteria community richness, distribution and diversity indices

After quality filtering , a total of 499,300 nucleotide sequence reads were obtained across the sample types used in this study. Number of sequences per sample ranged from 2,156 to 124,766 reads, while the number of OTUs ranged from 5 to 57 OTUs. The number of sequences in honey samples ranged between 2,156 to 36,872 reads,

and between 10 to 57 OTUs. Sequence reads in comb samples ranged from 10,254 to 124,766 reads, whereas OTUs ranged from 5 to 23. A total of 8,565 to 21,073 sequence reads were found in larvae samples, while the OTUs ranged between 6 to 12 OTUs. Among the adult bee samples, sequence reads ranged between 4,701 and 9,844 reads, while the OTUs ranged between 14 and 27 OTUs. In the frame samples, the sequence reads ranged from 3,015 to 10,881 reads, while the OTUs ranged from 22 to 38 OTUs. The comb samples S19 and S27 had the lowest chao1, Ace and Fisher's alpha indices indicating fewer taxa in the samples. Larvae sample S9 had the lowest simpson and shanon indices indicating dominance by a single taxa. Honey sample S16 had the higest diversity and community richness as indicated by the highest alpha diversity indices. Good's coverage index across the sample types ranged between 99% to 100% indicating significant capture of dominant communities during sequencing. Distribution of OTUs, sequences and diversity indices across the samples is presented in (**Table 4.12)**.

Table 4.12: A summary of sample types and their alpha diversity indices

sample Id	Sample type	Location	Sequences	OTU	chao1	Ace	Simpson	Shan-non	Fisher alpha	Goods Coverage
S1	Honey	Makima	13008	16	37.00	24.93	0.35	1.09	2.37	1.00
S2	Honey	Kamama	5417	12	19.00	25.98	0.02	0.12	1.70	1.00
S3	Combs	kianjokoma	12598	23	26.00	25.64	0.77	2.76	3.65	1.00
S4	Frames	Ishiara	5845	22	23.50	26.07	0.64	2.38	3.46	1.00
S5	Honey	Nthawa	9639	10	10.00	10.37	0.53	1.24	1.37	1.00
S6	Larvae	Kibugu	12931	12	12.00	12.35	0.30	1.02	1.70	1.00
S7	Combs	Muthatari	10254	17	24.50	24.76	0.33	1.01	2.55	1.00
S8	Frames	Kiangima	3015	38	60.75	51.81	0.32	1.26	6.66	0.99
S9	Larvae	Kivwe	21073	6	7.00	9.75	0.01	0.04	0.76	1.00
S10	Honey	Gitumbi	36872	37	55.20	56.91	0.53	2.02	6.45	0.99
S11	Adult Bees	Karingari	4701	24	31.00	33.42	0.76	2.69	3.83	1.00
S12	Combs	Kiamuringa	13447	14	20.00	21.75	0.66	1.94	2.03	1.00
S13	Honey	Ivinge	10547	31	40.00	45.02	0.34	1.38	5.21	1.00
S14	Combs	Makengi	51807	11	14.75	18.92	0.02	0.09	1.53	1.00
S15	Frames	Kiethiga	7692	28	67.00	50.46	0.61	2.07	4.61	0.99
S16	Honey	Gatunduri	2156	57	62.50	67.37	0.86	3.71	10.93	0.99
S17	Honey	Kivoti	7372	39	44.00	49.21	0.70	2.46	6.87	1.00
S18	Honey	Karurina	7850	19	26.00	41.45	0.74	2.36	2.91	1.00
S19	Combs	Nemburi	12326	5	6.00	9.09	0.08	0.29	0.62	1.00
S20	Frames	Mutunduri	10881	31	40.43	50.52	0.62	2.11	5.21	0.99
S21	Honey	Manyatta	15272	45	55.50	58.67	0.32	1.39	8.18	0.99
S22	Combs	Kigari	124766	7	8.00	8.88	0.53	1.19	0.91	1.00
S23	Adult Bees	Kairuri	9844	27	40.75	52.28	0.59	2.09	4.41	0.99
S24	Combs	Gikuuri	20518	6	7.00	7.91	0.02	0.11	0.76	1.00
S25	Adult Bees	Kianjuki	9548	14	15.50	15.97	0.77	2.34	2.03	1.00
S26	Larvae	Kangaru	8565	11	14.00	14.63	0.50	1.46	1.53	1.00
S27	Combs	Kavutiri	13402	6	6.00	6.00	0.62	1.51	0.76	1.00
S28	Larvae	Mukangu	14144	12	15.00	15.15	0.64	1.82	1.70	1.00
S29	Combs	Kanja	12813	11	11.33	12.85	0.57	1.44	1.53	1.00
S30	Combs	Ugweri	10997	10	25.00	44.33	0.09	0.36	1.37	1.00

OTU: operational taxonomic units, ACE: abundance-based coverage estimator

4.3.1 Bacterial taxonomic composition

From the composition analysis of the 16S rRNA predominant bacterial phyla were represented by Firmicutes (55.1%), Proteobacteria (44.4%), Bacteroidetes (0.3%), Actinobacteria (0.1%) and Cyanobacteria (0.1%) (**Figure 4.4**). The most abundant bacteria classes comprised of Actinobacteria, Bacteroidia, Oxyphotobacteria, Bacilli,

Clostridia, Alphaproteobacteria and Gammaproteobacteria (**Figure 4.5**). Firmicutes phylum was represented by the Lactobacillales (52.5%), Clostridiales (1.5%) and Bacillales (12.2%) orders respectively. Order Lactobacillales which was the most abundant comprised of seven genera, while Bacillales and Clostridiales had five and two genera respectively. Phylum Proteobacteria was represented by the orders Acetobacterales, Rhizobiales, Sphingomonadales, Betaproteobacteriales, Cardiobacteriae, Enterobacteriales, Oceanospirillales, Orbales, Pseudomonadales and Xanthomonadales. Orders Bifidobacteria, Micrococcales and Propionibacteria predominated the Actinobacteria phylum while the phylum Bacteriodetes was represented by orders Chitinophagales, Cytophagales, Flavobacteria and Sphingobacteria.

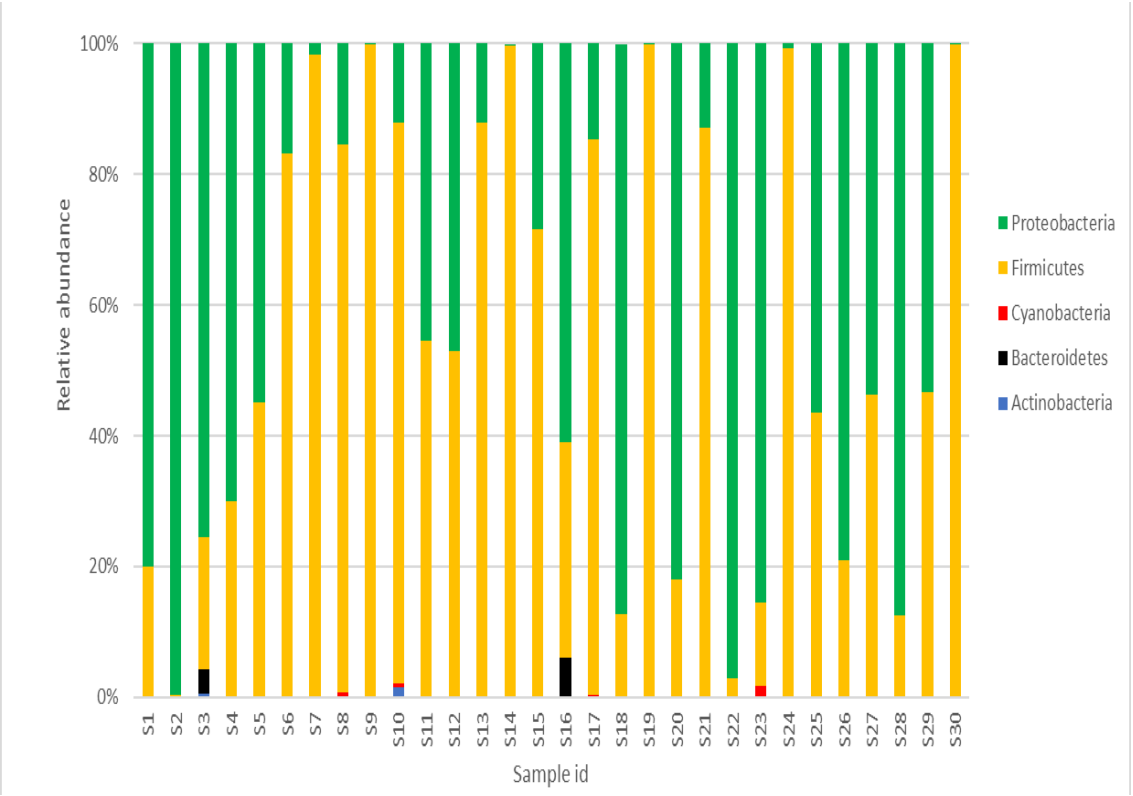

Figure 4.4: Relative abundance of the most dominant phyla across the sample types represented by the sample Id (S1-S30).

Figure 4.5: Relative abundance of the most predominant prokaryotic classes in different sample types collected in Embu county, Kenya.

4.3.2 Bacteria composition across the sample types and the sampling environment

To visualize the most abundant genera represented in the study across the sampling environment, the agroecological zones, habitat types and hives, a box plot based on the number of nucleotide sequence reads was used to illustrate the disparities in diversity and composition.

Most abundant genera represented in the study were visualized using a boxplot based on total nucleotide sequence reads for each sample type. These comprised of *Fructobacillus*, *Lactobacillus*, *Gilliamela*, *Bombella*, *Frischella*, *Enterobacter*, *Lactococcus*, *Serratia*, *Leuconostoc* and *Bacillus* (**Figure 4.6**)

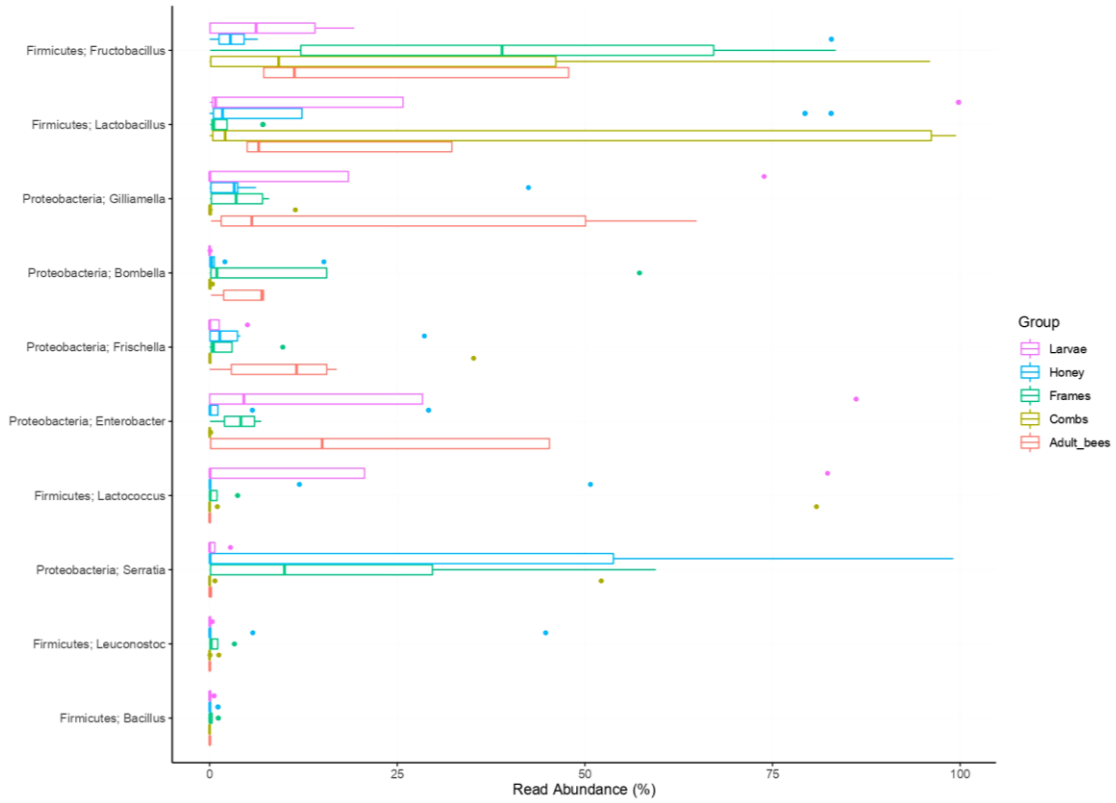

Figure 4.6: Boxplot showing the percentage read abundance of the ten most abundant genera observed in the study.

Plot colors represent the sample types, that is, purple-larvae, blue-honey, green-frames, yellow-combs and red-adult bee samples.

4.3.3 Differences in bacteria composition across the sampling locations in Embu

Bray-Curtis dissimilarity analysis was used to assess the variations in microbial community composition across the sampling environment. Analysis based on principal coordinate analysis (PCoA) demonstrated that the samples were separated into five clusters that were clustered together (**Figure 4.7**). This indicating a similar OTU composition. The PCoA1 and PCoA2 described 20.1% and 16.1% of variance in bacterial communities.

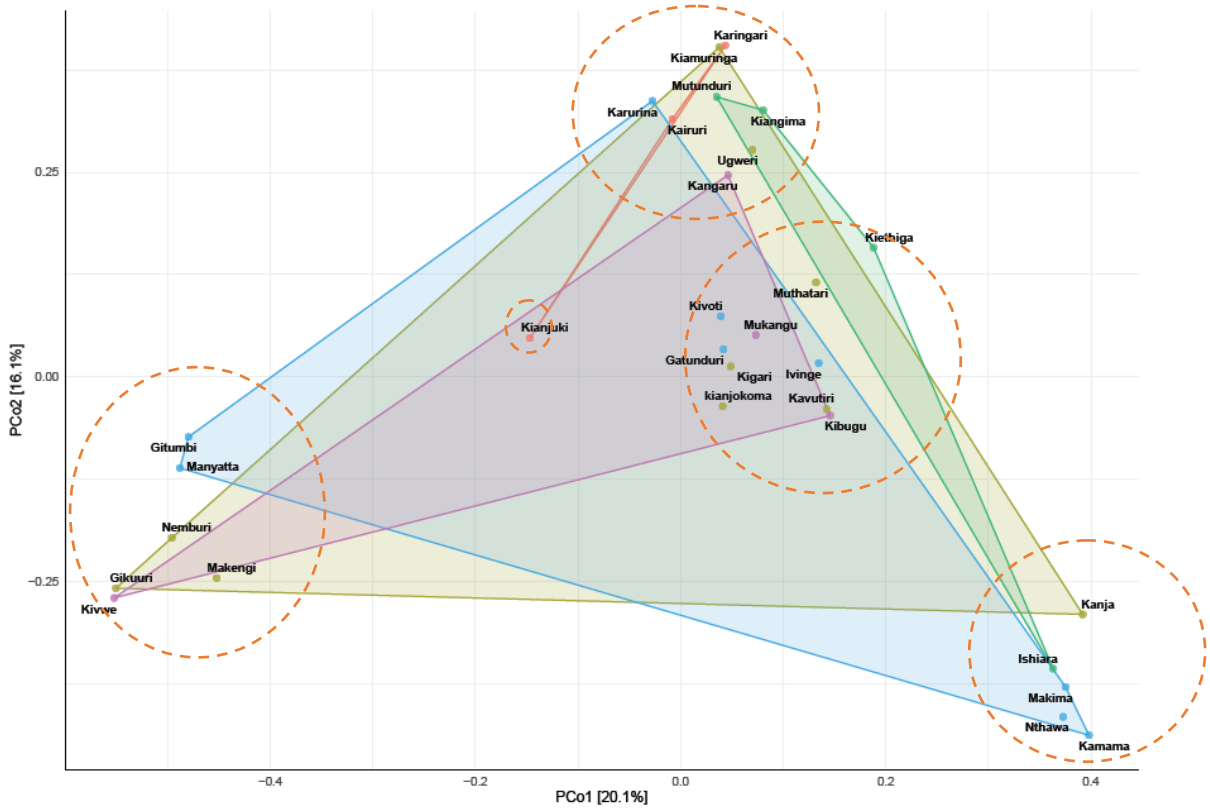

Figure 4.7: The Bray–Curtis dissimilarity analysis showing the clustering of the samples.

Sample types were plotted as polygon points in different colors. Blue indicating honey samples, green frame samples, red adult bee samples, yellow comb, samples and purple indicating larvae samples.

CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

5.1.1 Characteristics and constraints associated with beekeeping in Embu

Beekeeping in Kenya plays a fundamental role in food production, ecosystem service provision and balance. Beekeeping is practiced not only in forested areas but also in arid and semi-arid regions which make up the largest land area in Kenya (Sagwa, 2021). Embu county is characterized by typical agro-ecological profile ranging from the hot and dry lower zones to the cold and wet upper zones (Jaetzold, 2007). This makes Embu county an ideal location for exploring beekeeping constraints.

The age distribution data indicated that majority of the beekeeepers (39.6%) were within the 36-50 year age bracket, followed by 33.3 % in the 51-65 years range while 14.6 % and 12.5% were the age brackets 20-35 years and above 66 years respectively. This pattern suggests individuals embracing beekeeping are predominantly in their productive years. Youthful generation is yet to embrace the practice fully and is consistent with the common rural trend where the youth migrate to urban centers in search of jobs where there is likelihood of better employment opportunities and high wages (Ofunya *et al*., 2015). Another reason could be low interest in agriculture where they indulge in agriculture as the last resort (Ouko *et al*., 2022).

Beekeepers attained different levels of education indicating the ease of venturing into the beekeeping practice. Majority represented by 90.6% of farmers had attained secondary and tertiary education. In terms of experience, 5-10 years category from the secondary and tertiary education accounted for the majority of experienced beekeepers. This might suggest that education could play a critical part in prolonged engagement. Education is also a positive indicator of ability to receive training and adoption of new technology when facilitated which in turn will increase beekeeping income and productivity (Schouten, 2020). Number of years in beekeeping practice however was not associated with education level, thus increased productivity and income might answer the need to stay in the practice.

Establishment and maintainance of bee colonies is very crucial in beekeeping and so is the colony source. Majority, 69.8% of farmers obtained their colonies by catching swams. Catching swarms is the widely preferred method since it is convenient and cost free (Bihonegn & Begna, 2021). Buying colonies was mostly reported among farmers with tertiary education training this might suggest awareness of commercial options to obtain colonies. This is also associated with assured quality and performances of the queens and colonies or lack of skill on beekeeping as beginners thereby preferring buying swarms from skilled beekeepers as a source of starter colony (Teweldemedhn & Yayneshet, 2014). Forests and caves as the source of colony was the least preferred source. This practice exclusively associated with farmers who had primary education level of training which suggests reliance on traditional methods.

There was a strong association between hive abscondment and colony decline where across the hive types colony decline reported was linked to absconding ($p<0.000^*$). Absconding has been reported to be among the major constraints of beekeeping (Gebreyohans & Gebremariam, 2017). On the other hand, where abscondment was not reported, the colonies were stable and no decline was experienced. This suggests that colony maintainance and not hive type plays a significant role in preventing abscondment. Thus strategies to reduce and prevent hive abscondment would be ideal in colony maintainance and curbing a myriad of colony decline causes (Diriba *et al*., 2023).

In addition, beekeeping experience had some influence on the colony stability however no clear trend was observed across the years. Colony losses were reported to be associated with beekeepers experience where beekeepers with little experience suffered colony loss as compared to higher experience (Jacques *et al*., 2017). Herein, colony decline was recorded to occur independent of the farmers experience in beekeeping. Farmers with <1 year experience recorded the most cases of stable colonies across the experiences. This suggests an interplay of many factors in colony management and survival in addition to farmers experience.

Honey harvesting frequency was associated with farmer's beekeeping experience where most farmers harvested honey twice and thrice a year. In the study, the lowest beekeeping experience category, that is; less than 1 year however recorded most farmers who had few harvests in a year. Frequency in honey harvest might depict availability of bee forage and maximum honey production. Harvesting thrice a year has been reported as an indicator of apicultural development (Kiros & Tsegay, 2017). Similarly, migratory beekeeping has been reported to allow 4-5 harvests per year which translates to a significant source of income (Sharma *et al*., 2013). This points out the crucial role of farmers experience in maximizing honey production and overall apiculture development.

Despite reporting most cases of hive abscondment, traditional hives also reported most multiple harvests across the hive types. This might suggest traditional hive are still a viable option for honey production and colony maintainance. Frame hives also reported high cases of abscondment consistent with what has been reported on high rates of abscondment in traditional and frames hive type while transitional hives associated with higher colonizational rates (Ande *et al*., 2008). Consistent and multiple honey harvest across the hives suggest appropriate hive management and harvesting methods were used minimizing colony stress and chances of abscondment.

Some of the identified constraint associated with honey beekeeping were colony decline associated with hive abscondment, pests and swarming. Honeybee pests are one of the major causes of honeybee population decline. Some of the insect pests of honeybee reported were the ants, wax moths, wasps and the small hive beetle. Ants (*Dorylus fulvus*) were the largest pest group associated with colony decline. Ants are among the most conspicuously diverse, ecologically significant and abundant insects (Jorge *et al*., 2024). They have been associated with initiating aggressiveness in honeybees, massive honeybee deaths and robbing of hive products, that is, the pollen, brood and combs with their contents (Wakgari & Yigezu, 2021). This eventually initiates hive and nesting abscondment, increasing risk of opportunistic parasites exposure and pathogens spread.

Ant populations have been reported to affect honeybees both directly and indirectly. Increased availability of resources might explain the ants abundance and role as the major threat in this study. High infestations by ants has been reported to be associated with increased availability of resources and food supplies particularly in the wet seasons (Tembong *et al*., 2024). Ants have been reported to act as reservoirs of honeybee pathogens more so the deformed wing and black queen cell viruses (Dobelmann *et al*., 2023). Ants also prey and rob honeybee colonies resources and attack the brood thereby affecting and influencing survival especially in cases of infestation (Tembong *et al*., 2024). This increases the risk of hive abscond and eventual colony decline.

Waxmoth were found to be the second largest group of pests of honeybee. Two species have been reported to indirectly damage hive products namely the lesser wax moth (*Achroia grisella)* and greater wax moth (*Galleria mellonella)* (Fathy *et al*., 2017). Wax moths lay eggs on hive cracks where larvae emerge build grayish webs for protection. The larvae burrow and make tunnels which destroy the combs and pollute honey with their excreta (Fombong, 2017). Furthermore, the larvae feed on the wax combs and casts of honeybee larvae food skin and the stored food in the hives. Active bees with strong colonies can reduce the infestation of waxmoth to manageable levels however on weak colonies waxmoth infestation results in colony collapse (Wakgari & Yigezu, 2021).

Small hive beetle, *Aethina tumida* are long known pests native to sub-Saharan Africa (Neumann *et al*., 2016). In this study they were recorded as one of the minor pests. They have been associated with invading honeybee colonies where they lay eggs in hives and their larvae consume wax and food stored in the honeycombs (Gonthier *et al*., 2019). Strong African honeybee colonies have been reported to withstand severe infestations with negligible impacts at the colony level and abscondment as a response to heavy small hive beetle infestations (Eumann *et al*., 2016). Successful hive beetle reproduction has been linked to weak colonies or in recently abandoned colonies. Previous studies in the region have also reported small hive bettle as uncommon in Kenya (Torto *et al*., 2010).

Wasps were also reported as honeybee pests however as least frequent. Wasps have been reported to prey on honey bees where they attack hives to steal the honeybee larvae and feed on honey citing a significant problem especially for already weakened colonies as a result of other causes (Baracchi *et al*., 2010). Their large size enables them to capture foraging bees in the fields and even at the hive enterance (Pusceddu *et al*., 2017). Wasps have been reported as a menace due to direct predation of the honeybees which disrupts biodiversity and harms the commercial beekeeping activities (Monceau *et al*., 2014). Predation of the honeybees by the wasps can be a serious problem more so in the already weakened colonies by other causes. This study recorded fewer predatory wasps which could be as a result of other preferred food sources.

5.1.2 Culture-depedent method for bacteria community analysis

Honeybee possess a unique and specialized gut microbiota which is crucial to their metabolism and general bee health (Raymann & Moran, 2018). The presence of microoganisms in honey more specifically bacteria in the hive materials could have its origin in the foodstores, gut, crop, and food sources. In this study, a blend of 16S rRNA gene sequences and culture-based methods to identify the abundance and diversity of bacteria found in the hive materials, that is; honeycombs, adult worker bees, frame scrappings, honey and larvae were used.

Based on the culture method a total of 17 isolates were identified from the samples collected. Different carbon substrates were supplemented in the TSA media to test their ability to utilize various carbon sources; xanthan, xylan, pectin, starch, cellulose and chitin. Some of bacteria isolates used in the study potentially produced extracellular enzymes including cellulases, xylanases, amylases, pectinases, chitinases and xanthanases indicating their crucial role for bee nutrition and energy source. Pollen and nectar contain a variety of complex carbohydrates and effective breakdown and utilization is thus essential for the bee diet (Thakur & Nanda, 2020).

Amylolytic activities were observed on all isolates, indicating utilization of starch which is a monosaccharide. Utilization of pectin which is a more complex polysaccharide was observed in one isolate. The metabolic activities of these bacteria facilitate the effective digestion and absorption hence forming a symbiotic

relationship and this highlights the importance of maintaining a healthy gut microbiota for overall wellbeing and productivity of bee colonies.

Out of the 17 sequences of bacteria isolates, 16 were without ambiguities, these were found majorly to belong to phyla Firmicutes, Proteobacteria and Actinobacteria. This is consistent with what has been reported in other similar honeybee gut microbiota studies (Ellegaard & Engel, 2019).

Firmicutes in the isolates were represented by genera *Bacillus* and *Staphylococcus*. *Bacillus drentensis* (99.77%) and *Bacillus cereus* (99.77%) had the highest percentage identity match. *Bacillus drentensis* has been identified and used as an endophytic bacteria with the potential of stimulating plant growth (Hernández-Pacheco *et al*., 2021). These could have been acquired from the foraging environment. *Bacillus cereus* is a bacteria found in rotting food substrates with wide range sources from the environment, however it has also been recovered in honey and has been reported to pose a risk to human health which could lead to food borne illnesses (Brudzynski, 2021).

Genus *Staphylococcus* had affliates of *Staphylococcus edaphicus* and *Staphylococcus arlettae* (99.77%) with the highest percentage identity match. *Staphylococcus edaphicus* has been described as a bacteria promoting growth found in rhizosphere of plants (Sitlaothaworn *et al*., 2022). The Actinobacteria phylum was represented by *Staphylococcus arlettae*, *Micrococcus luteus*, *Micrococcus yunnanensis* and *Microbacterium oleivorans* with the highest percentage identity (99.76%). Proteobacteria phyla on the other hand consisted of the closest relatives of *Serratia nematodiphila*, *Serratia marcescens, Devosia riboflavin*, *Klebsiella oxytoca* and *Erwinia tasmaniensis.* Genus *Erwinia* has been described to consist of pectinolytic microorganisms associated with degradation of the plant cell wall resulting in plant tissue maceration and cell necrosis (Abbott & Boraston, 2008). This might explain the pectinolytic activity exhibited by isolate J1 in the study whose closest strain was *Erwinia tasmaniensis.*

5.1.3 Hive bacterial community

Metagenomics was incoorporated in this study to provide an insight on the information regarding the resident bacterial community's functional potential and also identification of the role of bacteria in the maintainance of healthy hive environment. Bee nutrition is entirely based on pollen and nectar. Pollen is a source of lipids, proteins and micronutrients while nectar supplies honeybees with carbohydrates. The colony's growth and development depend on optimal and adequate nutrition which is ensured by a balanced intake of nutrients from these food sources (Negri *et al*., 2019).

The provision and the nest-building behavior in honeybees is associated with storage of large amounts of food within the nest which promotes interaction between the nestmates and inevitably spread of microbes within colony (Romero *et al*., 2019). This thereby assists in maintaining beneficial microbiota (Kaltenpoth & Engl, 2014; Salem *et al*., 2015). Across the hives, distinct microbial clusters haves been observed (Anderson *et al*., 2013). These microbial communities are a combination of those derived from host organism and those from the foraging environment (Powell *et al*., 2014).

The bacterial taxa abundance and richness varied among the microenvironments that were sampled. The comb and larvae sample types had the lowest diversity and richness across the sampling sites. The *A. mellifera* workers emerge ftom the pupal stage void of the core gut microbiota or few bacteria of any kind (Powell *et al*., 2014). Therefore larvae harbors a subset of gut microbiota similar to the core microbiota found in adult honeybees. This could also be possibly due to diet change as they become older and absence of defection during the larval stage (Vojvodic *et al*., 2013).

Adult bees, frame scrapping and honey samples on the other hand had the highest richnesss and diversity across the sampling sites. Frame scrappings are generaly composed of propolis, traces of honey and pollen. They are therefore expected to reflect the composition of microbiota acquired from the surrounding environment and from the bee gut. High diversity particularly of sporadic and non-core bacteria in gut is expected (Muñoz-Colmenero *et al*., 2020). Adult bee are characterized by

stable and modest core microbiota composition which is shared across the individuals (Moran *et al*., 2012). This was evident in the similar alpha diversity values across the adult bee samples. Honey is known to contain antimicrobial properties and therefore a low microbial diversity in honey is expected. Honey however has high diversity of microbiota sourced from the pollinating environment which is gradually reduced during honey ripening (Brudzynski, 2021). This might explain the high observed diversity in study.

Most abundant bacteria phyla identified in this study were Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Cyanobacteria. Previous studies have reported that honeybee consistently possess a set of core bacteria communities that dominate their gut communities. These bacteria species comprises *Gilliamella apicola*, *Bartonella apis*, *Frischella perrara*, *Parasaccharibacter apium*, *Snodgrassella alvi* and Alpha 2.1 belonging to phylum Proteobacteria (Regan *et al*., 2018), *Lactobacillus* spp. Firm 5 and *Lactobacillus* spp Firm 4 of the phylum Firmicutes and *Bifidobacterium* species, which are members of Actinobacteria phylum (Kwong & Moran, 2016; Tola *et al*., 2020). Other phylotypes have been identified but they account for a small propotion of the gut microbiota. Other phylotypes include *Serratia marcescens*, *Apibacter*, *Lactobacillus kunkeii* and other Enterobacteriaceae (Ahn *et al*., 2012; Corby-Harris *et al*., 2014). The abundance of these bacteria phyla in honeybees is vital in degrading intricate plant polymers consisting of pollen, a major source of lipids, proteins and micronutrients. These pectinolytic, hemicelullolytic and cellulolytic specific activities are thus crucial in honeybee metabolism and nutrition (Rudra Gouda *et al*., 2024).

Fructobacillus was the most prevalent genus across the various sample types and was mainly sampled from adult bees, combs and frame scrapping. A similar observation has been reported whereby there was increased absolute abundance of *Fructobacillus fructosus*, *Lactobacillus kunkeii* and *Bombella apis* in colonies treated with propolis (Dalenberg *et al*., 2020). Propolis, a plant resin, applied to the interior of the honeybee nest cavity has been positively correlated with antimicrobial activity of larval food, high brood survival rates, honey production, adult bee longevity, bee hygienic behavior and increased colony strength (Nicodemo *et al*., 2014; Borba &

Spivak, 2017). Together with *Lactobacillus kunkeii*, *Fructobacillus* species form the fructophilic lactic acid bacteria (FLAB) group that prefers fructose as a growth substrate over glucose (Endo & Salminen, 2013). *Fructobacillus fructosus* has also been reported to have a beneficial effect on other core honeybee gut bacteria and utilizing lignin, a complex plant carbohydrates (Rokop *et al*., 2015).

Genus *Lactobacillus* was abundant in combs and honey sample across the various agroecological zones. *Lactobacillus* is one of the core gut group with two phylotypes: *Lactobacillus* Firm 5 and *Lactobacillus* Firm 4 (Abdi *et al*., 2023). These phylotypes have been associated with maintaining good honeybee health (McFrederick *et al*., 2018). Additionally, honeybees also form an association with other *Lactobacillus* species. A common example is the *Lactobacillus kunkeii* found in the corbicular pollen, hive surfaces, honey, honeybee foregut and flowers. This indicates that the honeybee and foraging environment are a common niche for multiple *Lactobacillus* species (Anderson *et al*., 2016). *Lactobacillus kunkeii* has been linked to a lower proportion of larvae infected with *Paenibacillus larvae*, the American foulbrood disease causal agent (Truong *et al*., 2023). *Lactobacillus kunkeii* exhibits antimicrobial effects aganist *Melissococcus plutonius* that causes the European foulbrood disease (Vásquez *et al*., 2012;Endo & Salminen, 2013).

Gilliamella spp. has been reported as the most dominant bacteria in honeybee gut (Zheng *et al*., 2019;Tola *et al*., 2020). Here the genus *Gilliamella* was the third most abundant group where high abudance was observed in adult bee and larvae samples. Additionally they predominated the agriculturally active lands in the upper midlands and the upper highlands agricultural ecological zones in Embu county. *Gillliamella apicola* genome possess complete glycolysis pathways and many phosphotransferase systems encoding genes thus their key role in digestion of carbohydrate rich diets in bees (Zheng *et al*., 2016). These rich diets include the pollen which provides lipids, amino acids and vitamins, and floral nectar which provide sugars that are needed for bee growth and reproduction (Zheng *et al*., 2019). *Gilliamella* abundance is also positively correlated with humidity and negatively correlated with altitude (Tola *et al*., 2020).

In this study, genus *Frischella* was abundant in adult bee samples. *Frischella perrarra* has been described as an opportunistic pathogen influenced by early adult bee succession of the gut bacteria community and also the state of the consumed nutrient sources. *F. perrarra* has been reported to induce melanization response which leads to the scab phenotype along with activation of honeybee host immune system including effector functions and signal perceptions (Emery *et al*., 2017). Abundance of *F. perrara* in adult bee samples in the study could be associated with the consumption of aged diet as reported (Maes *et al*., 2016) or an indicator of strong activated host immune system.

The genus *Enterobacter* was abundant in larvae and adult bee samples. Enterobacteriaceae have been associated with unhealthy colonies and gut microbiota disruption (Kwong & Moran, 2016). Enterobacteriaceae are also among the main symbionts of *Varroa destructor* (Pakwan *et al*., 2018). A high number of Enterobacteriaceae was found in guts of parasitized bees, and this is facilitated by the horizontal transfer between mite vector of *Varroa destructor* and honeybees (Hubert *et al*., 2016).

The genus *Serratia* high richness was found particularly in honey and frame scrapping samples. Honey samples were the richest in *Serratia* spp. *Serratia* species have also been described as signifiers of a typical microbiome composition in honeybees along with other Enterobacteriaceae where they occur in low frequencies (Moran *et al*., 2012). *Serratia marcesens* has been described as an opportunistic bacterial pathogen associated with increased lethality in young honeybees following exposure to glyphosate (Motta *et al*., 2018). Similarly, *Serratia* has been implicated in increased mortality on bees treated with tetracycline a broad spectrum antibiotic (Raymann *et al*., 2017).

The genus *Leuconostoc* was abundant in frame scrapping samples. *Leuconostoc* species have been associated with healthy honeybee colonies where they exert probiotic effect on honeybees. *Leuconostoc mesenteroides* has been reported to possess a satisfactory carbohydrate metabolizing ability (Huang *et al*., 2021). Honeybees depend mainly on fructose, sucrose and glucose as the main carbohydrates for survival. Some sugars from the natural nectar are reported to posses strong toxicity and can lead to reduced lifespan. *Leuconostoc mesenteroides* has also been reported to aid in utilization of sugars; xylose, mannose, lactaose, raffinose, arabinose, melibiose and raffinose which are toxic to the honeybees (Huang *et al*., 2021).

The genus *Bacillus* was also recovered from the hive material samples. *Bacillus* are spore-forming gram positive bacteria and are found widely in soil and plants. Along with fungi, spore forming genus *Bacillus* have been described as the predominant microbes recovered from beebread and from the larval feces (Gilliam & Prest, 1987). Species of *Bacillus* including *B. amyloliquefaciens*, *B. circulans*, *B. cereus*, *B. pumilus*, *B. thuringiensis*, *B. licheniformis* and *B. subtilis* have been found in honey fermentation of both *Apis mellifera* and stingless bee (Beux *et al*., 2022). In adult honey bee, *Bacillus subtilis* and *B. amyloliquefaciens* have been reported as dominant *Bacillus* species sourced from the foraging environment (Wang *et al*., 2015). The occurrence of *Bacillus* in the honeybee gut is positively correrated to an increase in amylase in the nectar thus aiding in the transformation of nectar into honey following degradation of sugars.

Genus *Bombella* reads were abundant in frame scrapping samples signifying their important role in the hive environment. *Bombella apis*, a bacterial symbiont has been reported to aid in the protection of bee brood due to their antifungal properties. Their presence in the hives has been associated with reduced number of spores produced in cases of infection thus limiting disease transmission (Miller *et al*., 2021).

5.2 CONCLUSION

This study established that:

1. Honeybee keeping is still an essential agricultural practice in Embu and is key to ecological balance, food production and importantly a source of livelihood.
2. There is domination of the individuals in the productive age group 36-50 years and little participation from the youth in beekeeping. Future sustainability of the honeybee keeping should be taken into consideration by onboarding the youth.
3. Hive abscondment, non-reproductive swarming and pests are the major causes of colony decline thus management practices ensuring colony stability are critical.
4. The core honeybee associated bacteria were consistent with what has been reported in similar studies, indicating colony stability and their role in overall bee health.
5. Opportunistic bacterial pathogens were observed as represented by the genus *Serratia* and *Enterobacter*. However, there were no cases of the major notifiable bacterial diseases like American foulbrood and European foulbrood.

5.3 RECOMMENDATION

1. Constant and frequent hive inspections are important to ensure healthy colonies in the long run.
2. Management strategies should focus on eliminating potential stressors which result in hive abscondment like in the case of inevitable pest infestation and prevalence.
3. Some bacteria symbionts such as *B. apis* have the potential for use as natural biocontrol agents in controlling fungal pathogens. This is an area for further research.
4. Further research on the occurrence and distribution of bacterial, viral and fungal pathogens in beekeeping regions in Kenya.
5. Awareness creation through teaching and training about beekeeping at an early age to enhance interest among the youth.

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APPENDICES

Appendix 1: Metadata list

Table showing the list for the samples sources, sample types, collection sites , crops types and the location coordinates

Sub county	Coordinates of place	Sample id	Code	Hive type	Sample type	Habitat types	Agro-ecological Zone	Food crops	Cash crop	Fruit crops
Mbeere south	0°48'00.4"S 37°33'33.6"E	Makima	1	Top Bar	Honey	Grassland	Inland Lowland (IL)	Millet and Sorghum	Khat	Mangoes
Embu north	0°26'08.5"S 37°28'14.4"E	Kamama	2	Traditional	Honey	Forest	Upper Highlands (UH)	Beans	Macadamia nuts	Avocado
Embu north	0°24'16.2"S 37°31'22.1"E	kianjokoma	3	Top Bar	Combs	Forest	Upper Highlands (UH)	Maize	Tea	Bananas
Mbeere north	0°26'39.4"S 37°47'44.6"E	Ishiara	4	Traditional	Frames	Grassland	Lower Midland (LM)	Maize	Khat	Mangoes
Mbeere north	0°35'08.5"S 37°34'03.9"E	Nthawa	5	Top Bar	Honey	Grassland	Lower Midland (LM)	Millet and Sorghum	Khat	Mangoes
Embu west	0°25'55.5"S 37°25'41.9"E	Kibugu	6	Traditional	Larvae	Grassland	Upper Midland (UM)	vegetables	Coffee	passion fruit
Embu west	0°32'20.7"S 37°29'20.0"E	Muthatari	7	Top Bar	Combs	Grassland	Upper Midland (UM)	Maize	Coffee	Mangoes
Embu north	0°29'25.0"S 37°28'55.4"E	Kiangima	8	Top Bar	Frames	Forest	Upper Highlands (UH)	Maize	Coffee	Avocado
Embu west	0°29'12.5"S 37°30'44.1"E	Kivwe	9	Top Bar	Larvae	Grassland	Upper Midland (UM)	Maize	Coffee	Avocado
Embu east	0°25'11.6"S 37°23'29.1"E	Gitumbi	10	Traditional	Honey	Forest	Inland Lowland (IL)	Maize	Khat	Mangoes
Embu	0°28'02.5"S 37°30'22.7"E	Karingari	11	Top Bar	Adult	Grassland	Upper Midland	Maize	Coffee	Bananas

west					Bees		(UM)			
Embu east	0°34'09.4"S 37°32'19.0"E	Kiamuringa	12	Top Bar	Combs	Grassland	Upper Highlands (UH)	Maize	Khat	Mangoes
Mbeere south	0°48'53.0"S 37°40'16.3"E	Ivinge	13	Traditional	Honey	Grassland	Inland Lowland (IL)	Cassava	Khat	Mangoes
Embu west	0°26'26.1"S 37°30'42.7"E	Makengi	14	Top Bar	Combs	Grassland	Upper Midland (UM)	Maize	Coffee	Bananas
Embu west	0°31'13.6"S 37°30'40.0"E	Kiethiga	15	Traditional	Frames	Grassland	Upper Midland (UM)	Maize	Coffee	Bananas
Embu west	0°29'04.0"S 37°29'28.7"E	Gatunduri	16	Traditional	Honey	Grassland	Upper Midland (UM)	Maize	Coffee	Bananas
Embu north	0°43'00.9"S 37°40'47.7"E	Kivoti	17	Traditional	Honey	Forest	Upper Highlands (UH)	Maize	Coffee	Avocado
Embu west	0°31'13.4"S 37°28'35.0"E	Karurina	18	Top Bar	Honey	Grassland	Upper Midland (UM)	Maize	Macadamia nuts	Mangoes
Embu west	0°28'45.1"S 37°30'43.3"E	Nemburi	19	Top Bar	Combs	Grassland	Upper Midland (UM)	Maize	Coffee	Bananas
Embu west	0°28'14.3"S 37°27'34.4"E	Mutunduri	20	Top Bar	Frames	Grassland	Upper Midland (UM)	vegetables	Coffee	Avocado
Embu north	0°25'49.6"S 37°28'53.4"E	Manyatta	21	Top Bar	Honey	Forest	Upper Highlands (UH)	vegetables	Tea	passion fruit
Embu north	0°26'40.8"S 37°28'39.2"E	Kigari	22	Top Bar	Combs	Forest	Upper Highlands (UH)	Maize	Tea	Bananas
Embu north	0°25'13.5"S 37°28'05.3"E	Kairuri	23	Traditional	Adult Bees	Forest	Upper Highlands (UH)	Maize	Coffee	Avocado
Embu west	0°26'57.6"S 37°32'49.6"E	Gikuuri	24	Top Bar	Combs	Grassland	Upper Midland (UM)	Maize	Coffee	Bananas
Embu west	0°25'44.2"S 37°30'32.0"E	Kianjuki	25	Traditional	Adult Bees	Grassland	Upper Midland (UM)	Maize	Coffee	passion fruit

Embu west	0°28'34.8"S 37°25'53.9"E	Kangaru	26	Traditional	Larvae	Grassland	Upper Midland (UM)	Maize	Macadamia nuts	passion fruit
Embu west	0°24'49.9"S 37°30'38.7"E	Kavutiri	27	Top Bar	Combs	Grassland	Upper Midland (UM)	Maize	Coffee	Avocado
Embu north	0°42'13.2"S 37°04'33.0"E	Mukangu	28	Traditional	Larvae	Forest	Upper Highlands (UH)	Maize	Coffee	Mangoes
Embu north	0°21'36.9"S 37°32'25.7"E	Kanja	29	Top Bar	Combs	Forest	Upper Highlands (UH)	Maize	Macadamia nuts	Mangoes
Embu north	0°27'55.8"S 37°35'24.8"E	Ugweri	30	Traditional	Combs	Forest	Upper Highlands (UH)	Maize	Coffee	Avocado

Appendix 2: Questionnaire

PROJECT TITLE: ASSESSMENT OF BACTERIAL PATHOGENS DIVERSITY AND BEEKEEPING CONSTRAINTS IN *Apis mellifera* COLONIES OF EMBU COUNTY, KENYA

Interview questions (tick as appropriate)

Sub-county …….

1. **Age of the respondent** ☐ Years
2. **Education status**

a) Indicate your highest level of education

☐Primary ☐Secondary ☐Tertiary

3. **Beekeeping situation**

a) How long have you been keeping bees?

☐Less than 1 year ☐1-5 years ☐ 5-10 years ☐10-15years

b) Where do you get bees from?

☐ Buying ☐ Catching swarming bees ☐Caves and forest

c) Other (specify)

……

4. **Number of colonies owned**

a) Type of hive used

☐ Traditional ☐ Frame ☐ Transitional

b) How many hives are located in?

☐ Backyard ☐ Apiary site ☐ Tree near homestead

5. Colony decline

a) Is there colony decline in the area?

☐ Yes ☐ No

b) If yes, what are the reasons?

☐ Absconding ☐ Diseases ☐ Predators ☐ Swarming

☐ Lack of water ☐ Pests

c) **Other (specify)**

…….....

d) Is there absconding at your site? If yes, what are the reasons?

……

……

……

……

e) Which type of hive is frequently absconded?

☐ Traditional ☐ Frame ☐ Transitional ☐ None

f) Which are the major pests and predators in your area that are a threat to the colony?

☐ Ants ☐ Beetles ☐ Wasps ☐ Bee lice

☐ Lizards ☐ Birds ☐ Wax moth

g) Other (specify)

……

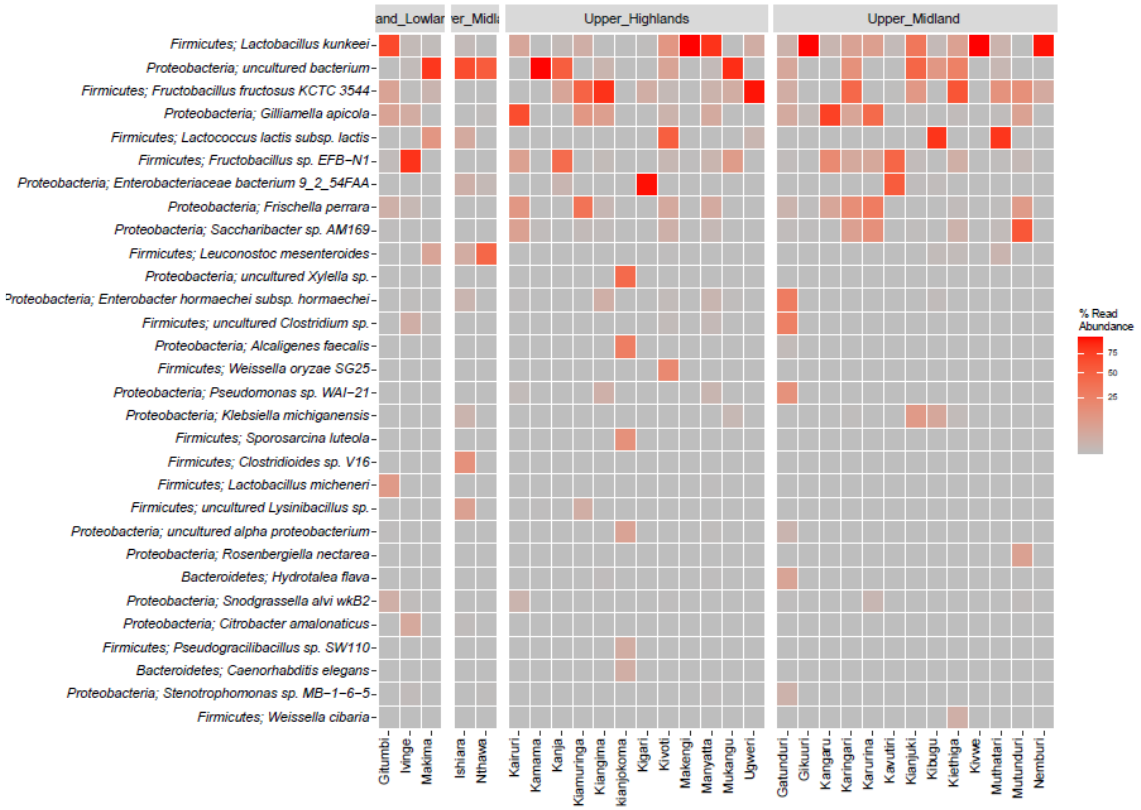
……

……

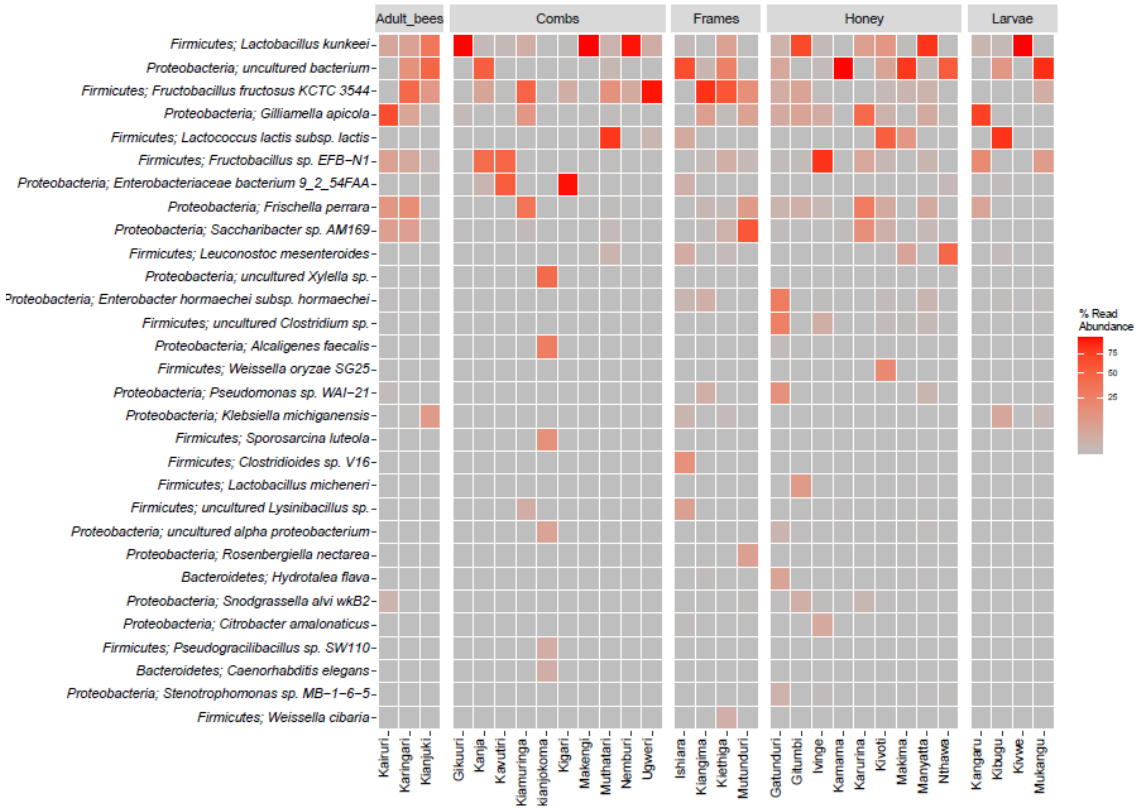
h) How many times do you harvest honey per year?

☐ Once ☐ Twice ☐ Thrice

Appendix 3: Heatmap showing 30 most abundant bacteria across the sampling sites as per the agroecological zones.



Appendix 4: Heatmap showing 30 most abundant bacteria across the sampling sites as per the sample types



Appendix 5: Network analysis of bacteria communities at the genus level based on the sample types.