EFFECT OF AGROECOLOGICAL ORIGIN ON HONEY ANTIBACTERIAL ACTIVITY AGAINST SELECT PATHOGENIC BACTERIA, ITS PHYTOCHEMICAL AND *BACILLUS* SPECIES PROFILES

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my beloved husband, Cyrus, whose unwavering support, encouragement, and love have been my constant source of strength. To my daughter, Amy, whose presence fills my heart with purpose and joy. To my parents, Mr. and Mrs. Gatero and Mr. and Mrs. Kaniaru for their endless support and belief in me.

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ABBREVIATION AND ACRONYMS

AMOVA	Analysis of Molecular Variance
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
GDP	Growth Domestic Product
GMP	Good Manufacturing Practices
HSD	Honest Significant Difference
KTBH	Kenyan Top Bee Hive
LAB	Lactic Acid Bacteria
LAPB	Lactic Acid-Producing Bacteria
LCMS	Liquid Chromatography Mass Spectroscopy
MBC	Minimum Bacterial Concentration
MRSA	Methicillin Resistant Staphylococcus aureus

ABSTRACT

Risks to human health posed by multidrug-resistant bacteria have been a menace to public health. Honey's content; bioactive organic compounds and bacteria population, have shown promise for the development of new antibiotics. Profiling *Bacillus* bacteria can serve as a scientific lead for discovering bioactive compounds with potential medical application. Honey's phytochemical composition and bacteria diversity play an essential part in its antibacterial activity. Research has enhanced our understanding of honey therapeutic antibacterial potential, but its application in clinical care is still limited. This study determined the influence of agroecological origin on antibacterial profile of honey, its diversity of *Bacillus* bacteria species and phytochemicals. A total of 54 samples were collected from Manyatta and Marigat regions of Embu and Baringo counties. Phytochemicals were extracted using the methanolic maceration method involving dissolution, filtration, and concentration with a rotary evaporator at 40 °C. They were quantified using Liquid Chromatography-Mass Spectrometry (LCMS). This was followed by an analysis of the honey extracts for antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Salmonella typhi*. To isolate pure *Bacillus* bacteria species, the honey samples were serially diluted to reduce bacterial concentration, heat-treated to select for spore-forming bacteria, and plated on HiChrome *Bacillus* agar. The isolates were identified using morphological and biochemical methods. The PCR products of the DNA from the isolates were shipped to the Netherlands' Macrogen laboratory for sequencing using the Sanger technology. Genetic diversity was determined based on the sequencing of 16s rRNA. The findings showed that both honey extracts from the two locations exhibited significant antibacterial activity against the tested microorganisms. The honey extracts from Manyatta Constituency, Embu County exhibited a noticeably greater activity ($p<0.05$) antibacterial activity than extracts from Marigat Constituency, Baringo County. The phytochemical composition and concentration varied between the two regions. LCMS results showed that Kaempferol, luteolin, lignans, triterpenoids, lanthionine, 2-hydroxycinnamic acid, coumarin, caffeic acid, *p*-coumaric acid, cinnamic acid, protocatechuic acid, myricetin, isocoumarin, ferulic acid, rutin, kaurene, gallic acid, tangeritin and chrysin were present in honey extracts from both regions, but in varying concentrations. There were secondary metabolites present in the identified bioactive compounds, including; myriocin, lanthionine, microlactin and phenazine. The bacteria identified were diverse and comprised 8 *Bacillus* species namely, *Bacillus velezensis*, *Bacillus atrophaeus, Bacillus siamensis, Bacillus cabrialensis, Bacillus halotolerans, Bacillus tequilensis, Bacillus inaquosorum* and *Bacillus licheniformis*. The results of this study proved that honey's antibacterial activity, phytochemicals composition and the profile of *Bacillus* bacteria species in honey is influenced by the agroecological origin. *Bacillus* bacteria species isolated in this study have produce potential active compounds that warrant isolation and purification for future use as antibacterial agents. Advanced screening and isolation of specific active compounds in the honey extracts to help profile the active compound in each isolate is recommended.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Honey has been studied for its antibacterial qualities, with different researchers investigating its capacity to prevent the growth of bacteria that are clinically significant (Albaridi, 2019, Almasaudi, 2021). Honey has been employed throughout history to treat ailments like burns, ulcers, cataracts, diabetes and for wound dressing (Naik *et al.*, 2021). In ancient Egypt, Greece, and Rome, honey was utilized as a cure for different ailments. In India, Ayurvedic medicine has been using honey for at least 4,000 years.

Several classical Greek classics, including Odyssey and Homer's Iliad, as well as the philosophical writings of Plato, Aristotle, and others, discuss the value of honey for human usage (Hardie, 2020). Very little was written about the usage of honey during the medieval times, and it was not a common topic in medical writings. Because of the advent of contemporary synthetic medicine in the nineteenth century, honey was overlooked. However, its resurgence can be seen as early as the turn of the 20th century, when honey was once again utilized as a cure for a number of ailments and as a superior wound healer (Naskar *et al.*, 2024).

Despite its historical uses, the application of honey in medicine has decreased since antibiotics discovery (Kuropatnicki *et al*., 2018). The rise of microorganisms resistant to antibiotics, however, generated a renewed interest in honey because of its strong antibacterial properties (Combarros-Fuertes *et al*., 2020). Despite its ancient use, honey has not garnered substantial recognition for its

medicinal benefits. Efforts are presently underway to integrate traditional and conventional medical practices to reduce reliance solely on antibiotics (Edwards *et al*., 2018).

Antibiotic resistance is the capacity of disease-causing microorganisms to endure the therapeutic impact of antibacterial medications (Sheikh *et al*., 2022). In clinical settings, abuse and overuse of antibiotics are generally considered to be the main ways that contribute to antibiotic resistance (Chokshi *et al.,* 2019; Muteeb *et al.,* 2023). Additionally, antibiotic contaminations and the emergence of pathogenic bacterial resistance have been significantly influenced by additional nonclinical large-scale antibiotic applications in aquaculture, cattle, and poultry farms (Zhao *et al.,* 2021). Antibiotic- resistant pathogen infections are typically more challenging to treat, have a high risk of recurrence, and result in substantial morbidity and mortality (Mancuso *et al.*, 2021).

Bacteria use three main strategies to resist antibiotics, with different variations. Antibiotic resistance exhibited by bacteria can be innate, acquired or adaptive resistance (Christaki *et al*., 2020). A bacterium acquires intrinsic resistance due to its inherent properties. Acquired resistance is the resistance that results from a bacterium that was previously susceptible developing a resistance mechanism by either genetic alteration or acquiring fresh genetic material from outside sources. The resistance to antibiotics brought on by environmental signals such as development condition, stress, for example, is known as adaptive resistance.

A study by Salam *et al*. (2023) estimated that in 2019, 1.27 million people died due to infections resistant to antibacterial treatments. Additionally, infections caused by antibiotic-resistant

bacteria, like methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii, and Klebsiella pneumoniae* lengthen hospital stays and place a heavy financial strain on national healthcare systems. An estimated $55 to 70 billion in losses are attributed to antibiotic- resistant illnesses in the U.S. every year. The losses exceeded €1.5 billion a year throughout Europe (Li and Webster, 2018). According to Njagi *et al*., 2020, in Kenya, 8.3% of those in need of outpatient or inpatient care reported not seeking treatment, and of these cost-related reasons accounted for 38.6%.

According to estimates, antibiotic-resistant illnesses cause over 30,000 deaths per year in the European Union alone, with Italy and Greece representing the vast majority of instances (Cassini *et al*., 2019). In low and middle-income countries in Africa, South America, and Asia, there is elevated morbidity and mortality from multidrug-resistant disease (Stewardson *et al*., 2019; Gandra *et al*., 2019). The number of deaths caused by antibiotic-resistant bacterial illnesses are projected to increase to 10 million by 2050 up from 700,000 in 2014, with a corresponding loss of $100 trillion in productivity and healthcare expenditure according to Jian *et al*. (2021). It has been reported that since 1990 over 8,000 deaths occur annually in Kenya due to AMR (IHME, 2023).

Many novel approaches based on the re-analysis of the resistance mechanisms by bacteria are being explored by scientists (Sammut, 2020; Uddin *et al*., 2021). Since ancient times, natural products have provided a promising platform for antibiotic development. Particularly in the last 20 years, natural materials and their semisynthetic equivalents have been essential in the creation of antimicrobial medications (Atanasov *et al*., 2021). Different terrestrial sources such as plants, fungi

and lichen which have presented above 80% of naturally derived antibiotics, have been discovered to inhibit multi-resistant infections through various ways.

Notwithstanding the significant effect on safety, compounds derived from nature have drawn particular interest because of their possible ability to combat various bacteria (Álvarez-Martínez *et al*., 2020). Numerous pure natural compounds and recently developed synthetic analogs have demonstrated their effectiveness as substitute antimicrobial agents against infections that don't respond to treatment (Hobson *et al*., 2021; Pancu *et al*., 2021; Breijyeh and Karaman, 2023). Natural antimicrobial compounds have also garnered a lot of attention as a potential substitute for ineffective antibiotics.

Rising healthcare costs have gained major worldwide concern, putting pressure on national economies, widening gaps, and preventing millions of people from accessing necessary medical care. Global health spending in 2016, the most recent year for which data were available, was $8.0 trillion (Chang *et al*., 2019). It is anticipated that U.S. healthcare spending will account for 26% of GDP by 2035 (Martin *et al*., 2021). Spending on health care rose globally to $7.9 trillion in 2017 as a result of the BRICS countries' Sustainable Development Goals (SGDs), and it is expected to reach $11 trillion by 2030 (Sahoo *et al*., 2023). According Kenyans spend about $150 billion shillings on healthcare annually (PBO, 2023; NTEP, 2023). Recent between January 2024 and January 2025, the cost of healthcare in Kenya rose by 3.3% (KNBS, 2025).

There’s a wide array of evidence of honey as a means for reducing the global economic burden of

healthcare. Honey, being an application treatment and particularly for wound and burn infections, there is a reduced need for using the more expensive last-resort antibiotics (Mokhtar, 2021). Recovery becomes quicker; hence, there are shorter hospitalizations. As the tendency to misuse antibiotics encourages bacterial resistance, this makes it necessary for the production of newer and much more costly antibiotics. The unique mechanisms of action of honey like low pH, hydrogen peroxide and bioactive compounds reduce the risk of bacterial resistance to honey, allowing many currently used antibiotics to be effective while minimizing the financial burden of new drug development (Almasaudi, 2021).

1.2 Problem Statement

Bacterial resistance to various antibiotics, including those considered as last-resort treatments, is escalating globally (Chandra *et al*., 2021). A concerning trend is the dwindling development of new antibiotics, as pharmaceutical firms have redirected their attention from the development of antibiotic drugs (Cook and Wright, 2022). This resistance phenomenon has eroded the efficacy of extensively used antibiotics over time, highlighting an urgent necessity for alternative antibacterial strategies. Additionally, the issue of cost and access to modern healthcare looms prominently, especially for individuals of modest and low incomes within our nation (Frank *et al*., 2023). Owing the persistence in the issue of price and availability of conventional treatment methods, there's a critical requirement for less expensive options. Honey can be seen as a potential alternative due to its low cost and there's no reported resistance by pathogenic microorganisms (Spoială *et al*., 2022). Nonetheless, the scientific community must aggressively undertake research so as to improve the standing nature of knowledge so that it may yield to its full potential.

1.3 Justification of the Study

A number of investigations have established that there is limited to no antibacterial resistance to honey because of its complex composition (Combarros-Fuertes, 2020; Bonsignore et al., 2021). Studies evaluating the antibacterial properties of the honey on multidrug-resistant bacteria have been shown to be very effective, with most experiments reporting that the honey showed an incredible antibacterial activity (Wasihun and Kasa, 2016; Gobin *et al*., 2018; Onyango and Liang, 2024). This indicates that honey may serve a rich alternative to the already existing antibiotics in dealing with the current antibiotic-resistant bacteria, which has brought a great deal of scientific attention on honey (Krishnakumar *et al*., 2020). Randomized and controlled trials have been used to demonstrate the ability of honey to treat wounds and burns, albeit, these trials are frequently limited by factors such as small sample sizes and poorly defined outcomes (Zhang *et al*., 2023).

Apart from this, when conventional therapies were ineffective, honey was primarily used as a last resort (Hungin, 2022). The use of honey in medicine is questioned due to the wide variability in antibacterial activity exhibited by honey from natural sources (Hossain *et al*., 2022). Furthermore, for the majority of low- and middle-class families, the cost of conventional treatment methods is certainly not affordable (Kookana *et al*., 2022). This critical condition demands immediate action to make treatment more affordable to decrease mortality rates. Since honey is available all year round and not too expensive, it is a suitable option.

Some of the drivers of honey antibacterial activity are its phytochemicals and bacterial composition (Johnston *et al*., 2018; Almasaudi, 2021). Particularly, the *Bacillus* species in honey are known to produce metabolites such as lactic acid and other organic compounds known to

induce antibacterial efficacy (Olofsson *et al*., 2016; Brudzynski, 2021). In several studies, *Bacillus* species have successfully been isolated from honey, and investigated for their bioactive potential and antibacterial activity (Hallaj-Nezhadi *et al*., 2022; Magdalena *et al*., 2020). Nevertheless, there is limited research of the diversity and antibacterial properties of *Bacillus* in honey in Kenya. Addressing this gap could provide locally sourced *Bacillus* strains with promising applications in medicine and biotechnology. Additionally, understanding how ecological zones influence these profiles can guide the selection of honey sources with the highest antibacterial potency, thereby expanding options for managing bacterial infections.

1.4 Hypotheses

i. Agroecological origin does not influence honey phytochemicals and metabolites composition

ii. Honey's agroecological origin does not significantly influence antibacterial activity against pathogenic bacteria.

iii. Marigat Constituency, Baringo County and Manyatta Constituency, Embu County honey does not exhibit different profiles of *Bacillus* bacteria species

1.5 Objectives

1.5.1 General objective

To determine the effects of agroecological origin on the diversity of phytochemicals, *Bacillus* bacteria species and antibacterial activity of honey on select human pathogenic bacteria

1.5.2 Specific Objective

i. To determine the diversity of phytochemicals and metabolites in honey from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County

ii. To determine the antibacterial activity of methanolic extracts of honey from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County

iii. To determine the diversity of *Bacillus* bacteria species in honey from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County

1.6 Significance of the study

Findings of this research will hold substantial implications for Kenya, particularly within the realm of medicine. Both contemporary and traditional medical practitioners stand to benefit, as the study's outcomes will facilitate the streamlined implementation of apitherapy, aided by the availability of pertinent effective data. The characterization of phenolic compounds and lactic acid-producing bacteria achieved in this investigation will offer valuable insights into identifying more honey harbouring these medicinal constituents. Such insights are of utmost significance to the pharmacological industry, as they can guide the development of therapeutic medicine. Moreover, the knowledge generated through this study will contribute to the enrichment of the existing awareness among local inhabitants about the advantageous medicinal utilities of honey. This augmented awareness, in turn, is poised to yield positive repercussions for beekeeping, enhancing it as a viable source of income and thereby elevating the livelihoods of individuals, driven by a heightened demand for honey.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Honey

Honey is a natural product made by bees from flower nectar or from exudates of plants, typically with assistance from insects that feed on plants (Machado *et al*., 2018). Honeybees gather nectar, change it by mixing it with certain ingredients of their own, place it inside the honeycomb, let it ripen and mature, and then dry and preserve it (Oliver, 2021). The Codex Alimentarius Commission emphasizes in establishing the guidelines that honey cannot be heated or treated to the point that either its quality is degraded or its core composition is changed (Balkanska *et al*., 2020). Honey's quality is influenced by several aspects, such as its chemical and physical characteristics, agroecological origin, botanical origin, entomological origin, and symbiotic relationships with beneficial bacteria (Cucu *et al*., 2021).

2.2 Properties and Composition of Honey

Female honeybees, according to Chapman (2024), suck nectar from flowers using their proboscis, a tubular tongue, and thin out the nectar with saliva and enzymes. The mixture is then evaporated down to about sixteen percent water and stored in cells where it serves as the primary food of the bees. As previous research shows, the taste and constitution of honey depend upon the flower origin, climate of the region, among other factors (Machado *et al*., 2018; Romero *et al*., 2024). Scripcă *et al*. (2019) indicate that quality of honey is measured based on physical, chemical and microbial parameters. Many scientific works have studied the complicated chemical composition and antibacterial characteristics of the honey (Maroof and Gan, 2020; Kunat-Budzyńska *et al*., 2023).

Physicochemical parameters such as moisture and pH vary among different honeys (Geană *et al*., 2020). The total honey acidity varies across different varieties, ranging from 8.68 to 59.49 meq/kg. Additional physicochemical factors include the ratios of mineral composition, water-insoluble solids, and reducing and nonreducing sugars, the amount of 5-hydroxymethylfurfural, diastase value, density, electrical conductivity, color, and refractive index (Sharma *et al*., 2023).

Honey is said to contain over 600 different compounds, including about 38.5% fructose and 31.0% glucose, as well as other types of carbohydrates (Johnson and Obi, 2024). It also contains vitamins and folic acid. Polyphenols, flavonoids, flavanones, phenolic acids and their derivatives, organic acids, and antimicrobial peptides are some of the substances found in honey (Valverde *et al*., 2022). Compounds found in propolis, bee pollen and wax can also be present in honey.

2.3 Microorganisms in Honey

Natural sources such as honey contain microbes capable of producing a broad variety of antibacterial compounds (Mullis *et al*., 2019). Microbes found in honey are from different main sources like pollen, honey bee digestive tracts, nectar, dust, and air (da Silva *et al*., 2024). In social insects like honey bees, oral-fecal and trophallactic transmission are the means by which colony members share and transfer intestinal bacteria. On the other hand, interaction with older bees in the hive, ingestion of hive material, stored pollen or bee bread during the mature phase are also part of the transmission and spreading of the bacteria (Pasho *et al*., 2021).

The most promising source of obtaining natural medicines with the use of microorganisms is fungi

and bacteria (Abdel-Razek *et al*., 2020). The microbiota of the gastro intestinal tract of honey bees is highly unique and specialized with predominant bacterial phyla being *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* (Nowak *et al*., 2021). It has been shown that the digestive tract of adult honey bees is most likely to be colonized with anaerobic bacteria as opposed to aerobic ones (Khan *et al*., 2020). Although the harsh parameters of the honey do not allow the growth or multiplication of certain bacteria, it has a high variety of microbes in minimal quantities and within a limited spectrum. Microorganisms in honey may affect its quality or safety. Microbes of concern in post-harvest management of honey include yeasts, coliforms and spore-forming bacteria. Coliforms are often used as indicators of the hygienic and commercial quality of honey.

Although microorganisms cannot multiply in honey, except for some yeasts and molds, they can grow and multiply when honey is used as a component in other foods, often causing damage (Scepankova *et al*., 2021). Microorganisms are involved in activities such as alterations, enzyme production, antibiotics and toxin production, promoting metabolic conversion and inhibition by microbial competition (García-Bayona and Comstock, 2018). Honey contains bacterial spores, especially those belonging to the *Bacillus* genus, for example *B. amyloliquefasciens* (Luca *et al*., 2024). However, honey has not been shown to contain vegetative forms of disease-causing bacterial species (Erler *et al*., 2022). Since bacteria cannot reproduce in honey, a high concentration of vegetative bacteria may be a sign of recent secondary source contamination. Secondary sources of contamination for honey, which take place after harvest, are similar to those that influence other types of food, including food handlers, air, cross contamination and equipment (Végh *et al*., 2024). These sources can be managed by good manufacturing practices (GMP).

2.4 Lactic Acid-Producing Bacteria (LAPB) in Honey

Honey contains various lactic acid-producing bacteria (LAPB), a group of gram-positive bacteria that produce lactic acid and other compounds such as acetic acid and ethanol (Leska *et al.*, 2023). The principal genera and widely studied include *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Walter, 2008; Mokoena, 2017). *Lactobacillus* is the largest genus, with more than 100 species, however in 2020 it was reclassified into 25 genera including 23 novel genera (Zheng *et al.*, 2020). Because of their probiotic qualities, LAPBs have also been the subject of in-depth research in both people and animals. This has allowed for their successful commercial use within the feed, food, and pharmaceutical industries (Abedin *et al.*, 2024). The discovery that LAPBs represented a portion of honey bee's gut microbiota has piqued scientists' curiosity about finding parallels and similarities with the probiotic bacteria that have been studied extensively in both humans and animals (Iorizzo *et al*., 2022).

Numerous scientific investigations have isolated and identified LAPBs in honey. A study by Abadi *et al*. (2023) isolated 13 strains of *Lactobacillus*, including *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*. Such strains are crucial for executing the fermentative processes associated with the conversion of pollen to bee bread and nectar to honey (Iorizzo *et al*., 2022). Lactic acid producing bacteria are of great importance in honey, since their activities relate to the health status of honeybee colonies and to the honey's antibacterial properties (Olofsson *et al.*,2016). These bacteria originate mainly from the pollen and nectar consumed by bees and from the contact occurring among individual bees within the hive (Vásquez and Olofsson, 2009).

The LAPB represent the normal inhabitants of the gastrointestinal tract of honeybees, whereby they contribute to their health by improving immune response and protection against pathogenic microorganisms (Motta and Moran, 2024). The presence of LAPB is highly important for balanced gut microbiota, protecting these insects against dysbiosis, a condition that is able to promote the development of various diseases (Leska *et al*., 2023). It is widely known that LAPBs produce lactic acid and other metabolites responsible for activity against the growth of pathogenic microorganisms. This antibacterial potential contributes to quality of honey and protection of bees from pathogens (Iorizzo *et al*., 2022). Different strains of LAPB have shown efficiency against different diseases and hence appear promising for probiotic applications.

Some of the LAPB like *Actinobacteria* species, although not broadly studied, have been identified in honey obtained from bees such as *A. mellifera*, *A. cerana*, *A. dorsata* and *A. florae* (Cui *et al*., 2022). They are popular for their metabolic abilities, and production of bioactive compounds. *Streptomyces* species, which is a member of the order *Actinobacteria*, has been isolated from honey (Al-Enazi *et al*., 2022). They are renowned for their capacity to generate antimicrobial compounds, which enhance the antibacterial characteristics of honey (Grubbs *et al*., 2021).

Bifidobacterium species have also been isolated from honey in several studies (Li *et al*., 2024; Nilsson *et al*., 2024). They primarily contribute to the health of honeybee colony, and also have possible health benefits to humans. Most of the bifidobacteria are found within honey bees' digestive systems, especially in larval and adult stages. Honeybee-related *Bifidobacteria* express

high strain diversity as a result of their long-term co-evolution with these insects (Mohan *et al*., 2017). Survival and successful operation within a beehive society depend on their ability to adapt themselves to live in various microhabitats of the hive, including nutrient pools created by larvae rearing and food storage.

Regarding honeybee health, *Bifidobacterium* species have been found to be implicated in many different processes that are considered important concerning honeybee health, including digestion, enhancing immune responses, and protection against pathogens (Schell *et al*., 2022). Certain strains, such as *Bifidobacterium asteroides*, have been associated with immune system activation in bees and therefore play a protective role against infections.

Fructophilic lactic acid bacteria (FLAB) have a growth preference for fructose, which makes honey an ideal habitat for them (Endo *et al*., 2018). *FructoBacillus fructosus* is among the most common bacterial species found in honey. It has been found to exhibit antibacterial properties (De Simone *et al*., 2023). This attribute is beneficial to both the bees as well as human beings consuming the honey. This antibacterial characteristic is derived from compounds that are produced by the *Fructophilic* species.

Notably, majority of the antimicrobial efficacy of microbial communities in honey has focused on the bacteria that produce lactic acid as the major secondary metabolite. Little research has been conducted on the efficacy of other microbial communities such as those that produce lactic acid as a minor secondary metabolite like the *Bacillus spp*, hence the background of this study.

2.4.4 *Bacillus* species

Bacillus bacteria are gram-positive, and spore-forming bacteria (Borriss *et al*., 2020). Their ability to develop spores enables them to endure honey's extreme conditions. They are fermenters, they can produce lactic acid by fermenting fructose and carbohydrates (Han *et al*., 2023). That's why they are called lactic acid-producing bacteria. The key feature that distinguishes lactic acid-producing *Bacillus* is that they are Gram-positive and give a positive catalase test (Ayodeji *et al*., 2017; Sharifi *et al*., 2017).

The different inherent elements of honey influence the ability of microorganisms to grow or survive through either bacteriostatic or bactericidal activity (Tamime *et al*., 2017). What is especially interesting in other studies is that *Bacillus* extracted out of various origins can produce various compounds that can inhibit the growth of microorganisms (Bielik *et al*., 2021). Among these are secondary metabolites that are bioactive, including bacteriocins, antimicrobial peptides and organic acids (Salwan and Sharma, 2020). Most antibiotic peptides and lipopeptide antibiotics are produced by bacteria from the genus *Bacillus*, according to a study by Baharudin *et al*. (2021). The peptides fall into one of two categories depending on whether they are synthesized non-ribosomally (polymyxins and iturins) or ribosomally (bacteriocins) (Stoica *et al*., 2019). *B. amyloliquefaciens*' functional unit encodes 4 to 5% of its genetic material for the synthesis of antimicrobial metabolites (Zeng *et al*., 2021).

According to Basi-Chipalu *et al*. (2022), *Bacillus* species are able to make a chemical known as bacteriocin-like inhibitory substance (BLIS). BLIS has been shown to be produced by *Bacillus*

strains such as *Bacillus thuringiensis, Bacillus megaterium, Bacillus subtilis, Bacillus licheniformis*, *Bacillus stearothermophilus,* and *Bacillus cereus* (Stoica *et al*., 2019). This goes to show that the *Bacillus* species may be used in enhancement of human health in food production and in curative medicine. Further studies on *Bacillus* species in honey and factors that influence their diversity are needed to enhance the knowledge content on sources, isolation and utilization of the antimicrobial properties of these microorganisms.

2.5 Antibacterial Potential of *Bacillus* Species

Although there is substantial research on the antibacterial properties of honey, few studies have been done on participation of *Bacillus* species in the antibacterial properties of honey (Zhang *et al*., 2021). *Bacillus* species are spore-producing organisms that can withstand extreme conditions like pressure, pH and desiccation (Logan and Vos, 2015). Additionally, by competing with other organisms for nutrients and producing a variety of antimicrobials like organic acids, and bacteriocins*, Bacillus* species are able to exhibit antagonistic activity against other microbes (Darband *et al*., 2022). Interestingly, it is possible to cultivate some *Bacillus* species for use as probiotics, because they produce bioactive compounds, such as lactic acid, acetate acid, and formic acid that act as antimicrobials against a variety of pathogens. The research on the antimicrobial capabilities of *Bacillus* strains is relatively limited, particularly in Africa (Baharudin *et al.*, 2021).

Numerous studies have reported presence of *Bacillus* species in honey including *B. megaterium, B. mycoides, Bacillus thuringiensis, Bacillus licheniformis, Bacillus pumilus, Bacillus megaterium* and *Bacillus coagulans* (Alippi et al., 2004; López and Alippi, 2007).

Bacillus sonorensis and *Bacillus licheniformis* with antimicrobial activity against *Micrococcus*

luteus and *Mycobacterium smegmatis* were isolated from honeybee samples (Martín-González *et al.*, 2023). *Bacillus safensis, Bacillus rugosus* and *Bacillus velezensis* isolated from will honey collected from Shushtar city, Khuzestan Province, Iran demonstrated significant antimicrobial efficacy against human and plant pathogens (Hallaj-Nezhadi *et al.,* 2022). *Bacillus* strains of B. laterosporus and B. megaterium strains isolated from honey samples and bee gut have been shown to demonstrate in vitro antimicrobial activity against *PaeniBacillus* and *Ascosphaera apis* honey bee pathogens (Alippi and Reynaldi, 2006). *B. subtilis* subsp. *subtilis* Mori2 strain administered in bee colonies was shown to inhibit microsporidian causes nosemosis (Sabate *et al.,* 2012). *B. subtilis* and *B. amyloliquefaciens,* which have found in honey (Wang *et al.*, 2015), have been reported to produce polyketides bacillaene, difficidin, and macrolactin which possess antimicrobial activities (Olishevska *et al.*, 2019). Therefore, the presence and role of *Bacillus* species in antibacteria efficacy of honey has been widely demonstrated. However, it's widely unrecognized how agroecological origin influence their populations and contribution to honey antibacterial efficacy particularly in Kenya, hence the gap this study seeks to address.

2.6 Antibacterial Properties of Honey

Many scientific studies have confirmed the wide-range antibacterial effects of honey (Hussain, 2018). Various factors have been proven to affect its antibacterial potency, including low pH, high concentration of sugar, osmotic impact, and presence of bactericidal and bacteriostatic agents (Almasaudi, 2021). Also, the immune-modulating capacity of honey and the actions of phytochemical compounds are described as beneficial to have an anti-bacterial action (Feknous and Boumendjel, 2022). Moreover, propolis and bee pollen are honey elements with antimicrobial activity themselves, playing their individual role in the therapeutic efficacy of honey (Karadal *et*

al., 2018).

2.6.1 Osmotic Activity

Honey consists of glucose and fructose, which constitute about 84% of the honey composition, which makes bacterial survival difficult (Afroz *et al*., 2023). Due to the high sugar saturation, honey is left with water level of 15% to 20% (Kumari *et al*, 2021). Very few water molecules are left available for bacteria because of the intense interaction of these water molecules with sugar molecules (Chen, 2019). Water activity ($_aW$) is a measurement of this "free" water. The optimum water activity for most bacteria range from 0.98 to 0.99 (Erkmen and Bozoglu, 2016). It has been found that the range of mean values for honey's water activity is 0.562 to 0.62 (Mădaş *et al*., 2019). There isn't enough water activity in this range for any species to flourish.

Nonetheless, it has been documented that certain yeasts can survive in honeys with elevated water content, which leads to the honey becoming spoiled (Silva *et al*., 2017). Fermentation does not take place if the water concentration in honey is less than 17.1%. Many species of bacterial are inhibited if the $_aW$ falls below 0.90 (Tapia *et al*., 2020). Conversely, certain species attain their maximum growth rate when their $_aW$ reaches 0.99. This indicates that the type of bacteria involved determines whether diluted honey solutions will suppress growth.

2.6.2 Acidity

Honey is characterized as acidic due to its pH range of 3.2 to 4.5 (Apriceno *et al*., 2018; Almasaudi, 2021). This pH range is sufficiently low to suppress a wide variety of diseases. Many bacterial species grow best in pH values between 7.2 and 7.4 (Allen and Waclaw, 2018). To grow, some common wound-infecting species require a minimal pH of 4.3; *Salmonella* sp. requires a minimum

pH of 4.0; *Pseudomonas aeruginosa* requires a minimum pH of 4.4; and *Streptococcus pyogenes* requires a pH of 4.5 (Yupanqui *et al*., 2022). This means the low pH is an important antibacterial factor for undiluted honey. However, the pH level will rise if honey is diluted, for example, by bodily fluids when consumed or applied topically, and its acidity will not be a strong enough inhibitor of many bacterial species (Scepankova *et al*., 2021).

2.6.3 Hydrogen Peroxide

The main process by which most honey exhibits antibacterial characteristics is the enzymatic synthesis of hydrogen peroxide (Bucekova *et al*., 2018). This therefore means that a significant portion of honey's antibacterial action comes from hydrogen peroxide (Farkasovska *et al*., 2021). The bee's hypopharyngeal gland secretes the enzyme glucose oxidase into the nectar, which aids in the production of honey (Bucekova *et al*., 2018). Glucose oxidase becomes active upon dilution, leading to the generation of hydrogen peroxide and gluconic acid (Brudzynski, 2020). This peroxide is mild in nature and exhibits antimicrobial properties without causing tissue damage (Oti, 2021).

When transition metal ions and ascorbic acid are present, hydrogen peroxide has a brief half-life because they catalyze its breakdown into water and oxygen (Yupanqui *et al*., 2022). This indicates very little hydrogen peroxide in full-strength honey. When honey is at its most concentrated, glucose oxidase is essentially dormant, and because of this, pure honey only produces hydrogen peroxide when it is diluted (Albaridi, 2019). This is because the acidity the enzyme creates during activity lowers the pH to a threshold where the enzyme is unable to function. When diluted, honey's effect increases by a factor of 2,500 to 50,000, creating a "slow-release" antiseptic that is

antibacterial without causing tissue damage. Notably, certain honeys, such as Manuka honey, maintain antibacterial effects regardless of the absence of hydrogen peroxide activity (Roberts *et al*., 2015).

2.6.4 Phytochemical Activity

The inability of the peroxide-generating machinery to adequately account for honey's antibacterial properties suggests the presence of other antibacterial agents (Bucekova *et al*., 2018; Bazaid *et al*., 2022). There have been reports of the separation of additional antibacterial compounds in addition to hydrogen peroxide (Almasaudi, 2021; Hossain *et al*., 2022). Moreover, it has been proven that heating honey reduces its effectiveness against some bacterial species while retaining it against others. This is because heating honey inactivates the enzyme glucose oxidase (Scepankova *et al*., 2021).

The best direct evidence that honey contains non-peroxide antibacterial components comes from reports of bacterial inhibition persisting in catalase-treated honeys thus lowering the hydrogen peroxide activity (Hussain, 2018). Compounds responsible for antibacterial activity have been discovered in honey across numerous studies, including pinocembrin, 2-hydroxybenzoic acid, and benzyl alcohol (Wani *et al*., 2020; Tanuğur *et al*., 2024). However, these are present in far too little levels to be responsible for any appreciable amount of activity on their own.

2.7 Agroecological influence on honey antibacterial activity

Various studies confirm that honey antibacterial activity is mostly dependent on its agroecological origin (Almasaudi, 2021; Yangoua *et al*., 2024). Honey, as stated by Tafere (2021), is composed of more than 180 distinct compounds like organic acids, minerals, vitamins, trace elements, proteins,

and enzymes. Various factors affect honey content, including the origin of nectar, seasonality, and climate conditions (Becerril-Sánchez *et al*., 2021).

Polyphenols, which are derived from nectar are a heterogeneous chemical family. They play an important part in honey's antibacterial potency (Al-Khayri *et al*., 2022). They are categorized into flavonoids (anthocyanidins, chalcones, isoflavones, flavonols, flavones, and flavanols) and non-flavonoids (phenolic acids) (Bento *et al*., 2017). These compounds are produced by secondary metabolism of plants and are identified by a number of phenolic groups that are linked to intricate structures (Tsimogiannis and Oreopoulou, 2019). As a result, the antibacterial effectiveness of honey differs based on its phytogeographical origin (Ciucure and Geană, 2019; Nakib *et al*., 2022). Flavonoids and phenolic acids serve as honey's biological markers, helping to determine its agroecological origin (Santos *et al*., 2021).

In contrast to primary metabolites that include amino acids, chlorophyll, and simple sugars, secondary metabolites play important roles in addition to being active during the processes of plant absorption, transport, respiration, and differentiation (Erb and Kliebenstein, 2020). Phenolic composition of honey is mainly based on the flowers the honey is made of and therefore a significant trait to define and classify honey, particularly in unifloral honey.

Flavonoids are low molecular weight substances that occur naturally and are predominantly water-soluble (Brodowska, 2017). They consist of two benzene rings connected by a linear carbon chain, often reorganizing into three rings containing fifteen carbon atoms. These compounds are typically

associated with sugars such as glucose and galactose. Flavonoids that are not bound to sugars are called aglycones. Based on the oxidation degree of the C ring, flavonoids are classified into isoflavones, anthocyanins, anthocyanidins, flavones, flavanones, flavonols, and flavanones. Flavones, flavonones, and flavanols are the most common forms of flavonoids found in honey (Bento *et al*., 2017). Though, other studies have demonstrated that agroecological origin affects honey phytochemistry and antibacterial efficacy, no study has been conducted to compare how different agroecological zones in Kenya affect these variables.

2.7.1 Effects of environmental conditions and vegetative coverage on honey antibacterial activity

The antibacterial properties of honey are significantly influenced by its floral origin and the plants from which bees get nectar. Naturally, the dissimilar floral species provide different quantities of bioactive constituents such as phenolics, flavonoids, and hydrogen-peroxide-associated compounds, which are responsible for the antibacterial activity of honey. As an example, buckwheat and avocado flower honeys have proven to be highly effective in terms of antibacterial properties compared to honeys produced from other flowers, therefore, highlighting the role of plants in the therapeutic potential of honey (Sharaf El-Din *et al*., 2024). Similarly, studies in Saharan areas found out that honeys rich in *Fabaceae* pollen displayed increased activity against bacterial pathogens (Almasaudi, 2021). All these observations point to the fact that the floral composition of honey is essential in determining the sources with the highest antibacterial activities.

In addition to the floral origin, the environmental and climatic conditions have a critical impact on

the antibacterial qualities of honey. The moisture content of honey, its enzyme activity, and the level of bioactive compounds depend on variables like altitude, temperature, seasonality, and the variety of plants in the region, which influence the antimicrobial activity of honey (Oyedokun *et al*, 2025). Seasonal variations, for instance, have been shown to alter the antibacterial activity of honey, with samples collected during the dry season sometimes exhibiting stronger activity than those from the rainy season (Lavinas *et al*., 2025, Rikohe *et al*., 2023). Mechanistically, such differences are explained by the differences in hydrogen peroxide production and non-peroxide mediators like methylglyoxal and flavonoids which respond to environmental stresses experienced by the source plants (Lavinas *et al*., 2025). Combined, these studies highlight that both the floral and ecological environment of honey is a critical determinant of its antibacterial capacity.

2.7.2 Vegetative coverage in the study areas

The semi-arid Marigat region receives only 450 - 700 mm of annual rainfall on average, which is split into two rainy seasons determined by the Intertropical Convergence Zone (ITCZ) movement (Okuku *et al*., 2024). A large portion of the precipitation falls in brief, intermittent showers during the months of April/May and October/November. The area's distinctive clay and clay loam soils are severely eroded by these downpours.

Marigat is dominated by drought resistant crops, adapted to hot and dry climate (Okuku *et al*., 2024). A diverse range of woody plant species, dominated by *Acacia*, combine to form the dominant layer of vegetation, with an understory of moderate to dense perennial grasses and forbs making up the vegetation beneath it (Kandie, 2022). Diverse woodlands with Acacia species like *A. melifera*, *A. nirotica*, and *Azadirachta indica* comprise the natural vegetation. There’s a vast

vegetative coverage of *Prosopis julifora* in the areas studied. Species of *Opuntia* are also present in Marigat. It is easy to see that there has been a broad conversion of vegetation areas into degraded locations and bare surfaces with grazing meadows and human settlements. Marigat's landscape consists of patches of evergreen forest, shrub, and grassland (Adimo, 2016).

Marigat Constituency is a major honey-producing region in Kenya (Chepkemoi *et al*., 2021). Notably, in this region, beekeeping provides a sustainable source of income since agricultural activities like crop farming and livestock keeping are limited by aridity. Additionally, the warm and semi-arid climate of Marigat constituency provides bees with the perfect habitat for growth and honey production. The vegetation present in Marigat is able to tolerate drought, and thus is rich in nectar and pollen, aiding the production of honey. Most of the honey produced in Marigat comes from conventional Tugen log hives constructed from materials found locally, like hollowed-out tree trunks (Yator, 2021). However, there's a gradual shift towards modern methods using Langstroth and Kenya Top Bar Hives (KTBH).

Manyatta Constituency in Embu County is located east of Mount Kenya, with a sizable chunk of its territory extending into the foothills. Most of the area that is now cultivated in Embu on the slopes of Mount Kenya used to be forest coverage. Exotic trees such as like camphor, African olive, and East African cedar are present in the higher elevations, while bamboo thickets dominate the midlands (Waiganjo *et al*., 2024).

Fertile soils and ample rainfall support extensive agricultural activities. Food crops that are

frequently grown are tea, coffee, beans, maize, potatoes, and vegetables (Nthiga, 2024). There's also a presence of fruit trees such as avocado, bananas, macadamia, mangoes and citrus fruits. More vegetative cover observed in Embu is low-growing grasses, cushion plants, and scattered shrubs (Waiganjo *et al*., 2024).

Manyatta's varied flora, temperate environment, and enterprising locals make it an ideal place for beekeeping. In Manyatta, beekeeping is a developing agricultural industry that boosts household income and promotes environmental preservation. This region, due to an abundance of vegetation, provides a rich nectar and pollen source for the bees throughout the year.

2.8 Test Microorganisms

2.8.1 *Staphylococcus aureus*

Staphylococcus aureus is a bacterial pathogen which leads to human diseases (Rasheed and Hussein, 2021). It majorly causes nosocomial bacteremia, infections of the cardiovascular system, prosthetic joints, surgical sites, pneumonia, and other respiratory tract illnesses (Chalmers and Wylam, 2020). A review from 2017 estimated the number of fatalities in the U.S. resulting from *S. aureus* to be 20,000 annually (McGuinness *et al*., 2017). However, the rate of mortality caused by *S. aureus* in developing countries surpasses that of developed countries. Treatment of ailments caused by *S. aureus* remains difficult due to the rise of multi-drug-resistant strains such as Methicillin-Resistant *S. aureus.*

Honey has shown remarkable activity against *S. aureus,* including MRSA (Gambo *et al*., 2018; Mama *et al*., 2019; Mudenda *et al*., 2023). Unlike traditional antibiotics, honey targets bacteria

through multifaceted actions like high sugar content, low pH, phytochemicals, and hydrogen peroxide production, reducing the likelihood of resistance development. Research has proven that honey is effective in treating infection, wound care, and can prevent bacterial colonization of medical devices. Its potential as an adjunctive treatment to antibiotics and as a natural alternative to resistant infection treatment makes it a valuable tool in the struggle against *S. aureus* infections.

2.8.2 *Salmonella typhi*

It is a gram-negative pathogen that causes human forms of typhoid fever (Khan and Shamim, 2022). Typhoid fever still remains one of the most contagious infections worldwide and a major cause of mortality in some regions (Crump, 2019). It is estimated that Typhoid and paratyphoid fever infections affected 17 million individuals across the world in 2015 with most of the occurrence being experienced in Southeast Asia, South Asia, and sub-Saharan Africa (Dai *et al*., 2020). The most prevalence of the ailments was observed in South Asia. Without treatment, both typhoid and paratyphoid fever may result in fatal outcomes; in 2015 the number of fatalities caused by the two diseases worldwide was estimated to reach 178,000 (Radhakrishnan *et al*., 2018).

Studies have proven that honey and its extracts inhibit the growth of *Salmonella typhi* (Hussain *et al*., 2015). This has been tested even in cases where *Salmonella typhi* has shown resistance to the conventional antibiotics (Wang *et al*., 2021). Also, it has been found that honey plays a significant role in breaking down the production of biofilm, which is one of the main ways *Salmonella typhi* circumvents therapy (Khataybeh, 2023). Moreover, differences in honey phytochemical content influenced by agroecological origin and geographical origin of honey, were linked to differences in antibacterial activity of honey towards *Salmonella typhi* (İstanbullugil *et al*., 2023).

2.8.3 *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped, facultatively anaerobic bacterium frequently found in warm-blooded species' large intestines (Geurtsen *et al*., 2022). For the most part, these bacteria are benign or even helpful to people. Naturally occurring in the stomach, the majority of *E. coli* strains are not harmful, but virulent strains can cause Crohn's disease, hemorrhagic colitis, newborn meningitis, urinary tract infections, and gastroenteritis (Cohen, 2022). In human medicine around the world, multidrug resistance in *Escherichia coli* is becoming a concerning problem. Although *E. coli* is naturally susceptible to practically all therapeutically relevant antibiotics, it has a high potential for resistance gene accumulation, primarily by horizontal gene transfer.

Honey, a natural and efficient substitute for traditional antibiotics, has demonstrated strong antibacterial action against *Escherichia coli* (Combarros-Fuertes *et al*., 2020). It works especially well against resistant strains because of its modes of action, which include oxidative stress induction and membrane disruption. Honey is also positioned as a possible treatment option for *E. coli*-related infections since it has a wide antimicrobial activity spectrum and low possibility of resistance development.

2.8.4 *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, spore-forming, aerobic and rod-shaped bacterium majorly found in soil and the gastrointestinal tracts of some animals. This bacterium is well known for its ability to survive extreme conditions due to its ability to form endospores. This characteristic also complicates the treatment of illnesses caused by *Bacillus subtilis*. Though generally considered a non-pathogenic bacterium, *Bacillus subtilis*, in rare cases acts as an opportunistic pathogen,

particularly in immunocompromised individuals, or those having underlying conditions (Lotte *et al*., 2022). *B. subtilis* has been linked with wound infections, endocarditis and bacteremia in immunocompromised patients. Research has indicated the efficacy of honey against *B. subtilis* (Brudzynski, 2021).

The ability of honey to act against multi-drug-resistant bacteria particularly *B. subtilis* and *E. coli* has been attributed to its synergistic activity (Noori *et al.*, 2012; Combarros-Fuertes *et al.,* 2020). Essentially, the synergistic effect of various biological potent ingredients such as phenolics and flavonoids contained in honey drive its ability to inhibit multi-drug resistant bacteria (Santos *et al.,* 2002). It has been found that honey can simultaneously damage bacteria cytoplasmic membrane, inhibit enzyme activity, bacterial motility, cell division and protein synthesis (Takaisi-Kikuni and Schilcher, 1994; Mirzoeva *et al.,* 1997). Caffeic acid and Galagin contained in honey can inhibit enzymatic activities in bacteria (Koo *et al.*, 2002; Jantakee and Tragoolpua, 2015). Propolis from honey is known to inhibit RNA-polymerase account for honey ability to entirely inhibit protein synthesis in bacteria (Grinn-Gofroń *et al.,* 2025).

2.9 Research Gaps

Although numerous studies have succeeded in isolating various *Bacillus* species from honey and explored their antibacterial and bioactive potential, these investigations have largely been conducted outside Kenya (Hallaj-Nezhadi *et al*., 2022; Magdalena *et al*., 2020). Honey has been recognized as an ecological reservoir of compounds produced via microbial and plant-bee interactions, supporting its broad antibacterial effects (Brudzynski, 2021). Nevertheless, very little is known about how the distinct agroecological zones of Kenya influence honey's antibacterial potency, its phytochemical

richness, and the profiles of resident *Bacillus* species. Furthermore, although phytochemical variation and antibacterial activity have been linked to honey's botanical and geographical origin in other countries (Pham *et al*., 2022; Mahmoodi-Khaledi *et al*., 2017), comparable data for Kenyan honey are lacking. By addressing this gap, we can better understand which honey sources in Kenya harbor the most antibacterial strength and beneficial bacterial isolates.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

This research study was carried out in two distinct geographical locations within Kenya: Marigat Constituency in Baringo County and Manyatta Constituency in Embu County (Figure 3.1). These regions are renowned for honey production. The choice for the two areas was influenced by their agroecological difference, which is due to varying rainfall patterns, altitude, and soil type among other factors, which in turn affect the vegetative coverage.

3.1.1 Marigat, Baringo County.

Marigat is located at 0.47°N 35.98°E. It is an arid to semi-arid region, with an annual rainfall of between 450- 700mm. Marigat has hot weather for the majority of the year, ranging from 26℃ to 35℃.

3.1.2 Manyatta, Embu County

On the other hand, Manyatta is located 0.4295° S, 37.4775° E. It experiences temperatures ranging from 18℃ to 28℃. Manyatta receives an annual rainfall of 1,400-1,600 mm (Mutua and Ranguma, 2020). Due to high altitude, a wetter climate and proximity to Mount Kenya, Manyatta features a diverse vegetation.

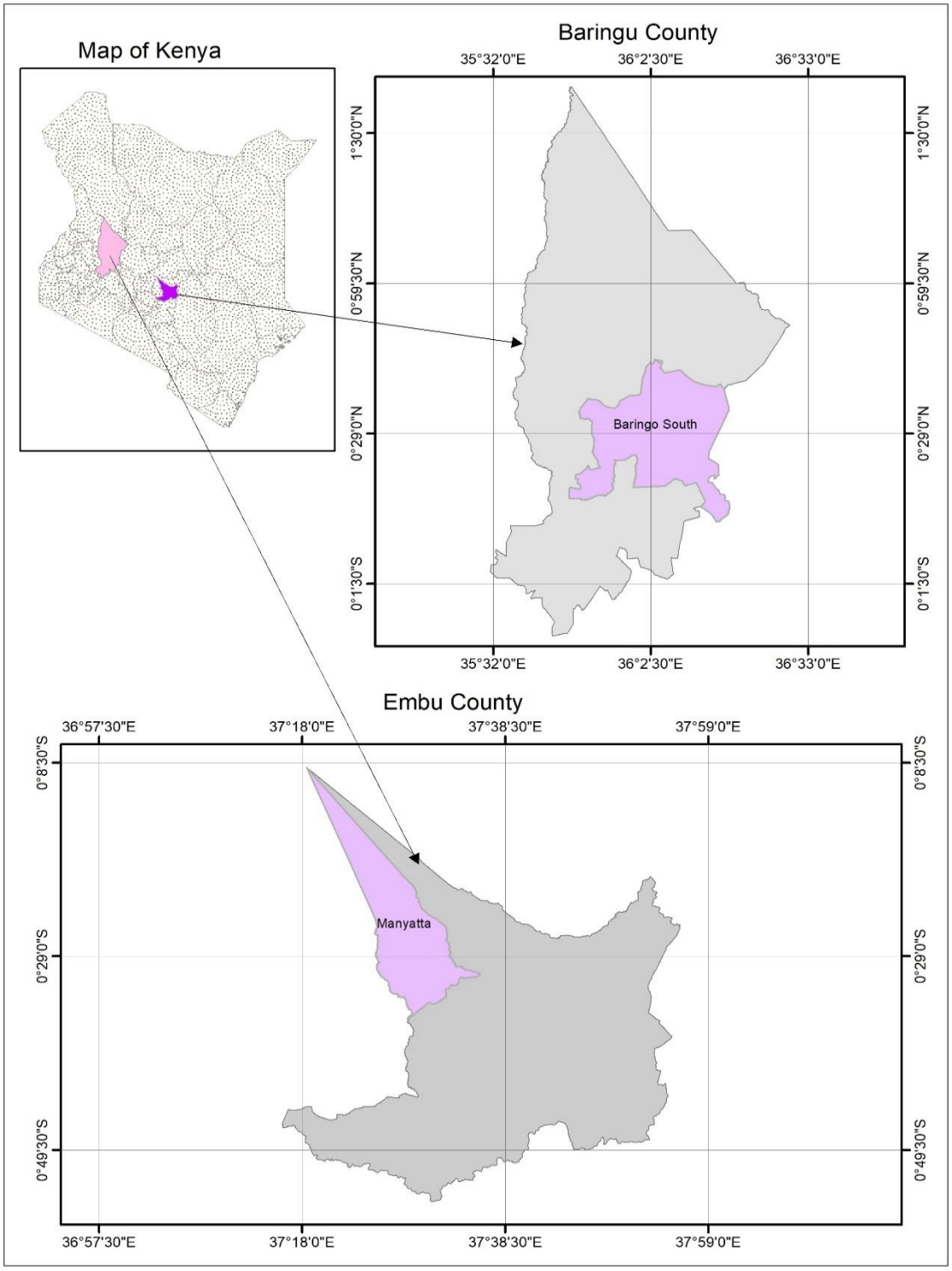

Figure 3.1: Map of Kenya showing areas where samples were collected (Source: Author).

3.2 Collection of Samples

The samples were collected by systematic sampling. Each agroecological region was subdivided into nine (9) sub-regions based on bee foraging distance. Within the sub-regions, the samples were collected at intervals of 4km radius, which corresponds to the average distance a bee travels for foraging activities. Beekeeping farms were identified in each region based on whether ripe honey was available in their hives. Honey samples from each of the nine regions were collected in triplicate, a total of 27 from each constituency.

All of the samples were acquired between the months of July and August, which are the months of crop harvest in Embu and Baringo respectively. The samples were obtained directly from the farmers and placed in 100 mL sterile airtight universal bottles. A clean sieve was used to filter the gathered samples to get rid of any extraneous objects, including wax, comb fragments, and dead bees. The samples were carefully stored at room temperature, and tested within 48 hours to prevent any physical changes caused by prolonged storage.

3.3 Extraction of Phytochemicals from Honey

The extraction was done following a method describe in Ben Amor *et al.* (2022) study. The collected samples of honey were prepared by dissolving 50 g of honey into 300 mL of analytical grade methanol. This mixture was continuously stirred with a shaker and left to stand for 48h at room temperature. It was decanted after 48 hours, and the sediment was filtered with the use of a Whatman paper No. 1. The extracts were concentrated with a rotavapor (R-200, BUCHI) and the methanol evaporated at a temperature of 40 °C. Then the methanol was allowed to further evaporate from the extract in a fume hood chamber to dryness at room temperature. The honey

extract was divided into two portions after weighing. The first being utilized for quantitative analysis of phenolic compounds, flavonoids and flavanols and the second portion for the antibacterial analysis. The concentrated extract was then stored at 4°C awaiting further analysis.

3.3.1 Liquid Chromatography-Mass Spectrometry Analysis Conditions LC-MS

Liquid Chromatography – Mass Spectrometry analysis was carried out in the KU National Phytotherapeutic Research Centre (NPRC) according to the protocol provided by Li *et al*., 2019, with some adjustments. A high precision weighing balance was used to weigh 1mg of each extract which was then dissolved in 1 mL double distilled water containing 95% methanol (v/v) and 0.1% formic acid. After 10 seconds of swirling, the mixture was sonicated for five minutes using Branson 2510E-DTE sonicator. The resultant mixture was centrifuged for two minutes at 1500 revs per minute, then poured into 1.5 ml sterile vials with labels awaiting analyzation. Centrifugation was crucial for eliminating all particles from the extracts in order to prevent column blockages.

Two microliters (2µl) of each of the extracts was injected into the Agilent Mass Selective Detector (MSD) 6120- single quadruple mass spectroscopy with an electrospray source (Palo Alto; California). In order to control the system, an Agilent Chemstation software (Hewlett-Packard) was used. The Zorbax SB C-18 column (1.8 µm × 2.1× 50mm) and Agilent Technologies 1200 infinite series were used for reversed-phase liquid chromatography. The gradient utilized were: 0 min, 5% B, 0 to 5 mins, 5 to 50% B; 5 to 10 minutes, 50 to 80% B; 10 to 15 minutes, 80 to 100% B; 15 to 25 minutes, 100% B; 25 to 30 minutes 5% B; 30 to 35 minutes 5% B (Water; acetonitrile). The flow rate used to inject a sample volume of 2µL was 0.5mL/min.

A positive full-scan ion-mode with a scanning range of 100–1500 m/z was used to collect the data. Each had a 50-milliseconds dwell period. The mass spectrum had the following conditions; 3.0 kV capillary voltage, 70V cone voltage, 5V extract voltage, 0.5V, radio frequency voltage, 110°C source temperature, 400L/h nitrogen gas flow, and 380°C nitrogen gas temperature for desolvation. The equation *y = 6008.9x - 5250.3 (R2 = 0.9987)* was obtained from a plot of peak area versus concentration. It was used to calculate the concentration of the extracts. For all analytes, every peak was subjected to the same process during quantification. Spectral database from the National Institute of Standards and Technology (NIST) was used to identify unknown phytocompounds, which were detected using mass spectra of LC-MS.

3.4 Antibacterial Sensitivity Testing

3.4.1 Nutrient Agar Medium

Nutrient Agar Media was reared according to the protocol provided by Wright (1934). One liter of distilled water was used to suspend 28 grams of dried nutrient agar powder. The two were mixed with slight heating and frequent agitation to completely dissolve. The dissolved medium was autoclaved at 15 psi (121^{o}C) for 15 minutes and then cooled to about 40-45^{o}C. Under sterile circumstances, the medium was transferred onto sterile petri dishes. Once solidified, the medium was stored in sterile plastic bags and stored at 4^{o}C awaiting use.

3.4.2 Muller-Hinton Agar

The Muller-Hinton agar was reared following a protocol described in Rimek *et al.* (2008) work. One-liter distilled water and 38 milligrams of Muller-Hinton agar powder were combined in a flat-bottomed conical flask. To fully dissolve the medium, the mixture was heated while frequently stirring and brought to a boil for one minute. A ball of cotton wool was used to firmly shut the flask,

and aluminum foil was then used to secure it. The mixture was left to cool to room temperature after being autoclaved for 15 minutes at 121ºC. A consistent depth of 3–4 millimeters was achieved by pouring the media into the petri dishes in a laminar flow. After that, the media-containing petri plates were put in a sterile plastic bag and kept at 4ºC until they were needed.

3.4.3 HiChrome Bacillus agar

Preparation this agar followed a protocol described in the technical data sheet provided by HiCrome (2023). Forty grams of HiChrome *Bacillus* agar powder was added to 1 liter of distilled water in a flat-bottomed flask. The mixture was stirred well until complete dissolution. It was heated gently while stirring ensuring that all the agar powder dissolved completely. The medium was autoclaved at 121°C for 15 minutes at 15 psi pressure. The media was left to cool to 45°C and then poured onto sterile petri dishes under aseptic conditions. It was left to solidify at room temperature and then was stored at 4°C awaiting use.

3.4.4 Bacterial Pathogens

Bacteria culture were prepared following a protocol provided by ATCC (2022). The four bacterial strains (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 11775, and *Salmonella typhi* ATCC 14028) were revived from lyophilized stocks at the Kenyatta University Microbiology Laboratory. The selected bacterial strains represent both Gram-positive and Gram-negative human pathogens commonly associated with clinical infections, making them ideal for evaluating the antibacterial efficacy of honey (Adole, 2023). Their inclusion provides a broad spectrum for assessing honey's potential effectiveness against diverse bacterial types of medical importance. Each strain was inoculated into Trypticase Soy Broth (TSB) and incubated at 37 °C for 18–24 hours to obtain primary cultures. The primary cultures were then sub-cultured

onto Nutrient Agar plates and incubated at 37 °C for 18–24 hours to ensure purity and active growth.

For preparation of working cultures, a loopful of each primary culture was inoculated into 10 mL of TSB and incubated at 37°C for 18–24 hours. The resulting cultures were used for antibacterial assays. For short-term storage, working cultures were maintained at 4 °C and sub-cultured as needed for subsequent experiments.

3.4.5 Antibacterial Sensitivity Testing of the Honey Extracts Against *E. coli, S. typhi, B. subtilis* and *S. aureus*

Antibacterial activity testing was done with the agar well diffusion technique as per the procedure outlined by Erhonyota *et al*. (2023). All honey extracts were screened against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria. The test organisms were acquired from the Kenyatta University Microbiology Laboratory, and revived by being inoculated on Nutrient agar plates, and incubated at 37ºC for 24 hours. A single colony was then later transferred to 5 mL sterile nutrient broth and incubated. A concentration of 1.0×10^8 CFU/mL was achieved by adjusting the bacterial inoculum with sterile saline to a 0.5 McFarland solution.

A bacterial lawn was created by spreading 100µL of the standardized bacterial inoculum on Mueller-Hinton Agar (MHA). Sterile 6mm cock borer was used to punch agar wells in the MHA agar. 30µL of the honey extracts were added into the wells. Ciprofloxacin (1 mg/mL), a conventional antibiotic, was utilized as a positive control, and 1% DMSO solvent, was used as a

negative control. The clear zone of inhibition in millimeters was used to assess the antibacterial sensitivity of the extract after they were treated for 24 hours at 37 °C. Every experiment was conducted in triplicate.

3.5 Isolation and Identification of *Bacillus* Bacteria Species in the Honey

3.5.1 Culturing and Purification

Honey samples obtained from the study sites were diluted by mixing 1g of honey with 9ml of sterile normal saline (0.85%) in a sterile test tube. The mixture was vortexed for 30 seconds in order to achieve homogeneity. To reduce the bacterial concentration, serial dilution (10^{-1} to 10^{-6}) on the mixture was performed with sterile normal saline. The mixture was then heat treated at 80 ºC for 15 minutes then rapidly cooled in an ice bath for 5 minutes to eliminate non-spore- forming bacteria.

HiChrome *Bacillus* agar was prepared in compliance with the manufacturer's guidelines (HiCrome 2023) and poured onto petri dishes and let to solidify. The plates were stored at 4ºC awaiting use. The heat-treated sample was measured using a sterile pipette (100µL) and placed onto the plates and evenly distributed using a sterile glass spreader, then incubated for 24-48 hours at 37ºC. After the incubation period, the bacteria were observed for colony morphology and color. Depending on the color and colony morphology, the colonies were isolated using a sterile loop and streaked onto fresh HiChrome *Bacillus* agar and incubated for 37ºC for 24 hours so as to obtain pure cultures. The confirmed *Bacillus* isolates were then stored in glycerol stocks at 4ºC awaiting further identification.

3.5.2 Gram Staining

The described by Smith & Hussey (2005) was followed. An isolate was streaked onto a glass slide,

which was then fixed by drying it over a tiny flame. The primary stain, crystal violet, the mordant, Lugol's iodine solution, ethanol 95% for quick decolorization, and safranin for counterstaining were then applied on top of the stain in that order. The suspension was left for ±1 minute before being washed under running water before the next solution was set. Finally, a glass deck was used to cover the glass slide, and it was examined under a microscope. Contrasting the color and form of the bacterium with observations was done.

3.5.3 Catalase Test

The protocol described by Reiner (2010) was followed. Fresh colonies were introduced into a glass slide, 5% H_2S solution was poured into it, and the combination was left for one minute to determine production of catalase. Ability of isolates to produce enzyme catalase that liberates oxygen from hydrogen peroxide was determined using the catalase test. A positive test is illustrated by gas production (bubbles), and a negative test by no gas production (absence of bubbles) (Zamanpour *et al*., 2023). According to Kaushal *et al*. (2018), a positive test indicates that the organism has the catalase enzyme, which decomposes hydrogen peroxide into oxygen and water. Positive tests illustrate that *Bacillus* species are present.

3.5.4 Indole Test

The test was conducted following the procedure described by MacWilliams (2019). After preparing the tryptone broth, it was transferred to test tubes and autoclaved. A few drops of a pure culture were put into test tubes, and they were incubated for 48 hours at 37°C. For indole production testing, 5 drops of Kovács reagent were added directly to the tube after incubation. The pink to red color development of the reagent layer overlying the medium in seconds when added to the reagent revealed the presence of the enzyme tryptophan. Bacteria belonging to the *Bacillus*

species are absent in the case where there is color development.

3.5.5 Motility Test

The protocol described by Shields and Cathcart (2011) was followed for motility test. Sulphide Indole Medium (SIM) was prepared, and needles inoculated with the bacteria isolates were punctured into it. They were incubated for 24 hours at 37°C. The bacteria growth was checked as to whether they had grown along the stab line or moved away from the stab line. *Bacillus* species are motile and therefore, a diffused growth pattern forming cloudiness in the medium is a positive indication of their presence.

3.5.6 Starch Hydrolysis test

The protocol followed is described by Lal and Cheeptham (2012). Using a sterile loop, a pure colony of the bacterial isolate was streaked onto a starch agar plate and incubated at 37 °C for 24–48 hours to allow growth. Following incubation, a solution of iodine was poured onto the plate, staining the starch dark brown. Production of amylase was identified by observing a halo or a clear zone around the bacterial growth where starch has been broken down. If no clear zone is present, the bacterium is negative for starch hydrolysis, indicating it does not produce amylase. On the other hand, production of a clear zone around the streak line of the colonies indicates starch hydrolysis, thus a positive test result.

3.5.7 DNA Extraction

The Zymo Research Quick-DNA^{TM} miniprep kit was used to extract DNA following manufacturers guide (Zymo Research, 2022). Genomic DNA was extracted from pure colonies of the bacterial isolates grown on nutrient agar media for 48 hours. Bacterial colonies were placed into 400 µl of sterile normal saline, thoroughly mixed by vortexing, then centrifuged at 12000 revs

per minute (rpm) for 10 minutes.

The cells were exposed to a lysis buffer which breaks the cell membrane to release DNA. The lysate was mixed with a binding buffer and transferred to a spin column for centrifugation at 12000 revs per minute (rpm) for 5 minutes for the DNA to bind to the silica membrane in the column. After that, the mixture was repeatedly washed to get rid of impurities like salts and proteins. The purified DNA was then eluted using an elution buffer to get it ready for use in subsequent processes.

3.5.8 Gel Electrophoresis

Gel electrophoresis was employed to check DNA integrity, estimate concentration, and detect contamination or degradation. Gel (1.3%) was prepared from agarose and placed in an electrophoresis chamber filled with a 1X TAE buffer solution. For visibility, a loading dye; 1µL of bromophenol blue was mixed with 4µL of DNA and loaded into the wells in the gel. An electric current (80V) was applied, which causes the molecules that are negatively charged to move in the direction of the positive electrode, with smaller molecules traveling farther and faster through the gel matrix. Once the run was complete, the gel was stained with Gel red dye to visualize the separated molecules under UV light.

3.5.9 PCR Analysis

The PCR was carried out using universal primers, 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5'GGTTACCTTGTTACGACTT 3'), which are complementary to the conserved sections of the bacterial 16S rRNA gene (Weisburg *et al*., 1991). A total of 24µl of the ingredients were used to make the PCR master mix: 1µl of 10µM dNTPs, 0.5µl of Taq polymerase, 2.5µl of 10X dream Taq buffer, DNase, RNase-free PCR water for top-up, and 0.5µl of the forward and reverse

primers. After that, 1 µl of DNA template was introduced into the master mix.

The amplification was done using an Applied Biosystems MiniAmp Plus Thermal Cycler. The cycle included a first denaturation phase that lasted three minutes at 94°C. The stages that were repeated were as follows: 40 seconds of denaturation at 95°C, 40 seconds of annealing at 51.7°C, and two minutes of extension at 72°C for 35 cycles. The cycle was extended one last time for five minutes at 72°C. In order to verify amplification and assess amplicon quality and size, these PCR products and the molecular ladder were run in 1.5% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer at 80V for 30 minutes. Then, 1µL of GelRed® stain was applied, and the samples were viewed under a UV trans-illuminator.

3.5.10 DNA sequencing

The PCR products were submitted for sequencing. Using both forward (27F) and reverse (1492R) primers, they were cleaned and sequenced from both ends using the Sanger sequencing method. This required making a master mix of taq polymerase, fluorescent-labeled dideoxy ribonucleotides (dNTPs), 27F and 1492R primers, and nucleotides. Following amplification, the products underwent capillary gel electrophoresis. A chromatogram displaying a fluorescent peak for every nucleotide throughout the amplicon's length was produced by using a laser to stimulate a fluorescent tag in each band (Crossley *et al*., 2020).

3.6 Statistical Data Analysis

LC-MS mass spectra were determined based on references published by NIST mass spectral database (Wallace and Moorthy, 2023). Agilent LC-MS-QTOF Mass Hunter data acquisition software version B.03.01 was utilized to conduct data acquisition in addition to LC-MS analysis.

The NIST Library was used to determine the molecular formula, chemical class, and compound names of the honey extract's constituents. The compound molecular weight, chemical class, structural formulae and name were identified. Similarly, comparative amounts per constituent were calculated as a percent with the peak-area normalized (Mohamad *et al*., 2021). The resultant data from this research was presented in tables and appendices.

After the results from the antibacterial assay was exported to an Excel spreadsheet, descriptive statistics were performed and expressed as means and standard error of the mean (SEM). Shapiro-Wilk test was used to ascertain whether the data was normally distributed. Statistical analysis of the data was done (Minitab 2022 Version) using one way ANOVA, followed by Tukey's Post Hoc for pairwise comparison of means. The significance level was set at $p \leq 0.05$. Un-paired T – test was used to compare the significant difference between antibacterial activity of honey extract from Manyatta Constituency, Embu County and Marigat Constituency, Baringo County.

The data on bacterial morphological characteristics were used for cluster analysis by coding into numeric values using Darwin version 6 software. A dendrogram was drawn based on the Jaccard similarity index using the Neighbor-joining method, while morphological diversity indices of the isolates were calculated with the help of PAST software version 3. Using BioEdit software, version 7.2.5, consensus sequences were created from the sequenced data.

In order to identify the bacterial strains, Basic Local Alignment Test was used to draw a comparison with the bacterial standard sequences available in NCBI GeneBank (NCBI, 2022).

MEGA X software was used to create the genetic phylogenetic tree. The genetic sequences were aligned using ClustalW, and the Neighbor-Joining method was used to infer evolutionary history, computed with the p-distances. DnaSP 6 software was used to calculate nucleotide diversity. Using Arlequin software version 3.5.2.2, sequenced data was transformed into haplotypes and utilized to compute genetic differentiation and Analysis of Molecular Variance (AMOVA).

CHAPTER FOUR

RESULTS

4.1 Phytochemical compounds from Manyatta and Marigat constituencies

Twenty- six (26) and twenty-three (23) compounds were identified in Marigat Constituency, and Manyatta Constituency honey extract, respectively. Flavonoids, phenolic acids, antibacterial peptides and metabolites were among the compounds identified in the extracts. The compounds present in both honey samples included kaempferol, luteolin, lignans, triterpenoids, lanthionine, 2-hydroxycinnamic acid, coumarin, isocoumarin, *p*-Coumaric acid, cinnamic acid, caffeic acid, protocatechuic acid, gallic acid, ferulic acid, myricetin, tangeritin, rutin, kaurene, and chrysin (Table 4.3).

The compounds salicylic acid, quercetin, microlactin and monoterpenes were present only in Marigat honey (Table 4.2) whereas 3-tert-butyl-2-hydroxy-6-methylbenzoic acid, sesquiterpenes, gentistic acid, amaranthine betacyanin, myriocin, pinobanksin and phenazine were contained only in honey from Manyatta Constituency (Table 4.1). The concentration of phytochemicals varied across the honey from the different agroecological zones as demonstrated by the relative abundance. Generally, the concentration of the identified phytochemicals varied between the two regions.

4.1.1 Phytochemical compounds identified from Manyatta Constituency, Embu County

The honey extracts from Manyatta Constituency, Embu County contained a variety of compounds (Table 4.1). The chromatogram (Figure 4.1) demonstrates peaks that represent the separation of various compounds, with each peak showing the presence of a specific analyte.

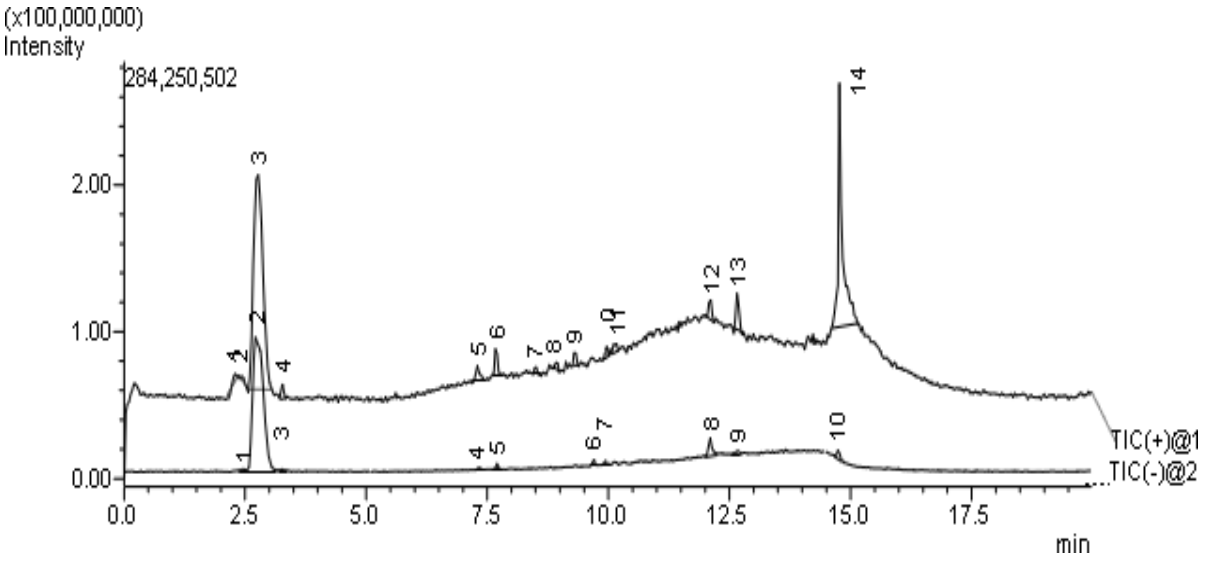

Figure 4. 1: A chromatogram showing peaks of compounds identified in honey from Manyatta using LCMS

Honey from Manyatta Constituency had considerably high concentrations of cinnamic acid and caffeic acid, which had a concentration of 6.16 ug/mg and 3.14 ug/mg respectively (Table 4.1). Myricetin was the most abundant flavonoid, at 8.13 ug/mg. Pinobanksin was also highly concentrated among the flavonoids with a 3.61 ug/mg concentration (Table 4.1). Among the terpenes identified, Kaurene was the most highly concentrated (2.891 ug/mg). Pinoresinol is a lignan that was identified in honey extract from Manyatta Constituency, Embu County.

Table 4.1: List of phytochemicals identified in honey from Manyatta Constituency, Embu County using LC/MS analysis

RT (Min)	Conc (ug/mg)	Molecular Formula	Molecular Weight	Compound Name	Compound Class
2.77	6.16	C9H8O2	148.16	Cinnamic acid	Phenolic Acid
14.76	3.14	C9H8O4	180.16	Caffeic acid	Phenolic Acid
2.89	0.32	C10H10O4	194.18	Ferulic Acid	Phenolic Acid
10.16	0.27	C9H8O3	164.16	*p*-coumaric acid	Phenolic Acid
7.31	0.26	C7H6O4	182.18	Gentistic acid	Phenolic Acid
14.73	0.21	C7H6O5	170.12	Gallic Acid	Phenolic Acid
9.69	0.20	C7H6O4	154.12	Protocatechuic acid	Phenolic Acid
7.71	0.19	C9H8O3	164.16	2-hydroxycinnamic acid	Phenolic Acid
9.32	0.17	C8H8O3	166.21	3-tert-butyl-2-hydroxy-6-methylbenzoic acid	Phenolic Acid
2.77	8.13	C15H10O8	318.23	Myricetin	Flavonoid
7.70	0.32	C27H30O16	610.52	Rutin	Flavonoid
12.11	3.61	C15H12O5	258.26	Pinobanksin	Flavonoid
12.67	0.82	C15H10O6	286.23	Kaempferol	Flavonoid
12.11	0.42	C15H10O4	254.24	Chrysin	Flavonoid
9.33	0.39	C15H10O6	286.24	Luteolin	Flavonoid
12.1	0.32	C17H16O6	372.38	Tangeritin	Flavonoid
3.27	0.26	C9H6O2	146.14	Isocoumarin	Flavonoid
7.34	0.16	C9H6O2	146.14	Coumarin	Flavonoid
9.42	0.39	C15H24	204.34	Farnesene	Terpenes
14.75	2.891	C20H32	272.45	Kaurene	Terpene
9.983	0.203	C30H500	426.7	Lupeol	Terpenes
10.32	0.274	C30H34N2O19	935.05	amaranthine betacyanin	Metabolite
2.313	1.014	C21H39NO6	401.54	Myriocin	Metabolite
3.241	0.156	C12H8N2	180.2	Phenazine	Metabolite
9.931	0.171	C6H12N2O4S	194.23	Lanthionine	Peptide
2.476	0.166	C20H22O6	358.39	Pinoresinol	Lignan

Key: RT – Retention Time; Conc- Concentration

4.1.2 Phytochemical compounds identified from Marigat Constituency, Baringo County

The honey extracts from Marigat Constituency, Baringo County contained a variety of compounds. The chromatogram (Figure 4.2) distinct peaks corresponding to the separated compounds, with each peak indicating the presence of a particular analyte.

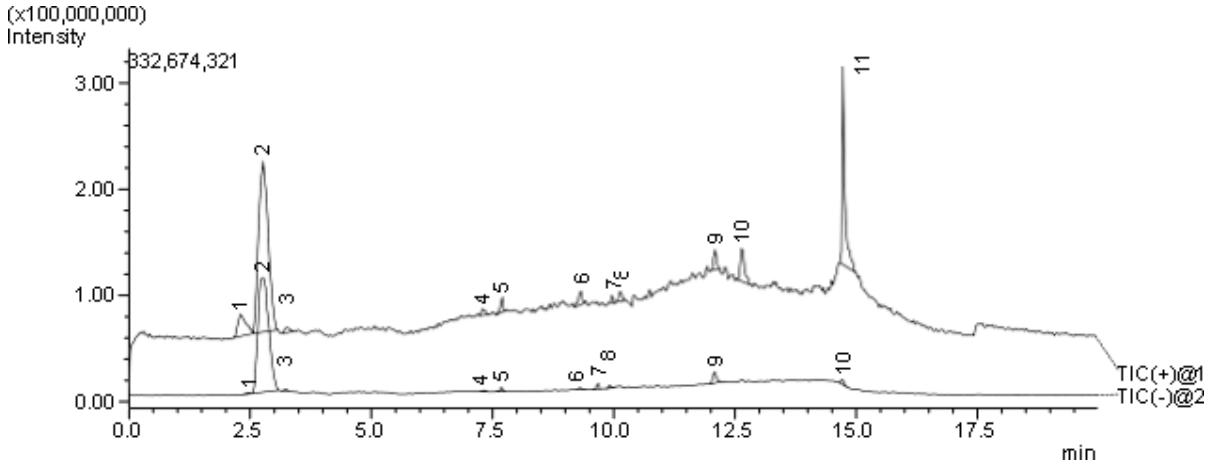

Figure 4. 2: A chromatogram showing peaks of compounds identified in honey from Marigat using LCMS

The most abundant compound in Marigat honey was 2-hydroxycinnamic acid with a concentration of 7.42 ug/mg, followed by *p*-coumaric acid at 5.14 ug/mg, while the least abundant was myricetin at 0.15 ug/mg concentration (Table 4.2). Kaempferol, at a concentration of 3.86 was the most abundant flavonoid identified in Baringo honey extract.

Table 4.2: List of phytochemicals identified in honey from Marigat Constituency using LC/MS analysis

RT (Min)	Conc (ug/mg)	Molecular Formula	Molecular Weight	Compound Name	Compound Class
2.76	7.42	$C_9H_8O_3$	164.16	2-hydroxycinnamic acid	Phenolic Acid
2.75	5.14	$C_9H_8O_3$	164.16	p-coumaric acid	Phenolic Acid
12.10	0.37	HOC_6H_4COOH	138.12	Salicylic Acid	Phenolic Acid
2.44	0.24	$C_9H_8O_4$	180.16	caffeic acid	Phenolic Acid
9.97	0.23	$C_{10}H_{10}O_4$	194.18	Ferulic acid	Phenolic Acid
7.70	0.20	$C_7H_6O_5$	170.12	Gallic acid	Phenolic Acid
9.93	0.18	$C_9H_8O_2$	148.16	Cinnamic acid	Phenolic Acid
7.32	0.16	$C_7H_6O_4$	154.12	Protocatechuic acid	Phenolic Acid
14.76	3.86	$C_{15}H_{10}O_6$	286.23	Kaempferol	Flavonoid
12.66	0.49	$C_{20}H_{20}O_7$	372.38	Tangeritin	Flavonoid
7.69	0.40	$C_{15}H_{10}O_7$	302.24	Quercetin	Flavonoid
12.09	0.34	$C_9H_6O_2$	146.14	Isocoumarin	Flavonoid
3.28	0.26	$C_{15}H_{10}O_6$	286.24	Luteolin	Flavonoid
8.94	0.24	$C_{15}H_{10}O_4$	254.24	Chrysin	Flavonoid
12.66	0.18	$C_9H_6O_2$	146.14	Coumarin	Flavonoid
2.47	0.16	$C_{27}H_{30}O_{16}$	610.52	Rutin	Flavonoid
3.26	0.15	$C_{15}H_{10}O_8$	318.24	Myricetin	Flavonoid
9.69	0.20	$C_{22}H_{22}O_8$	358.39	Pinoresinol	Lignan
8.51	0.20	$C_{30}H_{48}$	426.71	Triterpenoids	Terpene
9.32	0.29	$C_{20}H_{32}$	272.45	Kaurene	Terpene
2.30	0.26	$C_{10}H_{16}$	136.24	Pinene	Terpene
10.17	0.25	$C_{34}H_{48}O_{13}$	468.40	Microlactin	Metabolite
7.31	0.30	$C_6H_{12}N_2O_4S$	194.23	Lanthionine	Peptide

Key: RT – Retention Time; Conc- Concentration

4.1.3 Phytochemicals Identified in honey from both Marigat Constituency and Manyatta Constituency

Sixteen (16) substances were present in extracts of honey samples from both Marigat Constituency and Manyatta Constituency. These compounds include isocoumarin, cinnamic acid, gallic acid, *p*-coumaric acid, caffeic acid, ferulic acid, protocatechuic acid, 2-hydroxycinnamic acid, lanthionine, chrysin, kaurene, luteolin, kaempferol, myricetin, tangeritin and rutin (Table 4.3; Figure 4.3). The concentration of these compounds varied between the regions, as indicated by the relative abundance. Some compounds were found to be more abundant in Manyatta Constituency than in Marigat Constituency, and vice versa.

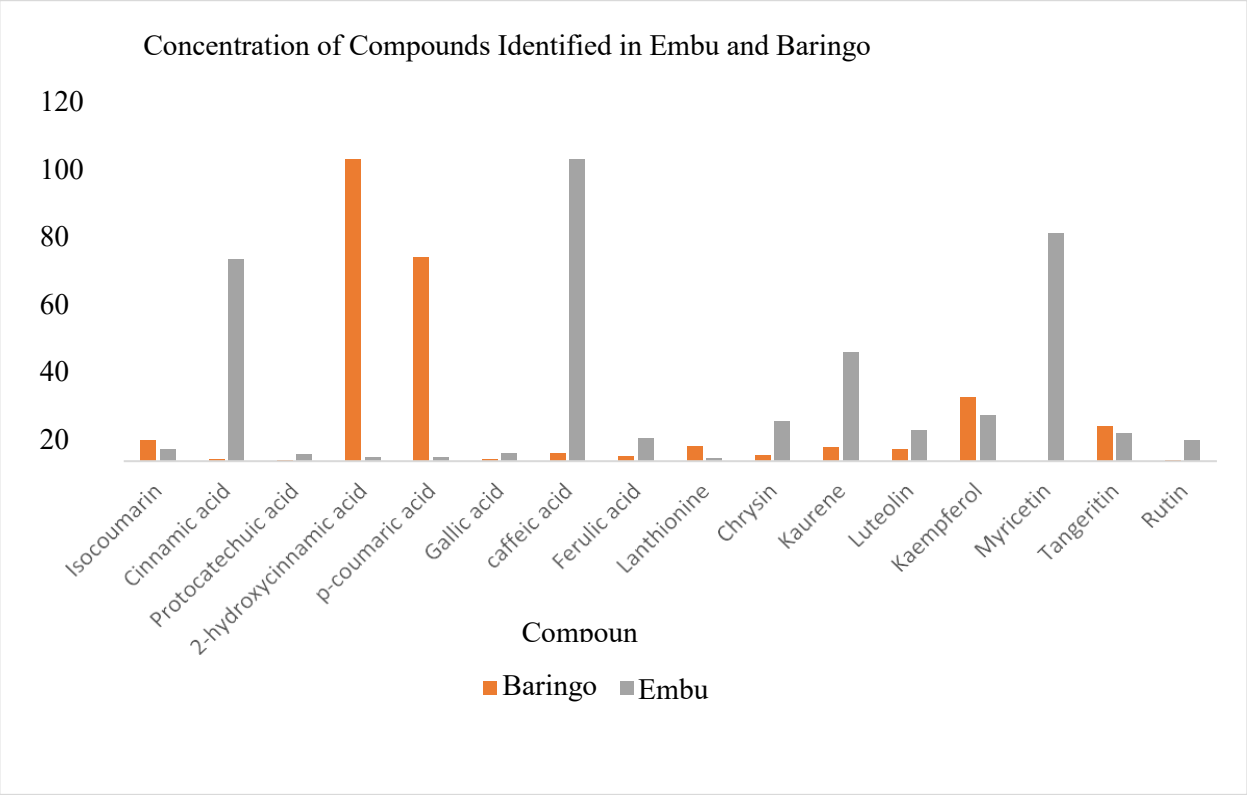

Figure 4. 3: Concentration of similar phytochemicals in honey from Manyatta and Marigat Constituencies

Notably, honey from Manyatta Constituency region featured a higher number of highly abundant compounds relative to honey from Marigat Constituency. High concentrations of cinnamic acid, protocatechuic acid, gallic acid, ferulic acid, caffeic acid, chrysin, kaurene, luteolin, myricetin, and rutin were found in Embu honey extracts (Table 4.3). Furthermore, isocoumarin, 2-hydroxycinnamic acid, p-coumaric acid, lanthionine, kaempferol and tangeritin were found to have a higher concentration in honey from Marigat Constituency as compared to honey Manyatta Constituency (Table 4.3).

Table 4.3: Compounds Identified in honey from both Marigat Constituency, Baringo County and Manyatta Constituency, Embu County

Compound Name	Compound Class	Concentration (ug/mg)	
		Embu	Baringo
Cinnamic Acid	Phenolic Acid	6.16	0.18
Caffeic Acid	Phenolic Acid	3.14	0.24
Protocatechuic Acid	Phenolic Acid	0.20	0.16
2-hydroxycinnamic Acid	Phenolic Acid	0.19	7.42
p-coumaric acid	Phenolic Acid	0.27	5.14
Gallic Acid	Phenolic Acid	0.21	0.20
Ferulic Acid	Phenolic Acid	0.32	0.23
Chrysin	Flavonoid	0.42	0.24
Kaurene	Flavonoid	2.89	0.29
Luteolin	Flavonoid	0.39	0.26
Kaempferol	Flavonoid	0.82	3.86
Myricetin	Flavonoid	8.13	0.15
Tangeritin	Flavonoid	0.32	0.49
Rutin	Flavonoid	0.32	0.16
Isocoumarin	Flavonoid	0.26	0.34
Lanthionine	Peptide	0.17	0.30

4.2 Antibacterial Activity of Marigat Constituency, Baringo County and Manyatta Constituency, Embu County

4.2.1 Susceptibility of Bacterial Isolates to Honey Extracts from Manyatta Constituency Subregions

Honey extracts from Manyatta Constituency showed excellent antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Salmonella typhi.* This was demonstrated by inhibition of bacterial growth as shown by the sizes of the zones of inhibition. There was an antibacterial reaction at every tested concentration. Honey extracts from the nine subregions in Manyatta Constituency demonstrated a dose-dependent antibacterial activity, whereby the highest concentration of the extract demonstrated the highest antibacterial activity across all the bacteria tested (Figures 4.4, 4.5, 4.6 and 4.7).

At 100 mg/mL, inhibition zones for all bacteria ranged from 14 to 19 mm, demonstrating substantial antibacterial activity. Conversely, lowest concentration (12.5 mg/mL) resulted in reduced zones of inhibition ranging from 7 to 11 mm. Ciprofloxacin, which was the positive control exhibited consistently higher inhibition zones across all bacteria, exceeding 20 mm, while DMSO, the negative control, showed no significant activity (6 mm across all cases) (Figures 4.4, 4.5, 4.6 and 4.7).

Among the tested bacteria, *B. subtilis* and *S. aureus* showed slightly higher sensitivity to the Honey extracts from Manyatta Constituency subregions at higher concentrations, with inhibition zones consistently exceeding 18 mm at 100 mg/mL (Figures 4.4, 4.5, 4.6 and 4.7). Conversely, *E. coli*

and *S. typhi* exhibited slightly lower sensitivity, having inhibition zones ranging from 15 to 16 mm at 100 mg/mL of honey (Figures 4.4, 4.5, 4.6 and 4.7). Notably, at every tested concentration, the antibacterial efficacy between extracts of the honey collected from various subregions in Manyatta Constituency, was not significantly different ($p>0.05$).

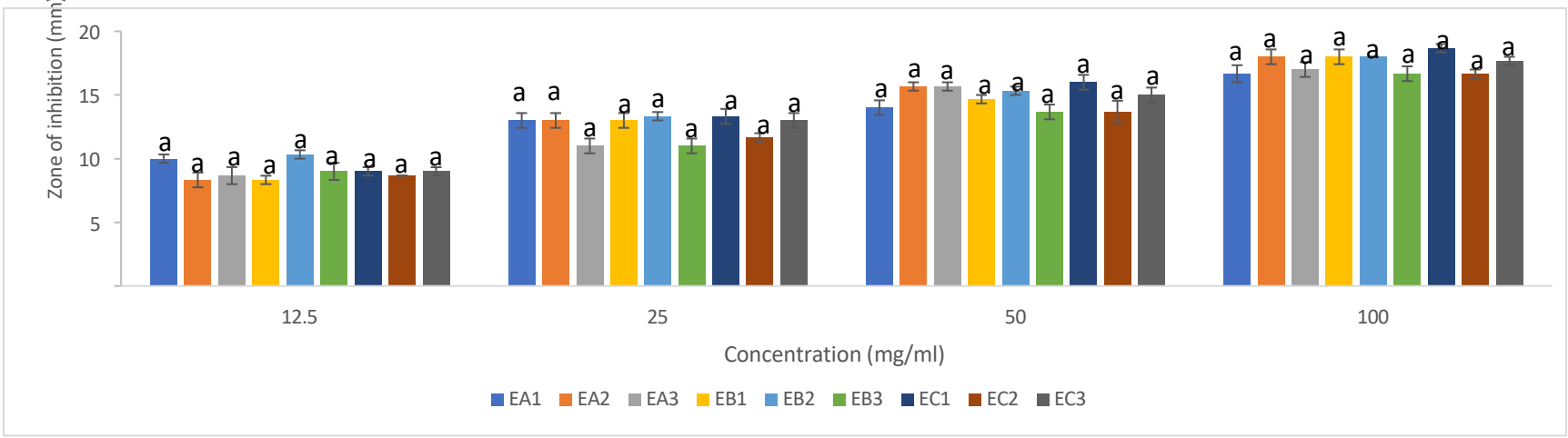

Figure 4. 4: Honey antibacterial activity against *S. aureus* within Manyatta Constituency subregions. Bars with the same superscript within concentration are not significantly different by one-way ANOVA ($p \leq 0.05$). KEY: EA, EB and EC - Manyatta Constituency Subregions

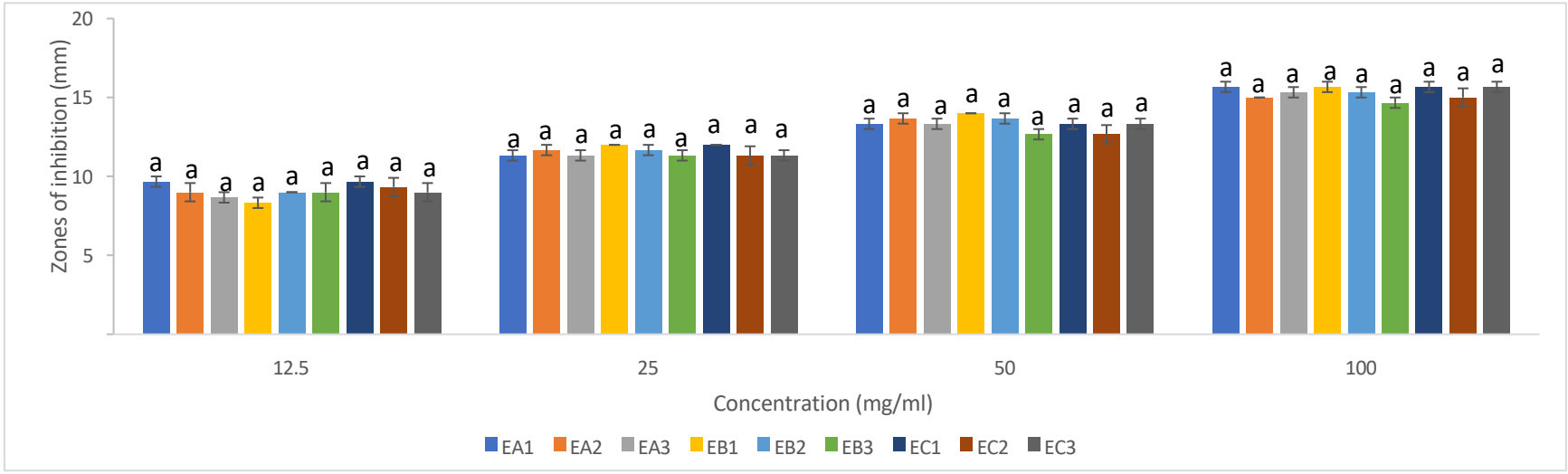

Figure 4. 5: Honey antibacterial activity against *E. coli* within Manyatta Constituency subregions. Bars with the same superscript within concentration are not significantly different by one-way ANOVA ($p \leq 0.05$). KEY: EA, EB and EC - Manyatta Constituency Subregions

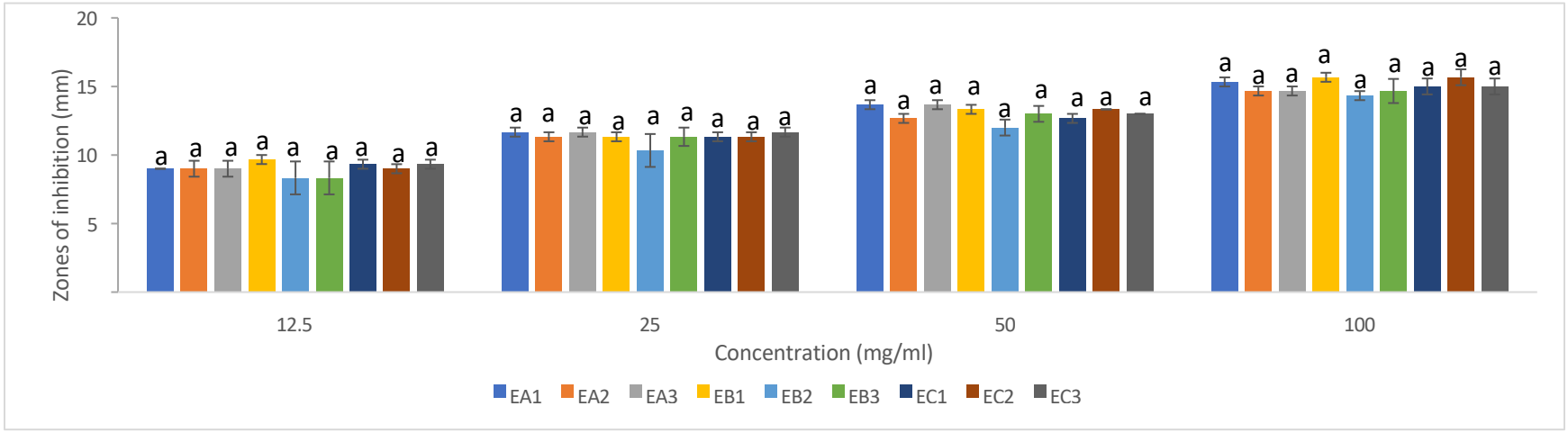

Figure 4.6: Honey antibacterial activity against *S. typhi* within Manyatta Constituency subregions. Bars with the same superscript within a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$) KEY: EA, EB and EC - Manyatta Constituency Subregions

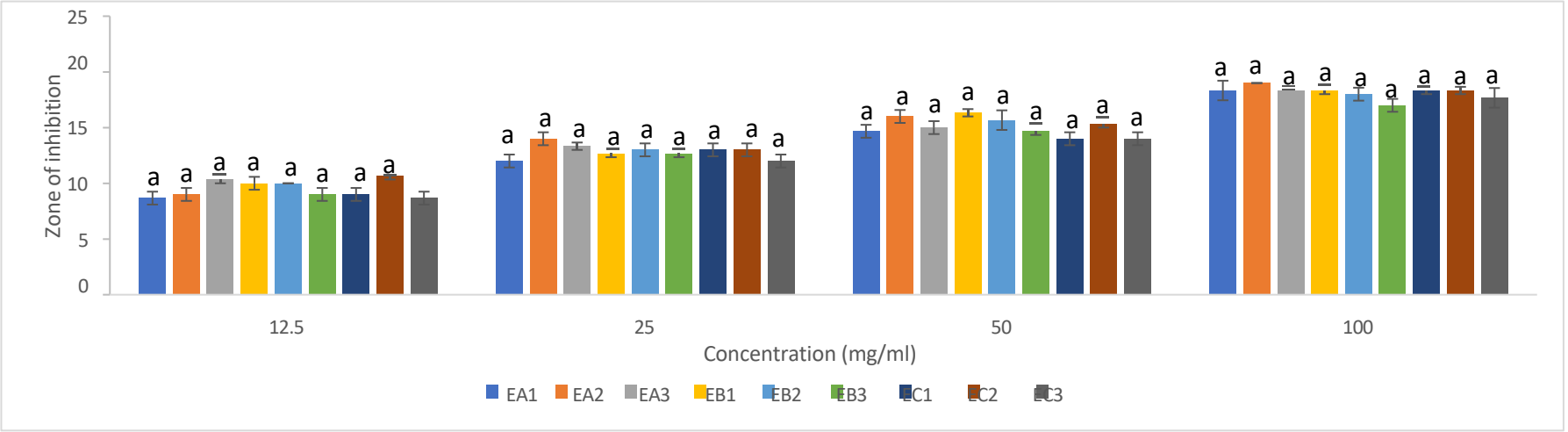

Figure 4.7: Honey antibacterial activity against *B. subtilis* within Manyatta Constituency subregions. Bars with the same superscript within a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$) KEY: EA, EB and EC - Manyatta Constituency Subregions

4.2.2 Antibacterial Activity of Honey Extracts from Marigat Constituency Subregions

Every honey extract gathered from various Marigat subregions showed antibacterial action against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella typhi.* This was demonstrated by inhibition of bacterial growth as shown by sizes of the zones of inhibition. Every honey sample that was gathered from various Marigat subregions showed antibacterial action (Figures 4.8, 4.9. 4.10 and 4.11).

At 100 mg/mL, the honey extracts showed moderate bacterial growth inhibition, having zones that ranged between 12.33 mm and 15.67 mm across all the bacterial species. At the lowest concentration, 12.5 mg/mL, low inhibition zones were observed (Figures 4.8, 4.9. 4.10 and 4.11). Ciprofloxacin, used as a positive control, exhibited the highest activity consistently above 19 mm across all the bacterial species. In contrast, DMSO, the negative control, showed no antibacterial activity, with zones of inhibition remaining at 6 mm.

Among the bacteria tested, *S. aureus* and *B. subtilis* showed relatively higher sensitivity to the honey extracts, maintaining inhibition zones above 15 mm at 100 mg/mL across most subregions (Figures 4.8 and 4.11). *E. coli* and *S. typhi* were less sensitive, with zones ranging from 12 to 13 mm at the same concentration. At every tested concentration, the antibacterial efficacy between extracts of honey collected from various subregions in Marigat Constituency was not significantly different ($p>0.05$).

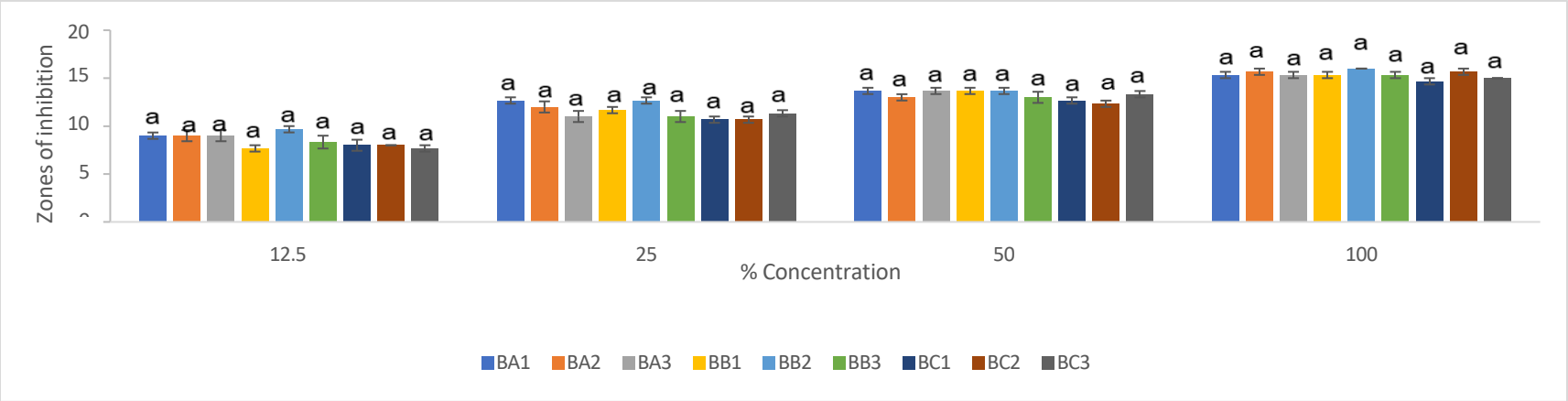

Figure 4. 8: Honey antibacterial activity against *S. aureus* within Marigat Constituencies subregions. Bars with the same superscript with a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$). KEY: BA, BB, BC - Marigat Subregions

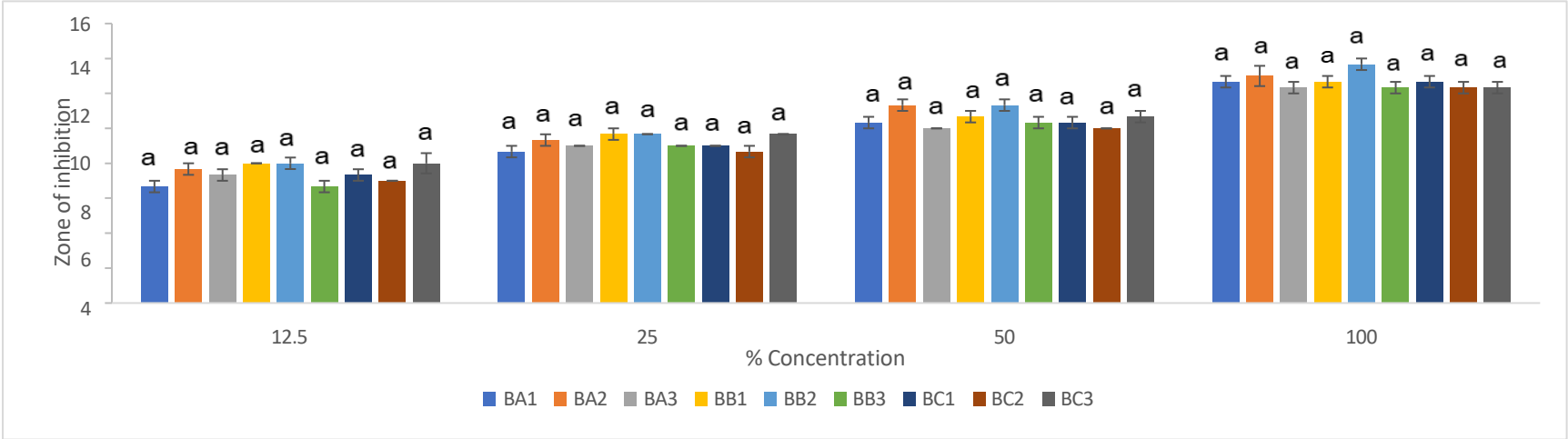

Figure 4. 9: Honey antibacterial activity against *E. coli* within Marigat Constituencies subregions. Bars with the same superscript with a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$). KEY: BA, BB, BC - Marigat Subregions

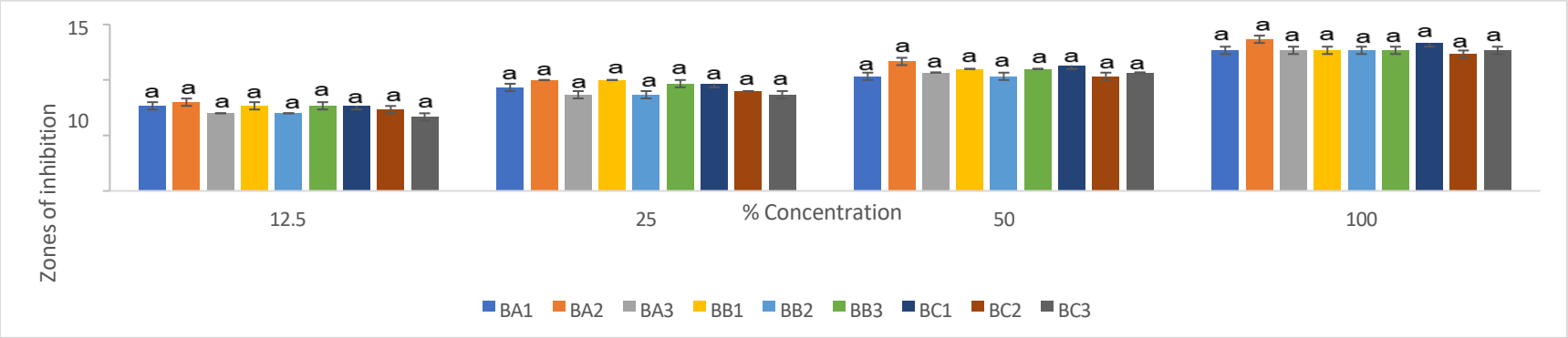

Figure 4. 10: Honey antibacterial activity against S. typhi within Marigat Constituency subregions. Bars with the same superscript with a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$) KEY: BA, BB, BC - Marigat Subregions

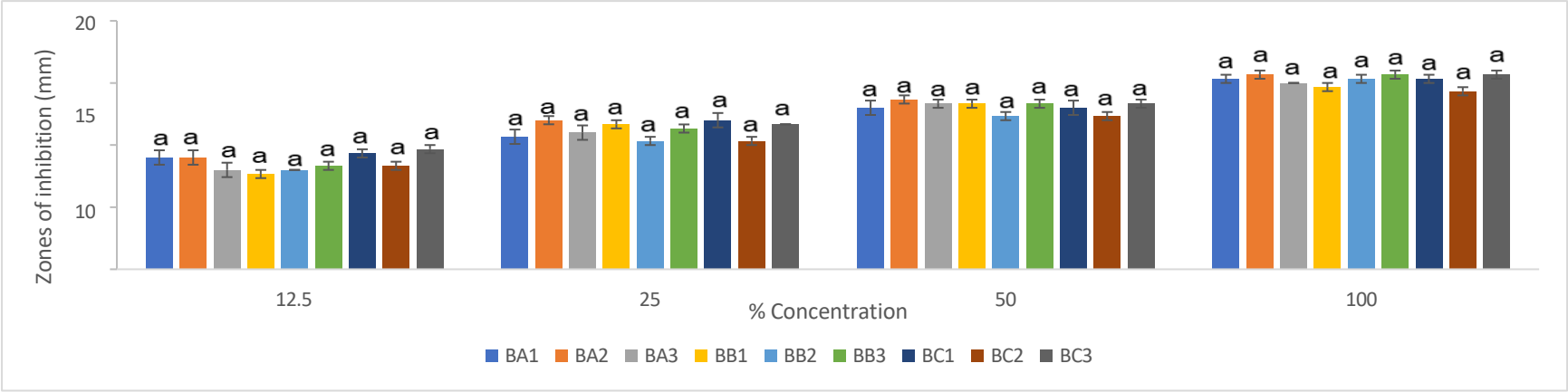

Figure 4. 11: Honey antibacterial activity against B. subtilis within Marigat Constituency subregions. Bars with the same superscript with a concentration are not significantly different by one-way ANOVA ($p \leq 0.05$) KEY: BA, BB, BC - Marigat Subregions

4.2.3 Antibacterial Activity of Pooled Honey Extracts from Manyatta Constituency Subregions

Since the honey samples from various subregions of Manyatta Constituency showed comparable antibacterial action, they were pooled. Thereafter, the antibacterial action of the pooled sample was established. As the resulted showed, the honey revealed antibacterial potency with zones of inhibition ranging from 9.07±0.14mm to 18.15±0.17mm (Table 4.4). In *S. aureus* the zones of inhibition ranges from 9.19±0.21mm to 17.48±0.19mm whereas for *B. subtilis*: 9.41±0.21mm to 18.15±0.17mm, *E. coli*: 9.07±0mm to 15.33±0.12mm and *S. typhi*: 9.41±0.21mm to 15.15±0.17mm (Table 4.4).

The findings demonstrated that antibacterial activity of honey extracts on *S. aureus* was significantly different ($p<0.05$) between all the tested concentrations. A similar trend was observed across *B. subtilis, E. coli* and *S. typhi.* This indicates that the efficacy of the honey extract differed depending on the level of concentration (Figure 4.12). Notably, the standard drug, ciprofloxacin, showed significantly higher ($p<0.05$) antibacterial activity than all the concentrations of the honey extract among all the bacteria (Table 4.4). This indicates that the standard has higher efficacy than honey at the tested concentrations in exerting antibacterial activity against the tested bacteria.

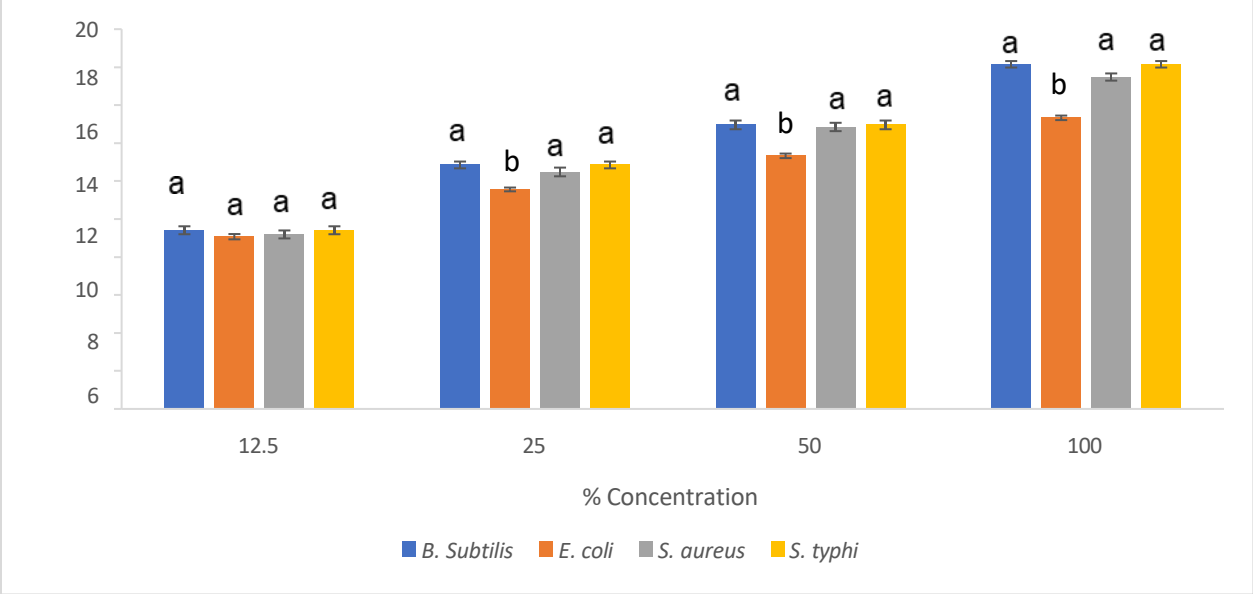

Figure 4.12: The effect of Pooled Honey Extracts from Manyatta on the four tested bacteria. KEY: Bars with the same superscript within a concentration are not significantly different by one- way ANOVA ($p>0.05$)

Notably, the extract, at all the tested concentrations revealed significant antibacterial activity ($p<0.05$) compared to the negative control (DMSO) (Table 4.4). This confirmed the antibacterial efficacy of honey. The honey extract's antibacterial activity was concentration-dependent; thus, the inhibition increased as the concentration increased and vice versa. At 12.5 mg/ml the antibacterial activity was the lowest, and increased with increasing concentration. The highest antibacterial activity was observed at 100mg/ml. This was the trend observed for all the bacteria tested (Table 4.4).

Among all the tested bacteria, *B. subtilis* was the most susceptible to the antibacterial activity of the honey extract (Table 4.4). At the concentration of 12.5mg/ml, there was no significant difference in the antibacterial activity of the honey extract across all the bacteria. At 25, 50 and 100mg/ml, the honey extract exerted significantly less ($p<0.05$)

antibacterial activity against *E. coli* than *B. subtilis, S. aureus* and *S. typhi*. There was no significant difference in antibacterial activity observed on *B. subtilis, S. aureus* and *S. typhi* across all the concentrations (Table 4.4).

Table 4.4: Antibacterial effect of Pooled honey sample from Manyatta Constituency Subregions

Concentration (mg/ml)	Zones of inhibition (mm)			
	S. aureus	***B. subtilis***	***E. coli***	***S. typhi***
DMSO	6.00±0.00^{f}	6.00±0.00^{f}	6.00±0.00^{f}	6.00±0.00^{f}
Cipro	19.96±0.17^{a}	20.00±0.15^{a}	23.15±0.12^{a}	22.85±0.14^{a}
12.5	9.19±0.21^{e}	9.41±0.21^{e}	9.07±0.14^{e}	9.41±0.21^{e}
25	12.48±0.23^{d}	12.85±0.18^{d}	11.56±0.01^{d}	12.85±0.18^{d}
50	14.85±0.22^{c}	14.96±0.23^{c}	13.33±0.12^{c}	14.96±0.23^{c}
100	17.48±0.19^{b}	18.15±0.17^{b}	15.33±0.12^{b}	15.15±0.17^{b}

Key: DMSO- Dimethyl sulphoxide; Cipro – Ciprofloxacin. Values with a different letter within a column are significantly different ($p<0.05$), Tukey's HSD test

4.2.4 Antibacterial Activity of Pooled Honey Samples from Subregions in Marigat Constituency

Extracts of honey samples from different subregions in Marigat Constituency demonstrated similar antibacterial activity; thus, they were pooled. The antibacterial action of the pooled sample was thereafter assessed. The results demonstrated that the honey extracts exhibited antibacterial potency with zones of inhibition ranging from 7.41±0.11mm to 15.37±0.11mm (Table 4.5). The results demonstrated that antibacterial activity of honey extracts on *S. aureus* was significantly different ($p<0.05$) between all the tested concentrations (Table 4.5). This trend was also observed across *B. subtilis, E. coli* and *S. typhi.* This is an indication that the efficacy of the honey extract differed depending on the level of concentration (Figure 4.13).

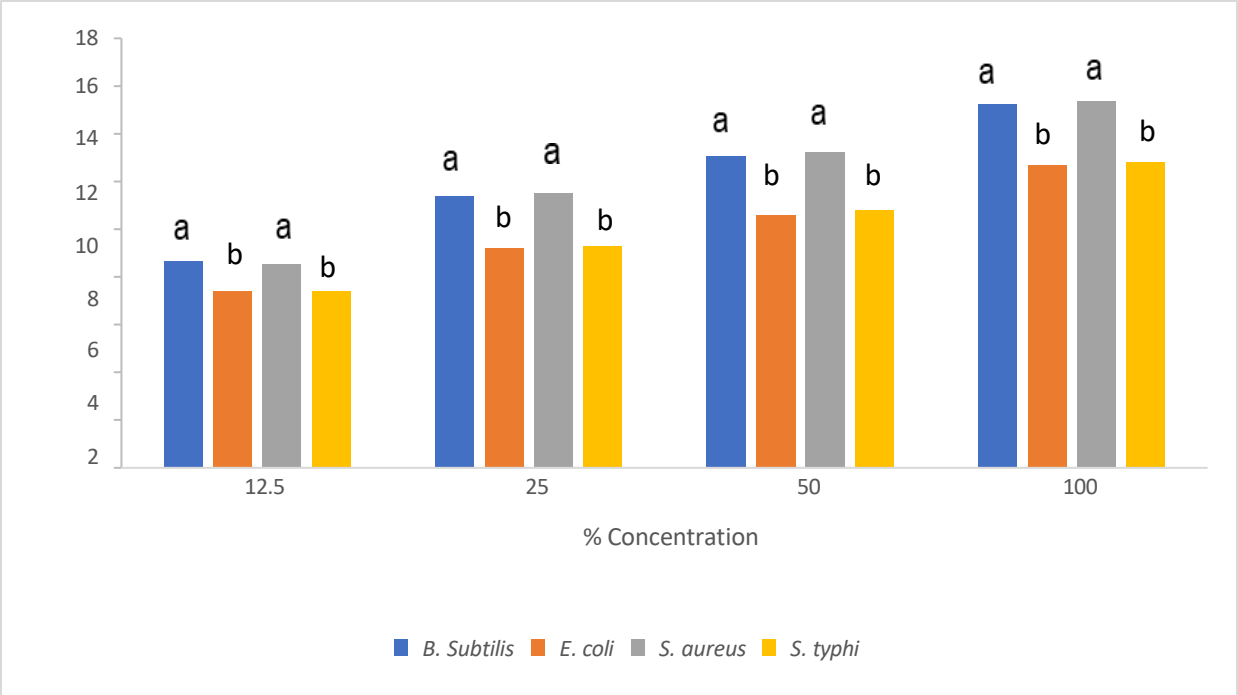

Figure 4. 13: The Effect of Pooled Honey Extracts from Marigat on the Four Tested Bacteria. Bars with the same superscript within a concentration are not significantly different by one- way ANOVA ($p>0.05$)

Markedly, the reference antibiotic, ciprofloxacin, demonstrated significantly higher ($p<0.05$) antibacterial activity than all the concentrations of the honey extract among all the bacteria (Table 4.5). This indicates that the standard has higher efficacy than honey at the tested concentrations in inducing antibacterial action against the tested bacteria. Notably, the extract, at all the tested concentrations revealed significant antibacterial activity ($p<0.05$) compared to the baseline and the negative control (DMSO). This confirmed the antibacterial efficacy of honey (Table 4.5). The antibacterial action of the honey extract was concentration-dependent, thus, the inhibition increased as the concentration increased and vice versa. At 12.5 mg/ml the antibacterial activity was the lowest, and increased with increasing concentration. The highest antibacterial activity was observed at 100mg/ml. This was the trend observed for all the bacteria tested (Table 4.5).

Among all the tested bacteria, *B. subtilis* and *S. aureus* were the most sensitive to the honey extract's antibacterial activity (Table 4.5). At all the concentrations, antibacterial activity of the honey extract against *B. subtilis* and *S. aureus* was significantly higher ($p<0.05$) as compared to *E. coli* and *S. typhi*. Honey extracts' antibacterial action against *E. coli* was statistically similar to the activity against *S. typhi*. Similarly, the antibacterial activity of the honey extracts against *S. aureus* was statistically similar to the activity against *B. subtilis* (Table 4.5).

Table 4.5: Antibacterial effect of honey from Marigat Constituency

Concentration (mg/ml)	**Zones of inhibition (mm)**			
	S. aureus	***B. subtilis***	***E. coli***	***S. typhi***
DMSO	6.00 ± 0.00^{f}	6.00 ± 0.00^{f}	6.00 ± 0.00^{f}	6.00 ± 0.00^{f}
Cipro	19.96 ± 0.17^{a}	20.00 ± 0.15^{a}	23.15 ± 0.12^{a}	22.85 ± 0.14^{a}
12.5	8.52 ± 0.18^{e}	8.63 ± 0.11^{e}	7.41 ± 0.13^{e}	7.41 ± 0.11^{e}
25	11.52 ± 0.19^{d}	11.37 ± 0.13^{d}	9.19 ± 0.11^{d}	9.30 ± 0.13^{d}
50	13.22 ± 0.13^{c}	13.07 ± 0.12^{c}	10.56 ± 0.12^{c}	10.81 ± 0.12^{c}
100	15.37 ± 0.11^{b}	15.22 ± 0.12^{b}	12.67 ± 0.13^{b}	12.81 ± 0.12^{b}

Key: Values with a different letter within a column are significantly different ($p < 0.05$), Tukey's HSD test.

Comparing the two regions, the findings of the study showed that the extract of the honey harvested from Manyatta Constituency, Embu County had significantly higher antibacterial activity than the extract of the honey harvested from Marigat Constituency, Baringo County, at each of the tested concentrations (Figure 4.14).

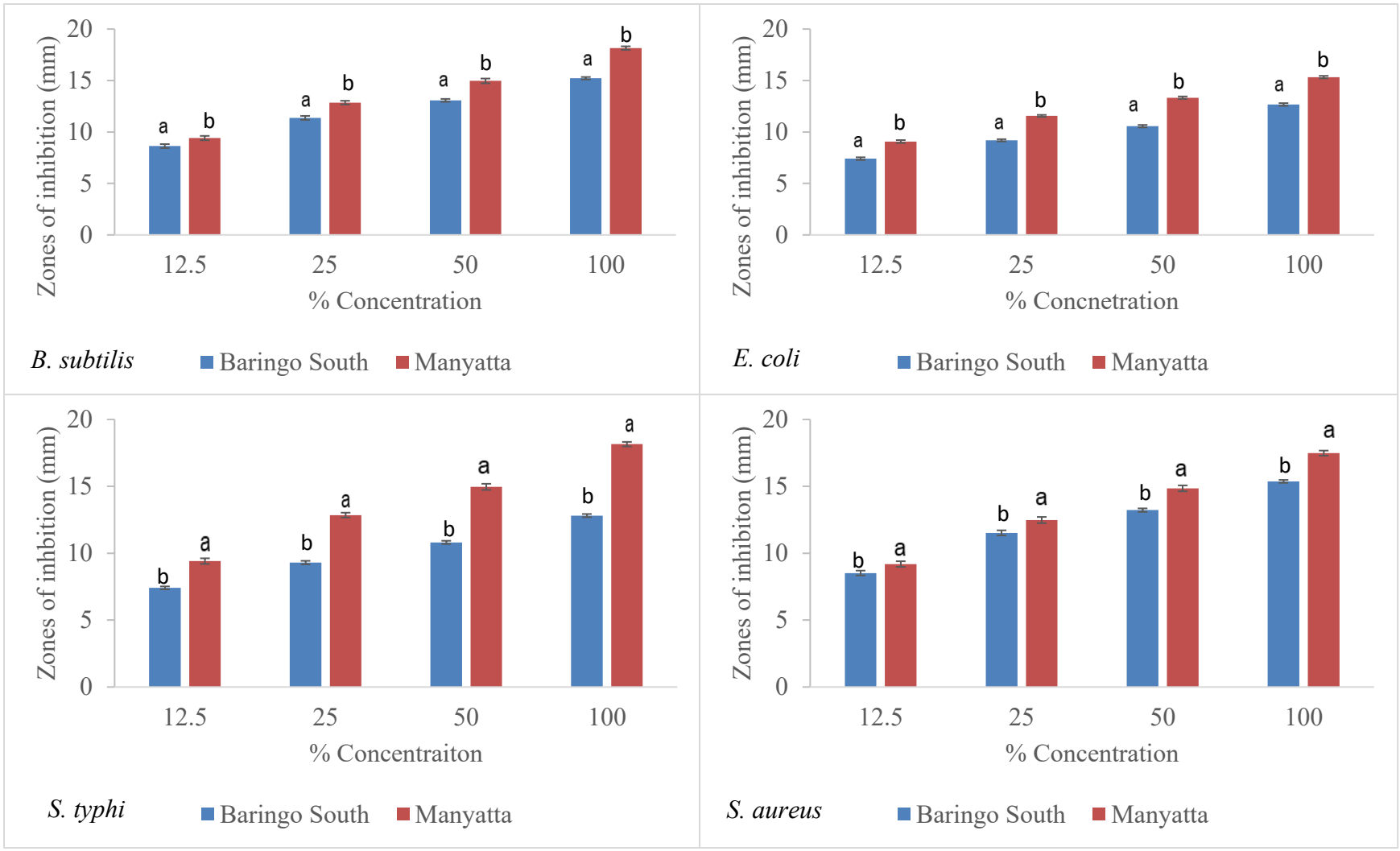

Figure 4. 14: Comparison of the antibacterial activity of between honey from Manyatta and Marigat Constituency. Bars with the different superscript within a concentration in each graph are significantly different by Un-paired t test ($p<0.05$)

4.3 *Bacillus* Species Identification

4.3.1 Morphological and biochemical characterization of *Bacillus* bacteria species in the honey samples

Following 48 hours of 37ºC incubation, the colonies' sizes were measured and morphologies observed on the plates. According to the similarity of their morphological characteristics, the isolates were placed into 8 groups (Table 4.6). Colony size ranged from small (1mm) to medium (3mm), with all the groups having a small to medium size (Table 4.6). Additionally, the majority of the colonies had pink to purplish color and were either circular or irregular in shape. Some of the isolates had opaque colonies, while some were translucent. Isolates E1 and E2 had a flat to raised elevation while the rest of the groups had a raised elevation (E3, E6, B3, B4 and B8). The texture of the colonies for the isolates was either smooth, smooth and moist or dry and wrinkled (Table 4.6).

Table 4.6: Morphological characteristics of the bacteria isolates

Group	Colony shape	Texture	Opacity	Elevation	Color	Size
E1	Circular	Smooth, moist	Translucent	Slightly raised	Light purple to bluish	Medium
E2	Irregular	Dry wrinkled	Opaque	Slightly raised	Dark pink to reddish	Small
E3	Circular	Smooth	Opaque	Raised	Pink to light purple	Small
E6	Circular	Smooth	Opaque	Raised	Light purple to bluish	Medium
B3	Circular	Smooth	Translucent	Raised	Light blue to purple	Small
B4	Irregular	Rough dry	Opaque	Raised	Pink to reddish-purple	Small
B8	Circular	Smooth	Opaque	Raised	Pink to light purple	Medium
B9	Circular	Smooth	Opaque	Raised	Light purple to bluish	Medium

Key: E1 – E6 Manyatta Constituency honey bacteria isolates; B3-B9 Marigat honey bacteria isolates. Small - (0.1 – 2mm); Medium (2.1 – 3mm)

Biochemical tests further helped in the identification of the isolates that were catalase-positive due to the production of bubbles after inoculation on hydrogen sulphide. All the isolates were Gram-positive rods (Table 4.7). The isolates were motile because on incubation, they moved away from the inoculation line, they did not just grow along the stab line (Table 4.7). Furthermore, the isolates were negative for indole since after the addition of the Kovac's reagent, there was no change in color (Table 4.7). The isolates were determined to hydrolyze starch by production of amylase due to the formation of a clear zone after addition of iodine to the bacterial colonies. After identification with morphological and biochemical methods, the isolates' identity was verified using PCR.

Table 4.7: Biochemical characteristics of the bacteria isolates

Group	Gram stain	Catalase	Motility	Indole	Starch Hydrolysis
E1	+	+	+	-	+
E2	+	+	+	-	+
E3	+	+	+	-	+
E6	+	+	+	-	+
B3	+	+	+	-	+
B4	+	+	+	-	+
B8	+	+	+	-	+
B9	+	+	+	-	+

Key: E1 – E6 Bacteria isolates in honey from Manyatta Constituency; B3-B9 bacteria isolates in honey from Marigat Constituency.

4.3.2 Genomic DNA and PCR products

The bacterial isolates' genomic DNA was extracted. Out of these, 16S rRNA genes of 8 isolates were successfully amplified. Gel electrophoresis led to the separation of good quality bands, approximately 1500 base pairs (plate 4.1).

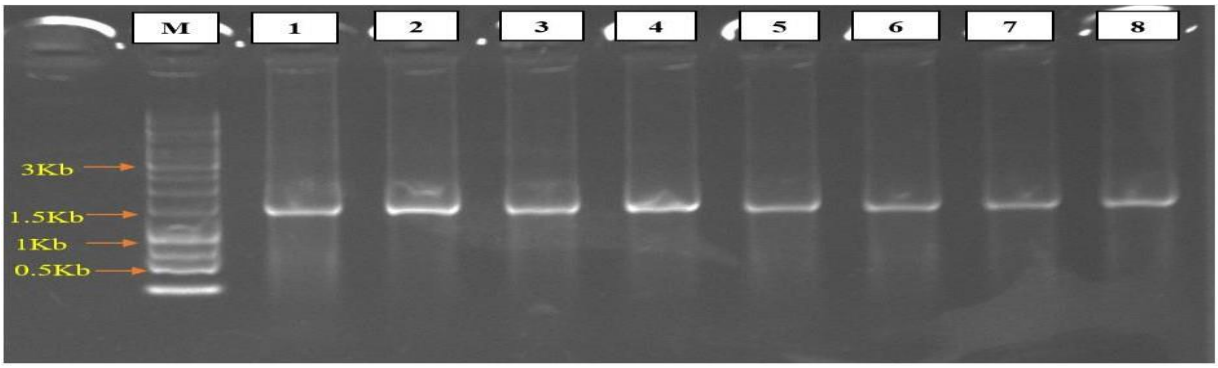

Plate 4.1: PCR amplified product for the 16s rRNA gene region of the bacterial isolates. 1.3% agarose gel, 0.5X TAE buffer, 80V for 1hour, gel red stain, Thermo Scientific O'GeneRuler 1 kb DNA Ladder, Lanes 1(EI), 2(E2), 3(E3), 4(E6), 5(B3), 6(B4), 7(B8) and 8(B9) -amplified samples of the isolates.

4.3.3 Identities of the isolates

The isolates' genetic sequences, which were analyzed using the amplified 16S rRNA gene, showed 97% to 100% resemblance to other genetic sequences stored in the NCBI GenBank database (Table 4.8). The particular species of *Bacillus velezensis*, *Bacillus atrophaeus*, *Bacillus siamensis*, *Bacillus cabrialensis*, *Bacillus halotolerans*, *Bacillus tequilensis*, *Bacillus inaquosorum*, and *Bacillus licheniformis*, all which fall within the *Bacillus* genus were the identities of the isolates. All of the isolates' consensus sequences were deposited in the NCBI GenBank and given GenBank Accession numbers, as shown in Table 4.8.

Table 4.8: Isolates Identities matches based on 16s rRNA sequencing and their GenBank Accession numbers.

Isolate	The organism with the most similar Match	Gene similarity	Accession number
E1	*Bacillus velezensis*	99%	PP838538
E2	*Bacillus atrophaeus*	98%	PP838539
E3	*Bacillus cabrialensis*	99%	PP838540
E6	*Bacillus siamensis*	97%	PP838541
B3	*Bacillus tequilensis*	99%	PP838534
B4	*Bacillus licheniformis*	99%	PP838535
B8	*Bacillus halotolerans*	100%	PP838536
B9	*Bacillus inaquosorum*	100%	PP838537

4.3.4 Phylogenetic analysis

The evolutionary history of the isolates was deduced using the Neighbor-Joining technique based on the sequence data. Using the p-distance approach, their evolutionary distances were calculated (Figure 4.15). The ideal tree's total branch length is equal to 0.776. Related taxa were clustered using the 1000-repetition Bootstrap test, and the percentage of duplicate trees is shown next to the branches. Eight *Bacillus* species were identified based on phylogenetic analysis, which divided the isolates into two major clusters (I and II) (Figure 4.15). Two subclusters, A and B, were generated by the main cluster I.

Subcluster A clustered together four isolates, E3, B3, B8 and B9 from the genus *Bacillus*, supported by a bootstrap value of 100. Isolate E3 had a 99% sequence similarity with *Bacillus cabrialensis* strain deposited in the NCBI database. Isolates B3, B8 and B9 had a 99%, 100% and 100% genus match with *Bacillus tequilensis, Bacillus halotolerans* and *Bacillus inaquosorum* respectively supported by a bootstrap value of 100. Subcluster B clustered together isolates E1, E2 and E6. The sequence homology for the isolates E1, E2 and E6 to *B. velezensis*, *B. atrophaeus* and *B. siamensis* was between 97% and 99%.

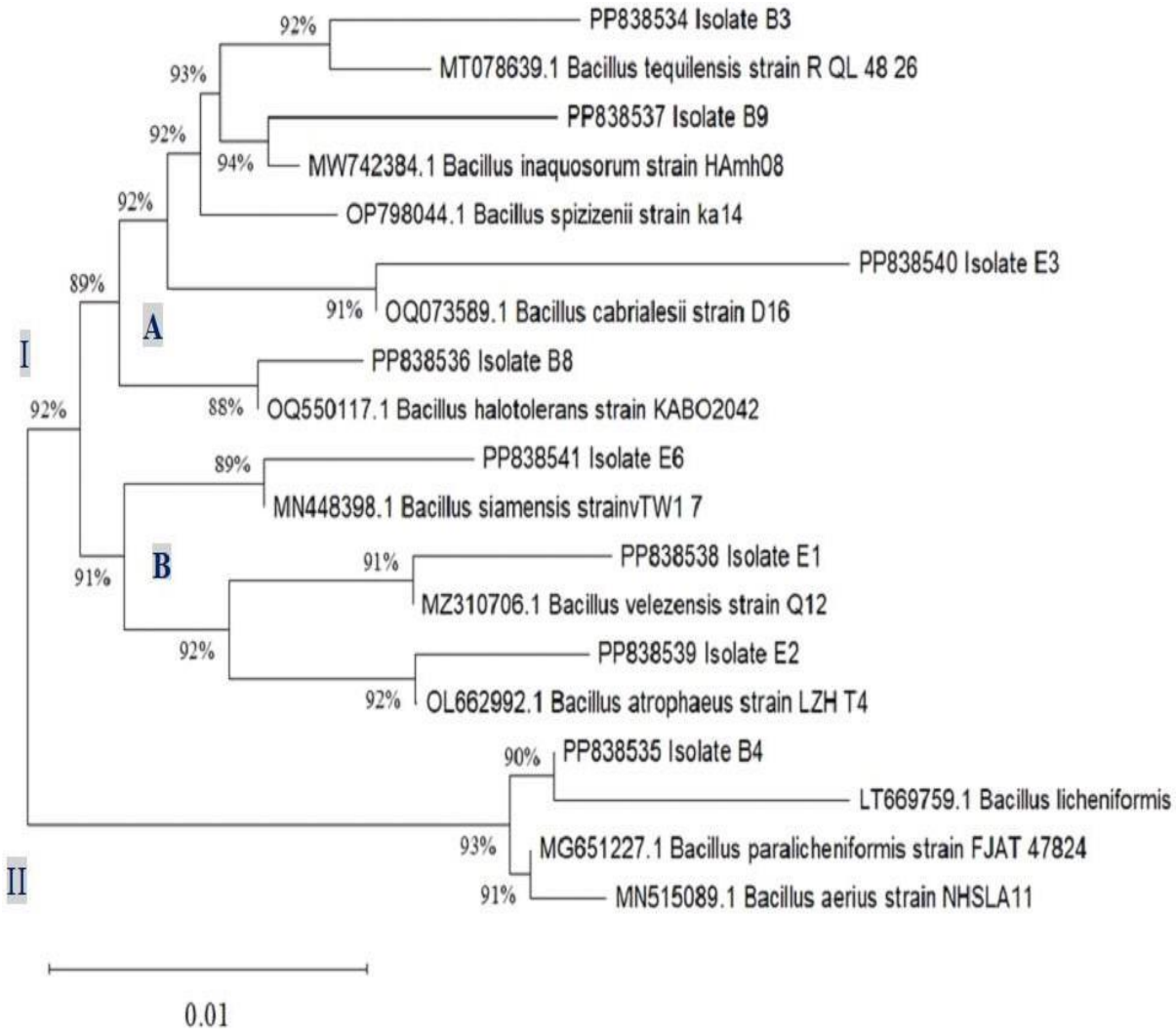

Figure 4. 15: Phylogenetic tree using the Neighbor-Joining method to illustrate the genetic relationships between isolates. Evolutionary distances are expressed in terms of the number of base changes per site and were calculated using the p-distance method. Red labels at the branches show the cluster levels.

4.3.5 Genetic Diversity and Differentiation

Table 4.9 presents the molecular diversity of the isolates. Isolates in honey from Manyatta Constituency had the greatest number of segregating sites (78), indicating a higher number of polymorphic sites (58) than in isolates from Marigat Constituency. There were four (4) unique haplotypes present in each population, indicating a similar haplotype richness in the two regions. Haplotype diversity was high in both regions (1.000). Isolates from Manyatta

had a higher nucleotide diversity of 0.031 compared to those in honey from Marigat at 0.223 (Table 4.9). According to Juke's Cantor, the corrected nucleotide diversity was higher than the uncorrected one, indicating that nucleotide mutations have occurred multiple times. Overall, the isolates in honey from Manyatta had the highest values of nucleotide diversity (0.31), while honey from Marigat had the lowest values (0.22) (Table 4.9).

Table 4.9: Molecular diversity of the bacteria isolates from the two regions

	Number of segregating sites	Number of haplotypes	Haplotype diversity	Average number of differences	Nucleotide diversity, Pi	Nucleotide diversity with Juke's Cantor, Pi
Marigat	58	4	1.000	30.500	0.02226	0.02265
Manyatta	78	4	1.000	42.167	0.03071	0.03145

Molecular variation at $p<0.05$ between isolates from various populations was significant, as indicated by Analysis of Molecular Variance (AMOVA) (Table 4.10). It was noted that most of the genetic variations were found within the populations (92.29%) but low among the population (7.71%). The low genetic variation among the populations is an indication that they are genetically similar. The variance within populations has a p-value of 0.02737 which is significant, therefore indicating substantial variation within populations, unlike that of among-population (0.13881), which is not significant**.** The fixation index was observed to be at 0.07710, which indicates a low genetic differentiation between the two populations.

Table 4.10: Analysis of Molecular Variance for 8 isolates from 2 populations based on 16S rDNA sequences

Source of variation	d.f.	SS	VC	% Mol Var	P values
Among populations	1	22.625	1.41667 Va	7.71	0.13881
Within populations	6	101.750	16.95833 Vb	92.29	0.02737
Total	7	124.375			
Fixation Index FST:		0.07710			

Key: d.f. -Degrees of freedom; SS- The sum of squares; VC- Variance components; % Mol Var. - percentage molecular variance.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATION

5.1 Discussion

5.1.1 Phytochemicals in the Honey Sample

The honey extracts from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County honey contained various phytochemical compounds. The compounds identified in honey extracts from both regions included phenolic acids, flavonoids, peptides, terpenes and secondary metabolites. The existence of phytochemicals in honey is mainly attributed to collection of nectar from plants by bees. These bioactive compounds originate from plants as part of their defense mechanisms against pathogens, and are transferred into honey during its production. Prior research has indicated the existence of various phytochemicals in honey. The findings of this study concurred with those reported in a study by Vazquez *et al*. (2021), who after assessing the presence and composition of phytocompounds in honey samples from Northwestern Spain discovered the presence of 25 phenolic compounds in varying concentrations. Similar outcomes were attained by Ben *et al*. (2022), in a study analyzing the phytochemical characterization and bioactivity of honey samples from various geographical regions of Algeria whereby an overall amount of 20 phytochemicals were identified.

The diversity and concentration of phytocompounds in honey extracts from Marigat Constituency and Manyatta Constituency honey varied considerably. In the honey extract from Manyatta Constituency, phenolics were the most abundant compound class, followed by flavonoids. This could be due to various factors like natural prevalence, their stability in honey as well as plants' response to environmental stress, which causes a higher

abundance in phenolic acids (Kumar and Goel, 2019). Manyatta, in Embu County, being located in the highlands has a wide diversity of dense flora including indigenous plants, agricultural crops and shrubs (Misonge *et al*., 2019) whereas Marigat in Baringo County, is a semi-arid area and is composed of sparse vegetation adapted to drier conditions such as acacia and succulents (Oliech, 2024). The difference in vegetative coverage influences the phytochemical profile of honey as different species produce different nectar and pollen compositions.

These results corroborate with those of a study by Vazquez *et al*., (2021) whereby the phenolic compounds concentrations displayed significant differences depending on the kind of honey. In a separate study by Yelin and Kuntadi (2019) it was observed that honey from monofloral sources (Java region) had differences in composition and concentration of phytochemicals form multifloral sources (Sumbawa region), which is in tandem with findings from this study. This observation also agrees with research conducted by Ranneh *et al*. (2018), whereby honey obtained from tropical multifloral rain forest region contained a higher concentration of phytochemicals than regions with lesser vegetation. Furthermore, similar to the present study, a study done in Kosovo by Ibrahimi and Hajdari (2020) also recorded that the lowest phenolic composition was observed in acacia honey, whereas the highest phenolic content was observed in honey samples that were obtained from forest regions.

Marigat honey extracts had *p*-coumaric acid and 2-hydroxycinnamic acid being highly abundant than Manyatta honey extracts. This could be influenced by the fact that Marigat

honey is mainly acacia honey, due to the abundance of acacia trees in the bees' foraging areas in the region of study. Several studies have demonstrated the abundance of *p*-coumaric acid and 2-hydroxycinnamic acid in acacia honey (Matkovits *et al*., 2023) This result corroborates with previous studies on the analysis of polyphenolic compounds in acacia honey, whereby *p*-coumaric acid was found to be present in high concentrations (Wang *et al*., 2020; Matkovits *et al*., 2023). In an investigation done by Stavrou *et al*. (2024), hydroxycinnamic acid was proposed as a biological marker for agroecological origin of acacia honey due to its abundance in acacia honey, which is comparable to what was found in this study.

Quercetin and Salicylic acid were only identified in Marigat honey, and were notably absent in Manyatta honey. This is due to the vegetation which the bees forage for nectar, influencing the phytocompounds present in the honey produced. This observation is in tandem with findings from a study by Farkas *et al*. (2023) on Hungarian honey, whereby quercetin was only identified in acacia honey. This study also demonstrated an abundance of Kaempferol in acacia honey, which corroborates with the findings from this study, whereby kaempferol was highly abundant in Marigat honey extract as compared to the Manyatta counterpart (Farkas *et al*., 2023).

Similar phytocompounds identified in both regions depicted a variation in the concentrations. Out of the 16 compounds identified in both Manyatta and Marigat (isocoumarin, cinnamic acid, protocatechuic acid, 2-hydroxycinnamic acid, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, lanthionine, chrysin, kaurene, luteolin,

kaempferol, myricetin, tangeritin and rutin), Manyatta region had a considerably higher number of highly abundant compounds. This could be due to the wider variety of flowering plants, and the availability of plants that produce nectar with a higher concentration of certain phytochemicals in Manyatta region, thus leading to honey with a richer phytochemical profile.

This is consistent with findings in research on Malaysian honeys by Maringgal *et al*. (2019), which showed a variation in the total amount of phenolic and flavonoid in the five regions, with the regions practicing agriculture having a higher level of contents, attributed to the vegetation diversity. A separate study evaluating the chemical constitution of Thai honey derived from various floral sources demonstrated that wild honey, which was presumed to be from multiple floral sources had the highest number of phytochemicals (Pattamayutanon *et al*., 2017), which aligns with the findings from this study.

5.1.2 Antibacterial Activity

In this study, honey extract from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County exhibited remarkable antibacterial activity. This antibacterial activity could be because of the existence of diverse phytochemicals present in the honey extracts. Bioactive compounds identified in the honey in both regions, including flavonoids, phenolic compounds and other secondary metabolites, are renowned for having antibacterial qualities. The remarkable honey's antimicrobial properties could be caused by the synergy of several chemical components. These extracts are complex mixtures that contain a broad variety of primary and secondary metabolites. Additionally, these extracts may have a range of pharmacological and biological activity mechanisms, like the capacity

to bind to protein domains, mitosis, immune response modulation, apoptosis, and signal transduction (Adamczak *et al*., 2019).

The discovery of this study is in tandem with the work of Bazaid *et al*., (2023) which showed the antibacterial properties of methanolic honey extracts against both Gram-positive and Gram-negative bacteria in a study conducted in Malaysia. In another research in Iraq, (Al-Hasani, 2018) the inhibition zone diameter of honey against common clinical pathogens ranged between 6-27mm, which aligns with the current study. A research by Hegazi *et al*. (2021) demonstrated the successful inhibition of five types of bacterial strains by honey. Similarly, Otmani *et al*. (2021) found that *S. aureus* and *S. typhi* growth was suppressed by honey extracts. Furthermore, Chuttong *et al*. (2023) reported that honey propolis extracts had antibacterial properties with the potential to replace antibiotics. *Melipona compressipes manaosensis* honey was found to exert antibacterial inhibition against a range of Gram-positive and Gram-negative bacteria in a study conducted by Ramón-Sierra *et al*. (2020), due to the presence of flavonoids and phenolic acids.

Honey extracts from Manyatta region demonstrated a higher antibacterial activity than extracts from Marigat region, demonstrating the agroecological origin's influence on antibacterial efficacy of honey. This can be credited to the higher number and phytochemical concentration identified in Manyatta honey extract. These results were comparable to those found in a study conducted on Saudi Egyptian honeys with varying geographical and agroecological origin, whereby a high antibacterial activity was depicted by the honey with the highest polyphenol content (Roby *et al*., 2020). This corroborates a

study by Syed *et al*. (2020) investigating the antibacterial activity of five varieties of Malaysian honey, whereby differences in the degree of activity against bacteria were noted between the honey from different regions. This was linked to the difference in the type, and concentration of phytochemicals in the honey, as they were obtained from different regions.

Caffeic acid was among the phenolic acids which was identified in both regions, and was notably abundant in Manyatta honey extract. Caffeic acid has been demonstrated to possess antimicrobial properties in various studies (Espíndola *et al*., 2019; Merlani *et al*., 2019). It exerts antibacterial activity using a number of methods, including disruption of bacterial membranes, generation of oxidative stress, prevention of biofilm formation and inhibition of key enzymes (Kępa *et al*., 2018). This shows that caffeic acid contributed to the antibacterial action of the honey extracts. This corroborates with a study by Park and Kang, (2019) whereby caffeic acid demonstrated antibacterial action against *E. coli* and *Salmonella enterica*. In a separate investigation by Khan *et al*., (2021) caffeic acid showed inhibitory activity against a range of microorganisms including *E. coli*, *S. aureus* and *P. aeruginosa*. Research by Kępa *et al*. (2018) also demonstrated the antibacterial efficacy of caffeic acid against *Staphylococcus aureus*.

Cinnamic acid was identified in honey from both regions, but was highly abundant in Embu honey extract. Cinnamic acid exhibits antibacterial activity by disrupting bacterial membranes, inhibiting key enzymes involved in DNA replication and metabolism, oxidative stress, and preventing biofilm formation (de Morais *et al*., 2023). This might be

one of the reasons behind the high antibacterial efficacy of Manyatta honey. This finding agrees with various studies whereby the antibacterial activity of cinnamic acid has been proven. A study by Ruwizhi and Aderibigbe (2020) successfully demonstrated the antibacterial activity of cinnamic acid against *B. subtilis* and *E. coli*. Additionally, findings on the study of cinnamic acid derivatives by Wang *et al*. (2021) demonstrated the ability of cinnamic acid and its byproducts to inhibit bacterial growth. In another research, cinnamic acid depicted antibacterial activity against *Staphylococcus aureus* (Ruwizhi *et al*., 2020).

The results of this research corroborate with prior research showing the presence of cinnamic acid as a phenolic compound in honey, and its significant antibacterial properties (Çobanoğlu *et al*., 2023). Cinnamic acid demonstrated antibacterial activity against Gram-negative (*Chromobacterium violaceum*) bacteria through disruption of the bacterial cell walls and compromise of cell integrity and function in a study by Cheng *et al*. (2020). These results corroborate findings by Malheiro (2019), who reported comparable antibacterial effects of cinnamic acid, although variations in susceptibility were observed depending on the concentration used.

The results of this investigation showed the presence of a combination of flavonoids in extracts from both regions. Flavonoids have different mechanisms of exerting antibacterial activity; thus, a combination of different flavonoids results in a synergistic effect whereby the antibacterial efficacy is enhanced beyond what individual flavonoids might achieve alone (Bouchelaghem *et al*., 2022). In this study, flavonoids such as luteolin and

pinobanksin which disrupt the cell wall of bacterial pathogens, were identified, together with biofilm-disrupting flavonoids like chrysin which is likely to amplify the antibacterial impact of the honey extract.

Myricetin and quercetin, found present in the two extracts, inhibits bacterial activity through oxidative stress (Imran *et al*., 2021; Veiko *et al*., 2023). These mutually beneficial relationships between the phytochemicals identified in the honey could explain the potent antibacterial effects of the honey extract observed in this study. A study by Kara *et al.* (2019) on Turkish honey reported myricetin and quercetin to be among the major phenolic compounds in oak honey, which corroborates the findings from this research, as the two phytochemicals were identified in high concentrations in Manyatta honey. Overall, the flavonoids' presence underscores the antibacterial potency of the honey extracts, suggesting it could serve as an effective natural antimicrobial agent.

The presence of antibacterial peptides, lanthionine and microlactin added a unique bioactive element to honey from these two regions. Other notable compounds include kaurene in honey extracts from both regions, which is a terpene, and may help explain honey's antibacterial and anti-inflammatory qualities. These findings are comparable to those in study by da Costa *et al*. (2018), whereby terpenes were identified in Brazilian honey. In a separate study Guimarães *et al*. (2019) terpenes were demonstrated to prevent the proliferation of *S. typhi*, *E. coli* and *S. aureus* through mechanisms such as loss of the cellular membrane integrity function.

Antibacterial activity of honey is linked to, among other factors, the availability of secondary metabolites (Cebrero *et al*., 2020). Microlactin, which is a secondary metabolite, was identified in Marigat honey extract. The presence of microlactin in Marigat honey could be a possible explanation for the antibacterial action observed against the bacteria tested, and more specifically, *Staphylococcus aureus.* This antimicrobial compound has demonstrated the ability to suppress bacterial growth of *Staphylococcus aureus* through inhibition of the enzyme peptide deformylase (PDF) (Tran *et al*., 2022). The inhibition of these enzymes causes the bacteria to be unable to synthesize proteins as their ability to hydrolyze polypeptides is hindered.

Comparison of Gram reaction of the bacteria indicated that, Gram-positive bacteria (*S. aureus* and *B. subtilis*) were highly inhibited with respect to Gram-negative bacteria (*E. coli* and *S. typhi*). This is attributed to the bacterial cell wall composition. Gram-positive bacteria have a thick peptidoglycan layer, with branched glycan chains. These layers make it highly permeable to a broad spectrum of phytochemical compounds, and therefore, they can readily pass through the inner cell wall, sensitizing the bacteria (Zgurskaya *et al*., 2019). These findings are in agreement with those reported by Bazaid *et al*. (2023), who determined that Malaysian honey antibacterial activity was more effective against Gram-positive than Gram-negative bacteria. In addition, the findings are consistent with the study of Didaras *et al*. (2020). This finding is also consistent with that of a study by Suhartatik *et al*. (2023), whose findings revealed that *S. aureus* was more sensitive than *S. typhi* and *E. coli*, and the results were attributed to the cell membrane and cell wall content of the different bacteria.

In addition, due to the fact that they have thick peptidoglycan walls, gram-positive bacteria are also more resistant to greater osmotic pressures (Wennerstrom *et al*., 2022). Gram-negative bacteria, on the other hand, have a thin peptidoglycan layer, but have an outer membrane rich in lipopolysaccharides, which acts as a protective barrier, limiting the entry of many phytochemicals (Paracini, 2019). The outer membrane can prevent the cytoplasmic invasion of the honey extract (Haktanir et al., 2021). As per Álvarez-Martínez *et al.* (2020), Gram-negative bacteria are generally resistant to plant-derived antimicrobials and sometimes show no response at all.

5.1.3 *Bacillus* Bacteria Species in Honey

This investigation successfully isolated *Bacillus* species from the tested honey samples. The identified species differed depending on the honey samples agroecological origin

. *Bacillus velezensis*, *Bacillus atrophaeus*, *Bacillus cabrialensis* and *Bacillus siamensis* were identified in honey from Manyatta Constituency. On the other hand, *Bacillus tequilensis*, *Bacillus licheniformis*, *Bacillus halotolerans* and *Bacillus inaquosorum* were identified in honey from Marigat subregions.

The difference in the species obtained from the two regions could be linked to different factors such as floral sources, geographical location, environmental conditions, beekeeping practices, and the inherent properties of honey itself (Xiong *et al*., 2022). Geographical origin greatly influences the diversity of the honey microbiome as different regions have variations in climate, soil type and local plant species (Brudzynski, 2020). As bees gather nectar and pollen, the distinct microbial communities that are present on the surfaces of

various plants can be transferred to honey. Since some microorganisms are linked to particular plants, floral variety affects the variety and quantity of bacteria, fungi, and yeasts in honey (Brudzynski, 2020). This could be an explanation for the variation in the microbiome isolated in honey from Manyatta and Marigat regions, as these regions have different climate, soil type and vegetation cover. The results of this study are in tandem with a study by Tsadila *et al*. (2021), whereby 38 honey samples from varied agroecological origin and geographical origin were assessed for microbial populations, and the study determined that these two factors greatly influenced the honey microbiome.

Additionally, honey produced in temperate regions may host different microbial communities than honey from tropical or arid areas due to differences in floral diversity, moisture levels, and temperature (Gaggìa *et al*., 2023). Marigat is an arid area, whereas regions in Manyatta where the honey was sampled from were wet regions, thus this could be an explanation for the variation in microbial diversity in honey from the two regions. A study by Gaggìa *et al.* (2023) on Italian and Maltese honey microbiome corroborates with the findings from this study, whereby the microbiome varied according to the climate of the regions they were sampled.

Antibacterial efficacy of honey is likely to be enhanced by the presence of *Bacillus* species through production of bioactive substances like organic acids, antimicrobial peptides and enzymes (Ratajczak *et al*., 2021). Magdalena *et al*. (2020) successfully characterized the antibacterial activity of *Bacillus* spp. isolated from honey against food-borne pathogens. This corroborates with the findings from this study that the antibacterial activity observed

in honey from the Manyatta and Marigat Constituents is likely to be influenced by the presence of *Bacillus* spp. isolated from the honey.

Bacillus species are recognized for either directly generate antibacterial substances, or indirectly through facilitating their biosynthesis in plants by promoting the expression of pathways leading to their synthesis (Dimkić *et al*., 2022). Myriocin and lanthionine, which are antimicrobial compounds identified in this study are produced by *Bacillus* species such as *Bacillus licheniformis*, which was isolated from Marigat honey. Research by Tran *et al*. 2022 identified antimicrobial peptides produced by *Bacillus* spp. and demonstrated their mechanism of action in antibacterial activity. This backs up the findings from this study that antibacterial activity by the honey could be influenced by the presence of these metabolites produced by the *Bacillus* spp.

According to Abdel-Nasser *et al.* (2024), *Bacillus* spp. especially those involved in plant interactions, can produce phenolic acids like cinnamic acid and its derivatives. Yuan *et al*. (2018) demonstrated the ability of *Bacillus amyloliquefaciens* and *Bacillus subtilis* to produce compounds related to cinnamic acid, which exhibit antimicrobial and antifungal activity. This stamps the possibility that the *Bacillus* spp. observed in tested honey samples could have produced some of the cinnamic acids present in the honey sample hence contributing to their antibacterial efficacy.

Bacillus velezensis is particularly notable for producing phenazine-like compounds (Wang *et al*., 2023), which was present in the honey from Manyatta Constituency, Embu County.

Phenazines and their derivatives have strong antibiotic properties (Guttenberger *et al*., 2017). This corroborates with a study by Xiong *et al*. (2023) in which *Bacillus velezensis* was identified in raw honey and phenazines antimicrobial compounds produced by this bacterium were identified. *Bacillus velezensis* is widely recognized in agricultural research for its production of various antimicrobials, including lipopeptides and polyketides, which help suppress a range of plant pathogens (Fazle *et al*., 2020).

5.2 Conclusions

i. The honey from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County contained similar and different flavonoids and phenolic acids. The phytochemicals concentrations varied between the honey from the two different regions.

ii. The honey extract from Manyatta Constituency and Marigat Constituency had varying antibacterial activity. Honey from Manyatta Constituency had higher antibacterial activity than honey from Marigat Constituency.

iii. The honey from Manyatta Constituency and Marigat Constituency contained eight (8) bacteria species of the genus *Bacillus*.

5.3 Recommendations

i. Future studies should focus on isolating specific phytochemicals in honey to determine which compounds contribute most significantly to its antibacterial activity, with the potential for these bioactive molecules to be developed into novel antibiotic agents.

ii. The honey extracts from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County are recommended as antibacterial agents for the management of mild to moderate bacterial infections, particularly those affecting

the skin, respiratory tract, or gastrointestinal system, and as potential natural alternatives in wound care and topical treatments.

iii. Different species of the genus *Bacillus* can be derived from honey from Marigat Constituency, Baringo County and Manyatta Constituency, Embu County, and they can be isolated and characterized for the production of bioactive metabolites which exhibit antibacterial properties. These metabolites have promising potential for development into natural antibiotic agents effective against pathogenic and drug-resistant bacteria.

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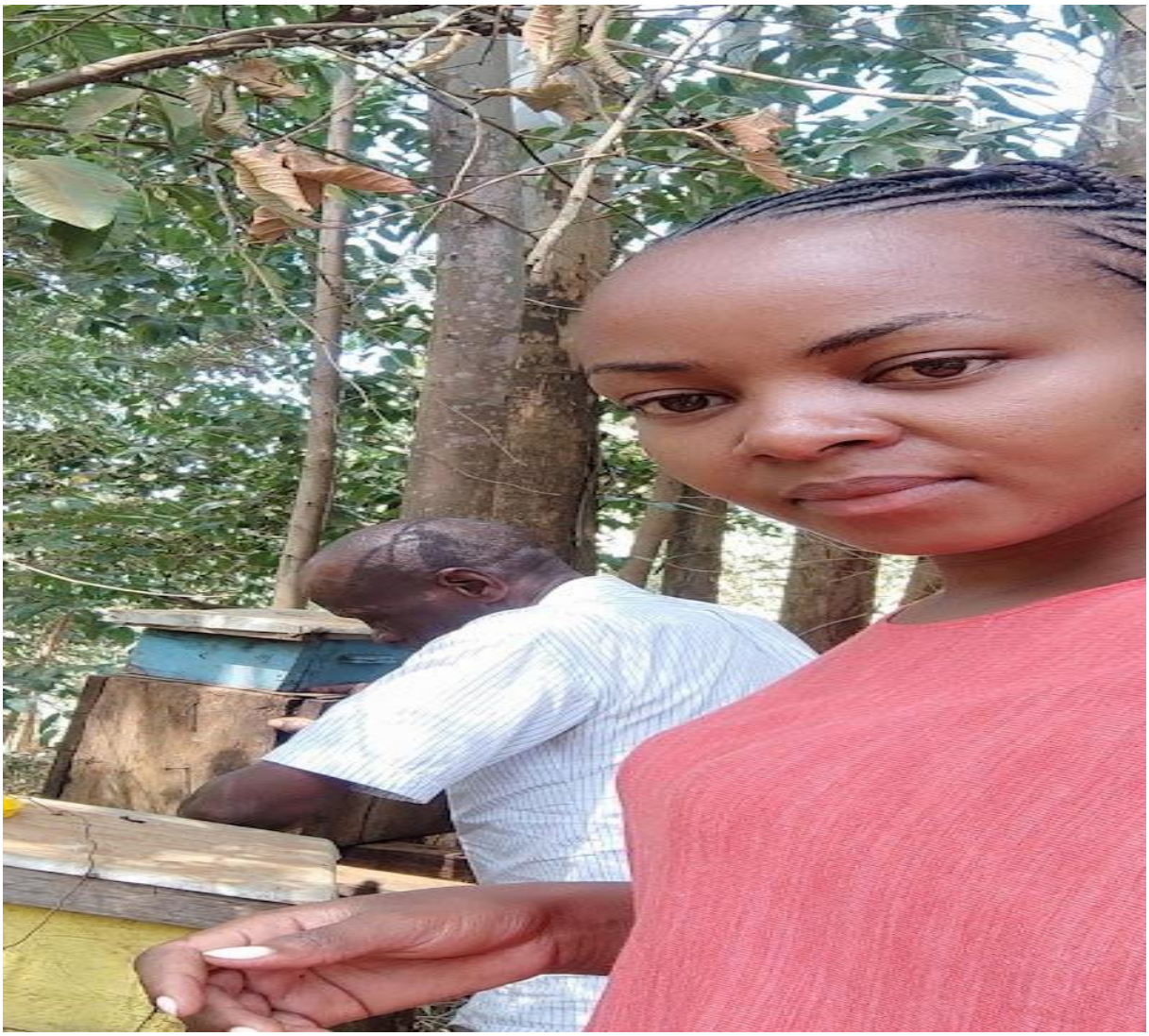
APPENDICES

Appendix I: Langstroth Beehives in Manyatta, Embu County

Appendix II: Collecting the Samples from a Farmer



Appendix III: NACOSTI Research Permit

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