UNIVERSITY OF NAIROBI

DETERMINATION OF HONEY QUALITY AND ITS ANTIBACTERIAL EFFECTS ON BEE HEALTH

BY

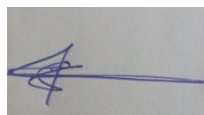**CHOGO SOLOMON INDIAZI**

I56/35049/2019

A Thesis Submitted for Examination in Fulfillment of the Requirements for Award of the Degree of Master of Science in Analytical Chemistry of the University of Nairobi

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the Faculty of Science and Technology, University of Nairobi requirements.

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DEDICATION

This thesis is dedicated to my loving parents Enock and Hellen Indiazi, my son Leo Indiazi, and my dear sisters Millicent and Babra Indiazi. Your immense support through the research and writing of this thesis is highly appreciated.

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ABSTRACT

The impact of pathogens on apiculture has led to the rapid decline in bee colonies in some parts of the world. Evidence in literature on pathogenic resistance to conventional antibiotics indicates that the use of antibiotics to treat bee bacterial diseases has led to development of pathogenic resistance genes. Additionally, trace amounts of these chemicals contaminate bee products, affecting their quality. There's need to investigate the possibility of employing natural forms of apitherapy to enhance bee health and colony sanitation. Honey is bees' source of carbohydrates and also a major candidate for apitherapy as it has demonstrated immense potential in treating human diseases. However, little research has been done on the benefits of honey to the actual producers, the bees. This study investigated the quality and the prophylactic and therapeutic antibacterial potential of honey on bee health as a natural defense line and an alternative to antibiotic use. Bees were inoculated with bacteria and the bacteria density in the bees' gut quantified over time in the prophylactic and therapeutic treatment groups using qRT-PCR (quantitative real time polymerase chain reaction). A choice experiment was used to determine the bees' choice of honey when healthy and diseased. Further, GC-MS (Gas chromatography mass spectrometry) analysis was used to characterize the honey's volatile and non-volatile extracts. Honey quality was assessed via phytochemical and physicochemical analyses. Honey influenced the bacterial density in the bees' gut over the sampled time. In both treatments, all the six honey suppressed the levels of *Serratia marcescens,* an opportunistic pathogen to bees, and *Escherichia coli* an environmental bacteria proving their prophylactic and therapeutic ability against the pathogens. The healthy bees had no preference for any specific honey but made a faster choice for Kitui honey. The diseased bees portrayed a diet change in response to infection and preferred Kitui honey. Overall, the diseased bees made a faster choice compared to the healthy ones. Lastly, quality analysis of the honey showed high phytochemical content; phenols 186 mg GAE/mg, and flavonoids 129.2 mg QE/100 g. Variable quantities of physicochemical parameters; carbohydrates 81.5% and pH 4.2 were also recorded, these factors contribute to honey's antibacterial activity. The volatile and non-volatile chemical profiles showed variability and similarities in the compounds present in each honey. Some of the compounds such as cedrol, 2,5-bis(1,1-dimethyl)-phenol, β-pinene, sibinene, 2,4-dimethyl-1-heptene and 2,5-bis(1,1-dimethylethyl)-phenol had proven antibacterial activity. This study's findings indicate that bees' diet plays a role in maintaining their health and they change their diet preference when diseased to seek honey with more therapeutic potential as evidenced by the chemical profiles, which contain antibacterial compounds. This new evidence provides an alternative form of treatment of bees' diseases through diet to enhance their health and product quality while mitigating the catastrophes of using antibiotics which pose the risk of development of resistance.

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ABBREVIATIONS AND ACRONYMS

Icipe	International Centre for Insect Physiology and Ecology
CFU	Colony forming unit
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
w/v	Weight per volume
GC-MS	Gas chromatography coupled mass spectrometry
STE	Saline Tris-EDTA
EDTA	Ethylenediamine tetraacetic acid
DNA	deoxyribonucleic acid
MGO	Methylglyoxal
MDR	Multidrug resistant
MRSA	Methicillin resistant *Staphylococcus aureas*
PSB	Phosphate saline buffer
°Bx	Degrees Brix

CHAPTER 1
INTRODUCTION

1.1 Background

The global food production depends on pollination to ensure food security, contextually, 87 of the main global food crops representing 35% of the world's food production volume depends on animal pollination (Sluijs & Vaage, 2016). Animals account for 90% of pollination for crop production and of that, 99% of the pollinating agents are insects leaving 1% to birds and bats (Hoshiba & Sasaki, 2008). Honeybees comprise approximately 70 – 80% of the insect pollinators representing their immense value in filling the global food basket and ensuring food security to the increasing human population (Hoshiba & Sasaki, 2008; Sluijs & Vaage, 2016).
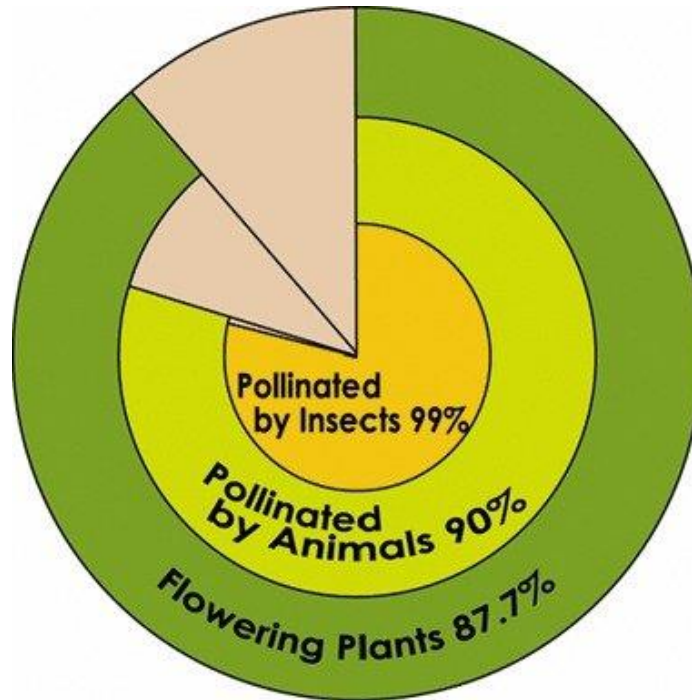
Figure 1: Percentages of flowering plants pollinated by animals *source* (Hoshiba & Sasaki, 2008)

Unfortunately, apiculture has experienced a decline in the population of both managed and wild bee colonies over the previous two decades in some parts of the world. That has caused a deficit in pollination and a reduction in the production of useful bee products (Thorburn *et al*., 2015). A 44% loss of honeybee hives was reported in the United States from 2015 to 2016, which was a 3.5% increase compared to the preceding year and a 12.5% increment in comparison to the 2013 to 2014 period (Cameron & Sadd, 2020).

Pathogen invasion into bee colonies is attributed as one of the significant causes of the decline in bees' population. Additional factors include; exposure to harmful agrochemicals like pesticides, fungicides and insecticides, climate change, predation, invasion of alien species, and habitat loss (Cameron & Sadd, 2020; Potts *et al*., 2010) (Figure 2). These factors occur singularly or in combination, causing a tandem effect, which further weakens the insects' immune response when infected bees get exposed to pesticides sprayed on flowering plants as they forage (Cameron & Sadd, 2020). The interaction between immune-compromised bees with pesticides and other harmful agrochemical agents in the field leads to death in most cases. The decline in bees' colonies has also been exacerbated by parasitism, lack of flowering plants, and land degradation and fragmentation (Goulson *et al*., 2015).
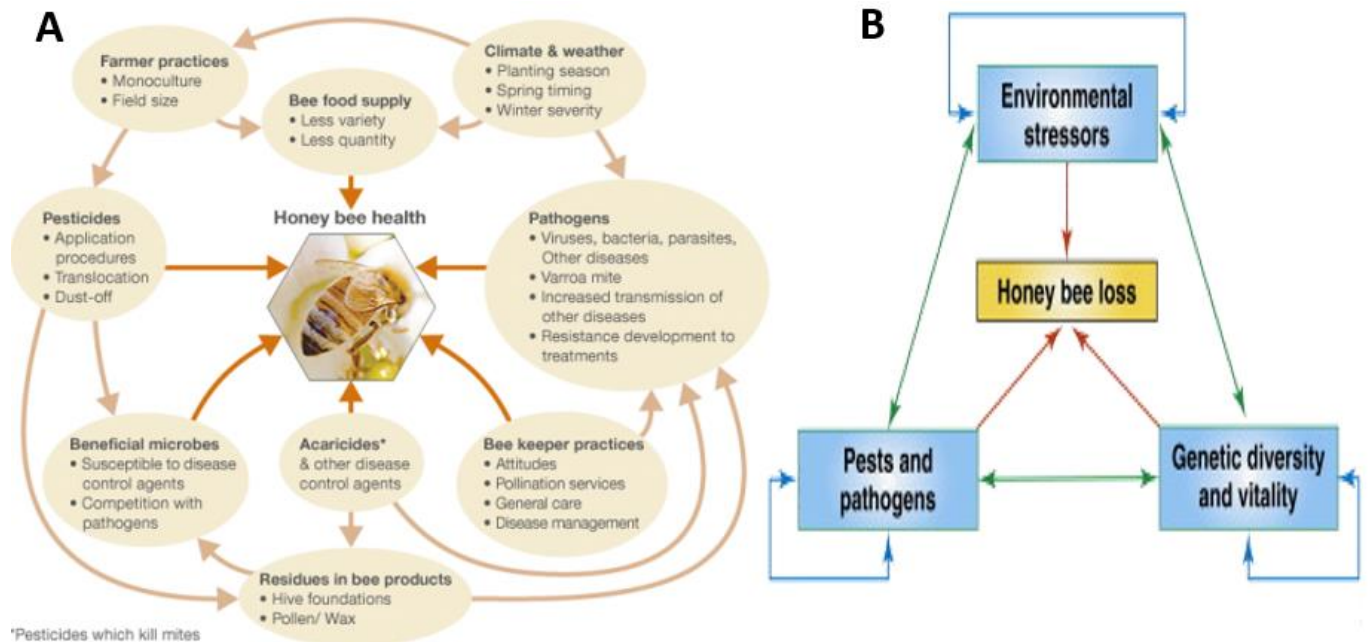
Figure 2: Multiple stressors that act independently and interactively affecting bee health (2A & 2B) modified from (Johnson & Lynne, 2015; Potts *et al*., 2010).

Artificial methods of reducing parasitism's effects on colony stability like the 'beehive bottom board' have been developed, hence slightly suppressing parasites (Ucak-Koc, 2014). Recent studies involving Italian and Africanized honeybees have shown that they have naturally developed resistance to some parasites like the *Varroa* mites via self-grooming mechanisms (auto grooming).

The parasitized bees get rid of the mites by dislodging them, thereby inhibiting their impact on brood collapse, which is a positive step for the bees to naturally sustain colony stability (Cheruiyot *et al*., 2018; Invernizzi *et al*., 2015). The persistent problem that still fosters colony collapse leading to the reduction in bee population is pathogen invasion.

1.2 Overview of the Harmful Microbes Affecting Bees

Microbial pathogens have a grave global effect on insects' health, population, and diversity (Potts *et al*., 2010). Besides other stressors such as pesticide exposure and lack of food sources, bees are adversely affected by the invasion of microbial pathogens into their colonies affecting their health and often culminating in death and loss of colonies (Doublet *et al*., 2015). Bees interact with and pick harmful pathogens as they forage for nectar, associate with infected nest mates, during an invasion of parasites in the colony, when they consume contaminated food or transmitted via mating from the drones to the brood through egg laying (Davis *et al*., 2019; Doublet *et al*., 2015).

Honeybees are susceptible to infection from an extensive pathosphere whose members are ubiquitous in the environment or transmitted by bee parasites. These include, viruses, protozoa, fungi, and bacteria (Dolezal & Toth, 2018). Honeybees are infected by over 30 viruses which have been reported in multiple studies, most viral induced diseases are latent in the hive existing in an asymptomatic state and only cause considerable harm to the bees' health in presence of other stressors (Amiri *et al*., 2020; Evans & Schwarz, 2011; McMenamin *et al*., 2016; Mutinelli, 2011; Ullah *et al*., 2021). Some of the prevalent viral infections affecting honeybees include; Black Queen Cell Virus, Deformed Wing Virus, Sacbrood Virus, Slow, Acute and Chronic Bee Paralysis virus, and Filamentous Virus (Grozinger & Flenniken, 2019; Mutinelli, 2011).

Additionally, bees suffer from protozoan infections like Amebiosis, and fungal infection such as Stonebrood, Chalkbrood, and Nosemiosis (Vojvodic *et al*., 2012). Honeybee parasites also spread infection when they infiltrate the colony, which include Varroatosis and Aethinosis (Lecocq *et al*., 2016) . Honeybees are mostly affected by bacterial infections, two of the most common and extensively studied being American Foulbrood, which is described in other studies as the most infectious and destructive (Locke *et al*., 2019), and European Foulbrood, which is equally virulent.

Beside these pervasive ones, the honeybee is susceptible to other bacteria which cause adverse effects to its health, amongst them *Serratia marcescens*, which has been linked to causing sepsis in the larvae and adult bees leading to their eventual death (Burritt *et al*., 2016). It is an opportunistic bacterium in bees whose effects manifest when the bees' immunity is compromised by other infections or stress factors (Fünfhaus *et al*., 2018; Locke *et al*., 2019). The other identified bee bacterial diseases include Powdery scale and May disease. Often, farmers use chemicals like antibiotics to treat colony bacterial infection.

The short-lived success in the use of pesticides and antibiotics to treat most of the honeybee infections has led to microbes mutating and becoming resistant to the chemicals. This has posed an even more significant challenge of resistance and left the burden of defense against disease solely to the bees' immune system (Locke *et al*., 2019; Mutinelli, 2011; Tian *et al*., 2012). Resistance to drugs by specific strains of pathogens due to the extended exposure during treatment has necessitated the search for alternative, more efficient treatment options to alleviate the defense workload on the bees' immune system (Tian *et al*., 2012).

Bees' immune system is the number one defense line in preventing or in response to an infection. Still, bees' diet is equally important as it is a source of antimicrobial substances, boosts the immune system, and increases its ability to ward off infection (Berenbaum & Calla, 2021). Research has shown change in foraging patterns of bees when anticipating or upon confirmation of possible infection to the brood, colony, or individual worker bee (Roode & Hunter, 2019). This implies that the primary food for bees – honey, has a role in disease prevention (prophylaxis) and infection treatment (therapeutic) (Erler *et al*., 2014; Gherman *et al*., 2014). The dietary composition changes shown by the shift in foraged plants proposes the possibility of using natural resources available to foragers to promote colony health and stability by preventing and treating bee diseases via diet as opposed to chemicals (Berenbaum & Calla, 2021; Roode & Hunter, 2019; Locke *et al*., 2019).

Nectar, which is a prerequisite to the processing of honey by bees, is composed of different components, including; water, sugar, organic acids, minerals, vitamins, amino acids, proteins, hydrogen peroxide, volatile compounds, phytochemicals like flavonoids, phenols, alkaloids and terpenoids (Santos-Buelga & González-Paramás, 2017).

All these components are obtained directly from floral sources. They individually and synergistically play a significant role in honey's fungicidal, virucidal, protozocidal, bacteriostatic, and bactericidal properties that enable its prophylactic and therapeutic ability, which is important in the self-medication of bees (Roode & Hunter, 2019; Koch *et al*., 2019; Santos-Buelga & González-Paramás, 2017; Simone-Finstrom & Spivak, 2012).

1.3 Problem Statement

Bees' are vital pollinators in the agricultural sector, with an annual estimated global pollination value of over \$361 billion (Hanley *et al*., 2015). Besides its economic value, pollination plays a significant role in food production to eliminate malnutrition and food insecurity (Potts *et al*., 2010). However, this service is threatened by the sudden and increasing decline of bees' colonies in some parts of the world due to pathogen infections (Goulson *et al*., 2015). The use of chemicals to treat these infections is proving ineffective as pathogens are developing resistance due to long term exposure (Tian *et al*., 2012). Secondly, antibiotics in extreme measures have also recorded detrimental effects on the bees' products and health. Furthermore, there is a risk of the antimicrobial resistance genes spreading into the environment and entering the human food chain via honey consumption as some studies have found antibiotic resistance genes (ARGs) in honey (Li *et al*., 2021). Lastly, chemical residue contaminate honey which serves as human food (Al-Waili *et al*., 2012; Tian *et al*., 2012).

Overdose of antibiotics to the bee colonies has a negative effect on the bees gut microbiota population density, which is essential in colony health, survival, and the regulation of the host-pathogen interaction concerning bacterial load and ultimately the host physiology (Kešnerová *et al*., 2020). Due to pathogen resistance to treatment and the adverse effects of chemical use on bee colonies, there is a need for a novel natural mechanism of preventing, managing, and treating brood and adult honeybee pathogenic infections with minimal effects on the bees' health, survivability and the quality of their products (Roode & Hunter, 2019).

Honey has demonstrated an excellent ability to cover this gap, as shown in Dixon, (2003); hence there is a need to study the self-medication behavior of bees concerning their foraging habits in response to the risk of, or infection and their dietary preferences when healthy and infected.

1.4 Objectives

1.4.1 General Objective

To determine the quality of different honey and their prophylactic and therapeutic antibacterial effects on bee health.

1.4.2 Specific Objective

    i.   To determine the prophylactic antibacterial effect of different honey.

    ii.   To determine the therapeutic antibacterial effect of different honey.

    iii.   To establish the choice of bees on the different types of honey depending on their disease status.

    iv.   To evaluate the components in the different honey possessing prophylactic and therapeutic antibacterial abilities through chemical analysis.

1.5 Justification and Significance of the Study

Pathogen infection often leads to the disintegration of the honeybee colony. That is worsened by the effects of repeated exposure to antibiotics, which causes development of resistance and interferes with the bee gut microbiota (Bulson *et al*., 2021; Kešnerová *et al*., 2020; Kwong & Moran, 2016; Raymann *et al*., 2017). Antibiotics pose an intricate problem if used in excess amounts or over a lengthy period (Palmer-Young *et al*., 2017). That has prompted investigations on into the effectiveness of apitherapy by studying the self-medication behaviors in honeybees (Erler & Moritz, 2016). Honeybee nutritional diet, majorly composed of honey, has a crucial role in maintaining the bees and colony health and sanitation. Worker bees collect nectar that is incorporated with secondary plant metabolites with varying degree of antimicrobial potential (Molan, 1992). When processing the nectar to honey, bees ingest and regurgitate it with additional enzymes then store it as honey for later consumption (Santos-Buelga & González-Paramás, 2017).

The interaction of the secondary plant metabolites and other bactericidal and bacteriostatic components present in the nectar during the processing and consumption enhances the bees' immunity against diseases, improves their health and ultimately reduces colony mortality (Simone-Finstrom & Spivak, 2012). Plants enrich their nectar with specific secondary plant metabolites hence creating a difference in the honey's antimicrobial ability.

This informs the bees' choice of foraging on different flowers when infected with different pathogens (Roode & Hunter, 2019; Erler & Moritz, 2016). Bees have demonstrated a change in foraging activity when at risk or when infected, by preferentially increasing the consumption of nectar from specific plants believed to have antimicrobial property (Roode & Hunter, 2019; Simone-Finstrom & Spivak, 2012). This self-medication ability of the honeybees if studied extensively and the preferred compounds and their floral sources determined. There is a potential of discontinuing the use of antibiotics in treating bees' bacterial diseases by promoting self-medication (Abbott, 2014).

LITERATURE REVIEW

2.1 The Effect of Pathogens, Pests, and Stressors on Honeybee Health

Honeybees are susceptible to multiple diffusive and contagious diseases, pests invasion, and environmental stressors which often co-occur and overwhelm the bees' defense mechanisms against infection leading to mortality in severe cases (McMenamin *et al*., 2016). The exposure of honeybees to stressors such as, insecticides, pesticides and poor nutrition are key determinant of the severity of infection which range from mild to deadly. It has been shown in a previous study that an interaction between the sub-lethal doses of pesticides and pathogens increased honeybee mortality in their life cycle (Doublet *et al*., 2015). Globally, honeybee have come under attack from deadly bacterial, fungal and viral infections that affect their health and curtail their normal functioning in regard to colony sanitation and foraging for food, two factors which ensure their survivability (Evans & Schwarz, 2011).

Bee pathogens are prevalent around the world as shown in (Ellis & Munn, 2005) in an extensive review highlighting the worldwide widespread of bacterial, fungal, and viral disease alongside the distribution of honeybee pests such as the parasitic mites like *Varroa*. Data from a contemporary research that evaluated the distribution and impact of pathogens and parasites on honeybee populations in East Africa by Muli *et al*. (2014) confirmed the presence of *Nosema apis* in some apiaries along with *Varroa*. Additionally, in a recent study conducted in Kenya for the surveillance of common honeybee pathogens, Ongus *et al*. (2018) reported the presence of multiple pathogens. They included *Varroa destructor virus*, which was the most abundant at 50% prevalence and others with lower prevalence like the *Deformed wing virus* at 44% and *Black queen cell virus* at 36% among others in the sampled apiaries around the country (Ongus *et al.,* 2018).

2.1.1 Honeybee Pathogens Manifestation and Classification

The occurrence or manifestation of maladies in colonies of honeybees depends on multiple factors such as the bees' genetic makeup, which is traced to the queen's genetic heritage. The bees' genetic makeup influences their resistance to certain diseases and the in and out of hive hygienic behavior.

Pathogens (fungus, bacteria, protozoa, and virus) are also a determinant in the occurrence of diseases since they need agents to manifest and the severity of the disease depends on the pathogen load (Fünfhaus *et al*., 2018; Research and Extension Unit, 2018). Environmental factors like temperature, humidity, and the availability of flowering plants to provide nectar for the bees also plays a role in disease occurrence among honeybee colonies. These have been found to be crucial triggers for the onset of diseases in some instances (Research and Extension Unit, 2018).

Honeybee maladies classification is done in two distinct ways, the first one being according to the causative agent of the disease, which is often accurate and the most commonly used and categorizes the disease into bacterial, fungal, viral or parasitic (Table 1). The other classification is according to target population in the honeybee colony as it is composed of bees at different developmental stages and duty specialization. This classification yields brood and adult bee diseases (Ellis & Munn, 2005; Research and Extension Unit, 2018).

Table 1: Honeybee pathosphere, their causative agent and classification

Disease	Causative agent	Type
Acariasis	*Acarapis woodi*	Parasitic
Aethinosis	*Aethina tumida (Small hive beetle)*	Parasitic
Varroatosis	*Varroa destructor*	Parasitic
Tropilaelapsosis	*Tropilaelaps spp*	Parasitic
American foulbrood	*Paenibacillus larvae*	Bacterial
European foulbrood	*Melissococcus pluton*	Bacterial
Chalkbrood	*Ascosphera apis*	Fungal
Stonebrood	*Aspergillus flavus*	Fungal
Nosemosis	*Nosema apis – Nosema ceranae*	Fungal
Amebiasis	*Malpighamoeba mellificae*	Protozoa
Sacbrood virus (SBV)	*Virus Picorna-like*	Viral
Chronic Bee Paralysis Virus (CBPV)	*Cripaviridae*	Viral
Acute Bee Paralysis Virus (ABPV)	*Dicistroviridae*	Viral
Deformed Wing Virus (DWV)	*Iflaviridae*	Viral
Black Queen Cell Virus (BQCV)	*Dicistroviridae*	Viral
Israeli Acute Paralysis Virus (IAPV)	*Dicistroviridae*	Viral
Kashmir Bee Virus (KBV)	*Dicistroviridae*	Viral
Kakungo Virus	*Iflaviridae*	Viral
Tobacco ringspot virus	*Secoviridae*	Viral
Invertrebrate Iredescent Virus type 6	*Iridoviridae*	Viral

2.1.1.1 Viral Honeybee Pathogens

Viruses take a significant share of the honeybee pathosphere and are linked to the colony losses of the Western managed honeybees (McMenamin & Genersch, 2015). A surveillance conducted in East Africa confirmed the presence of multiple viruses in some of the examined hives in Kenya (Muli *et al*., 2014). Viruses are prevalent and latent in honeybees globally and often exist in asymptomatic state. Independently, viruses cause minimal harm to the honeybees as the viral load is maintained at less severe levels (Research and Extension Unit, 2018). However, the presence of stressors and invasion of parasites and pests, triggers a rapid viral replication leading to lethal viral load that affect the bees' health (Grozinger & Flenniken, 2019; Tantillo *et al*., 2015; Ullah *et al*., 2021). Pest invasion and pathogen infection weaken the bees' immune system enabling the reactivation and replication of the pre-existing latent viruses in the bee to virulent levels affecting the bees' health and causing mortality of the bees or brood (Grozinger & Flenniken, 2019; Ullah *et al*., 2021).

Since the introduction of the *Varroa* destructor, there has been an upsurge in the number of viruses affecting honeybees, a justification of how pests and stressors exacerbate the severity of viral infections to honeybees (Zijlstra, 2013). In addition, the *Varroa* mite itself is a vector for bee viruses transmitting them through its saliva. Virus transmission in the honeybee colony occurs in two ways; vertically which is the primary mode of transmission where the virus is passed down from the queen to the brood. And, horizontally through the bees interaction in the hive, their fecal matter, *Varroa* saliva, beekeeper induced, or royal jelly (Amiri *et al*., 2020; Ullah *et al*., 2021; Yañez *et al*., 2020). The total number of viruses Amiri *et al*. (2020) found in honeybees is estimated to be slightly over 30 but whether or not all have detrimental effect to the honeybees health is still under scientific scrutiny (Amiri *et al*., 2020).

Bee viruses affect bees at different developmental stages in their life cycle. The Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Sacbrood Virus (SBV), and the Black Queen Cell Virus (BQCV) often affect the bees while in the initial development stage, referred as the brood. However, the BQCV exclusively affects the queen cells. The CBPV and Acute Bee Paralysis Virus (ABPV) affect adult bees (Research and Extension Unit, 2018; Ribière *et al*., 2010; Tantillo *et al*., 2015; Zijlstra, 2013).

2.1.1.2 Bacterial Honeybee Pathogens

American Foulbrood (AFB) is a deleterious and widespread bacterial disease that affects the bee brood (Figure 3). It is caused by a Gram-positive aerobic bacteria *Paenibacillus larvae* (Forsgren *et al*., 2018; Fünfhaus *et al*., 2018). AFB is highly contagious as it spread through spores of the bacterium which can remain viable and infectious on hive and beekeeping equipment for approximately 30 years causing recurrent reinfections (Research and Extension Unit, 2018).
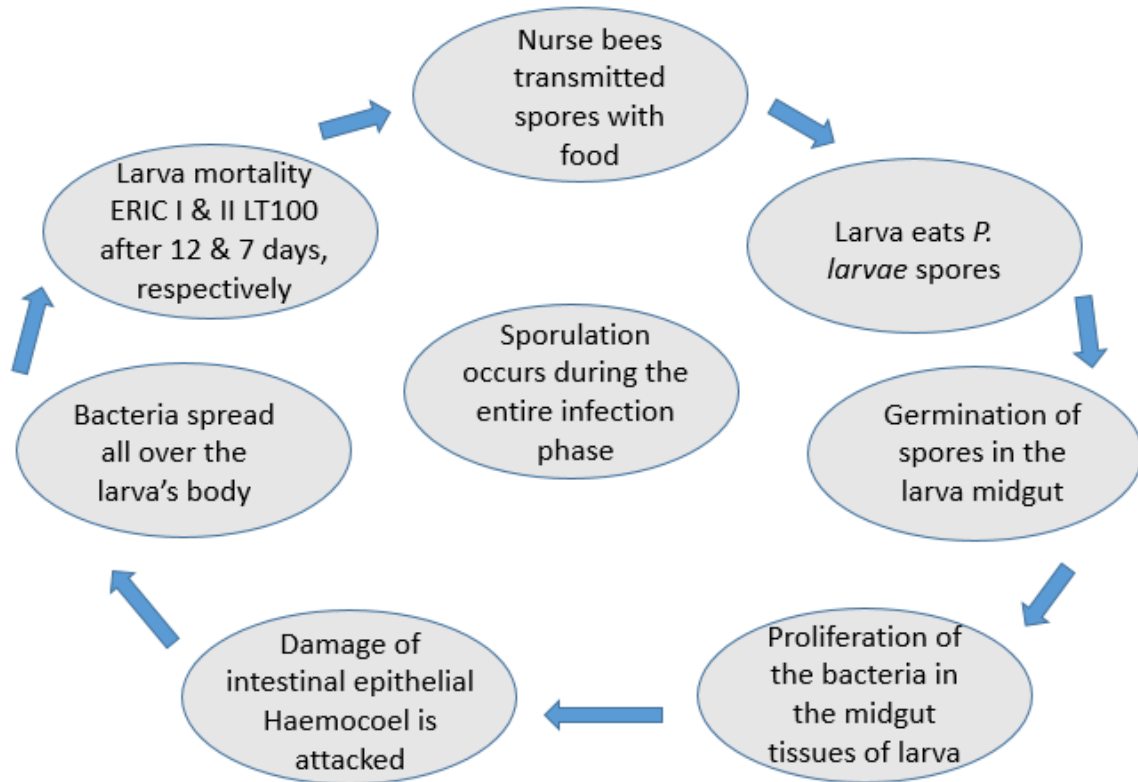
Figure 3: The transmission cycle of AFB modified from Jończyk-Matysiak *et al*. (2020)

AFB's attack on a healthy brood turns its color to chocolate-brown and melt to a gooey sac to the bottom of the cell with the tongue protruding to the cell's walls, a condition referred to as 'pupal tongue'. As the disease progresses the brood capping is punctured and sinks into the cell producing a foul smell. With time the dead larvae dries, hardens into a black scale sticking to the cell's floor while harboring the bacterium spores acting as a site for reinfection (Evans & Schwarz, 2011; Locke *et al*., 2019; Zijlstra, 2013).

The disease curtails the growth of a colony by killing the brood, which eventually decreases the colony numbers ultimately causing loss of the colony, if unchecked the disease could spread and affect the entire apiary (Jończyk-Matysiak *et al*., 2020).

American Foulbrood's therapy includes the use of veterinary antibiotics such as Terramycin whose usage is being strictly regulated in the United States due to the continued emergence of AFB resistant strains and contamination of bee products (Al-Waili *et al*., 2012; Genersch, 2010; Hansen & Brødsgaard, 1999; Jończyk-Matysiak *et al*., 2020). American Foulbrood's effects are amplified by the presence of stressors on the bees' colony, however, sufficient supply of quality food alleviates the disease's burden to the bees. Besides the bees' immune systems and food supplementation, the bees try to manage infection by conducting colony sanitation, which includes removing the affected larvae from the colony (Hansen & Brødsgaard, 1999).
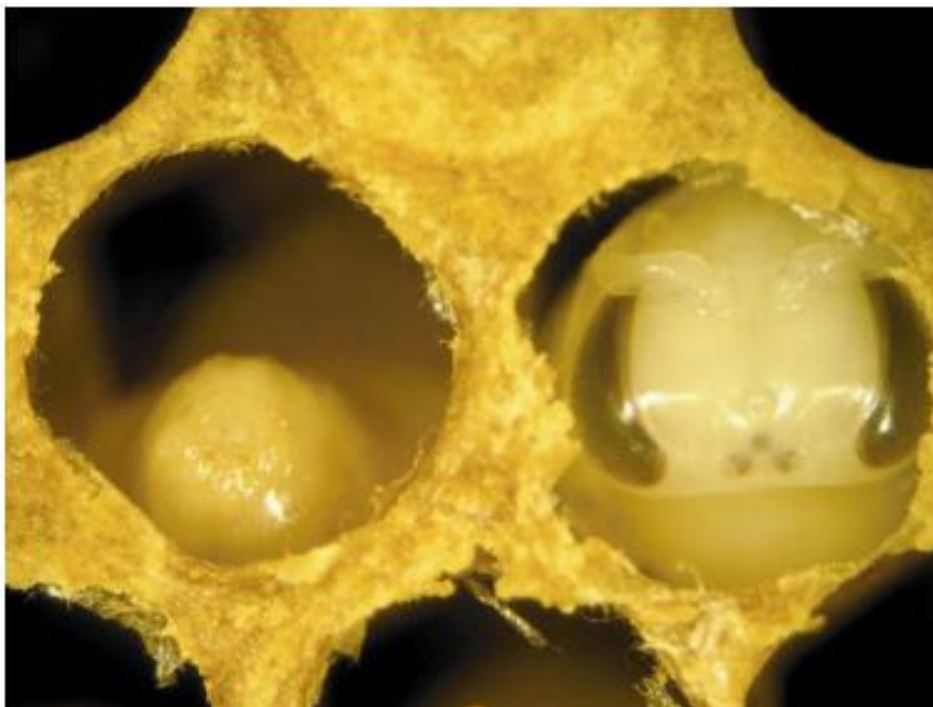
Figure 4: Roppy mass of ABF infected larvae on the left and a healthy black eyed larvae on the right, 13 days post-infection *source* (Genersch, 2010)

European Foulbrood is another destructive honeybee bacterial disease causing brood mortality. Its causative agent is a Gram-positive bacteria called *Melissococcus plutonius* often co-occurring with other bacteria such as *Streptococcus faecalis, Bacillus alvei, Paenibacillus alvei, Achromobacter*

eurydice, causing different symptoms. The major fluctuation is the presence or absence of a foul smell depending on the bacteria *M. plutonius* co-occurs with (Fünfhaus *et al*., 2018) . The disease is wide spread in different locations practicing apiculture around the world with variable severity and affects 4 - 5 days old larvae (Evans & Schwarz, 2011; Forsgren, 2010).

The affected brood appears brown and twisted in different positions in the cells and often die before the bees cap the brood cells (Figure 5). The pathogen *M. plutonius* spreads orally in the brood by nurse bees which get contaminated when cleaning out the dead larvae and transmit the bacteria to uninfected larvae when feeding them (Fünfhaus *et al*., 2018; Research and Extension Unit, 2018; White, 1920). Starved, genetically weak or colonies exposed to pesticides or any other stressor are at a high risk of infection and suffer severe effects from EFB. Food supplementation by restoring a constant nectar flow and pollen supply either artificially or naturally suppresses the effects and spread of this disease (Alaux *et al*., 2010; Locke *et al*., 2019; Research and Extension Unit, 2018; White, 1920).
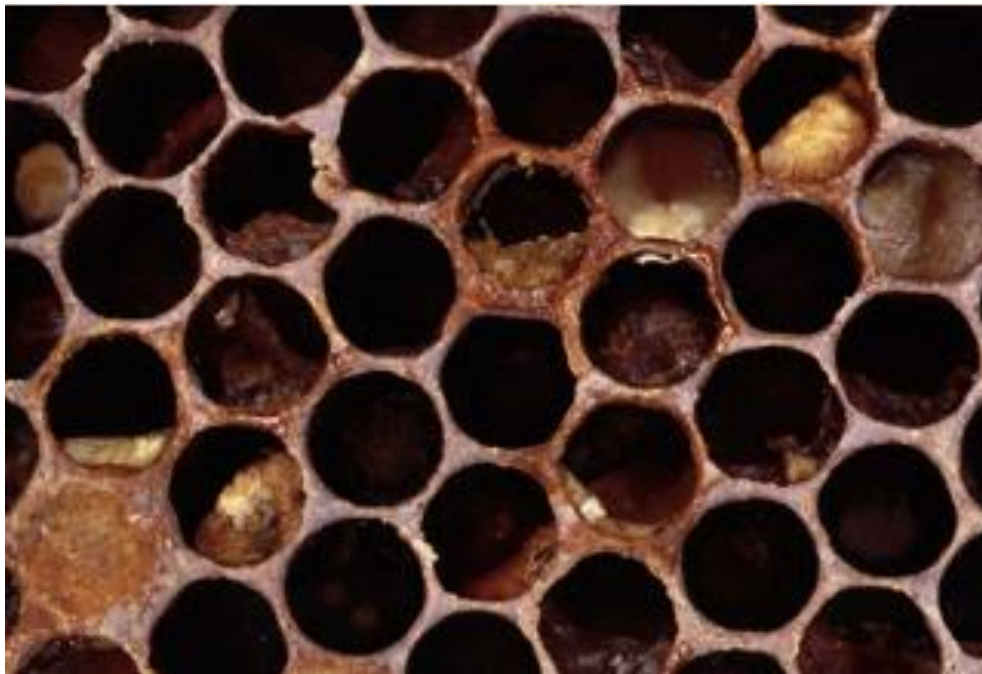
Figure 5: Honeybee comb with brood infected with AFB in uncapped cells *source* (Forsgren, 2010)

Serratia marcescens is a bacterial pathogen of family *Enterobacteriaceae* attacking an extensive range of insects, animals and humans.

It affects approximately 70 insect species including the honeybee. Despite receiving less attention compared to the widely studied AFB and EFB, this bacterial disease causes mortal septicemia in adult honeybees and possibly the brood (Burritt *et al*., 2016; Fünfhaus *et al*., 2018; Raymann *et al*., 2018). *Serratia marcescens* is an opportunistic Gram-negative rod-shaped, facultative anaerobic bacteria with a characteristic red pigmentation when cultured on agar (Figure 6) (Burritt *et al*., 2016; Fünfhaus *et al*., 2018; Gliński & Jarosz, 1990). Opportunistic bacterial pathogens only affect susceptible organisms or host i.e. when they're immunocompromised, subjected to stressors, or when the organism's microbiome is perturbed. *S. marcescens* has been reported as a member of the gut microbiome of honeybees at relative abundances of less than 5% and peacefully co-existing with the other bacterial community in the gut (Moran *et al*., 2012). However, when the honeybee experiences immunocompromising or gut microbiota disrupting stressors such as exposure to agrochemicals and antibiotics, the risk of infection from *S. marcescens* drastically increases (Fünfhaus *et al*., 2018; Raymann *et al*., 2018; Steele *et al*., 2021).

A study has shown increased mortality in adult honeybees when orally infected with a gut isolated *S. marcescens* bacteria upon exposure to antibiotics or pesticides (Raymann *et al*., 2017), proving the ability of *S. marcescens* to become virulent under favorable conditions. In another study which examined the pathogenicity of *S. marcescens* strains isolated from the honeybee's gut. It was found that the honeybee associated strains do not trigger an immune response during infection as the expression of phenoloxidase or antimicrobial peptides were low in the infection period. Thus suggesting that these strains are able to evade the bee's immune system and, therefore, having the potential of increased virulence (Raymann *et al*., 2018). Another *S. marcescens* strain, *Sicaria* has been associated with haemocyte loss and inducing Sepsis in honeybees (Burritt *et al*., 2016).

Figure 6: *S. marcescens* bacteria cultured on an agar plate

There are also other bacterial pathogens that have been documental to have detrimental effect on the honeybee immune system impairing their defense against pathogens and jeopardizing their health. These include *Bacillus pulvifaciens* bacterium which is the causative agent of Powdery Scale Disease, which has almost similar symptoms to AFB (Graaf *et al*., 2006; Raymann *et al*., 2018).

Additional honeybee pathogenic bacteria include two species *Spiroplasma apis* and *Spiroplasma melliferum* belonging to the genus *Spiroplasma* responsible for causing symptoms of a disease often occurring in May hence the name 'May Disease' (Burritt *et al*., 2016; Fünfhaus *et al*., 2018). The disease is characterized by flightless bees that quiver and crawl on the ground with a hard and swollen abdomen due to their intestines filled with undigested pollen (Fünfhaus *et al*., 2018). However, this bacterial infection in adult honeybees is often undetected as their occurrence triggers social immunity associated behaviors where bees work together to prevent disease transmission in the colony (Raymann *et al*., 2018). That sometimes include restricting access of infected bees to the colony by a special group of worker bees guarding the entrance. The bees succumb to the infection unnoticed outside the hive making it challenging to diagnose such bacterial infections (Simone *et al*., 2009).

There's a possibility of existence of other bacterial diseases affecting honeybees but their detections, diagnosis and documentation is curtailed by the bees' social and individual immunity and colony sanitation behaviors.

2.1.1.3 Fungal Honeybee Pathogens

The most common fungal diseases that affect honeybees worldwide are Chalkbrood, Stonebrood and Nosemosis. Chalkbrood is caused by a filamentous fungus *Ascosphaera apis* and it exclusively affects bee brood (Cornman *et al*., 2012). This invasive mycosis affects the brood individually posing a less significant threat to the entire colony. However, its continued and long-term effect deteriorates the colony health, reduces the colony population and productivity and subsequently the production of bee products. A study reported a 5-37% reduction in honey production in colonies affected by Chalkbrood (Aronstein & Murray, 2010). Depending on the disease severity, Chalkbrood can cause immense loses to a colony, the disease severity depends on inoculum dosage, age of the brood, temperature and humidity.

Chalkbrood thrives in cool and humid climates although its detection in parts of the world with hot and dry climates suggests its adaptability to change in environment (Aronstein & Murray, 2010; Research and Extension Unit, 2018). Chalkbrood often spread horizontally through diet when the larvae ingest spores of *Ascosphaera apis,* which develops in the larvae's gut, infects all its internal organs causing its death from systemic mycosis. However, its spread can also be fueled by poor bee keeping practices. The dead mummified larvae cadaver produces spores which remain infectious for extended periods in the hive (Cornman *et al*., 2012; Vojvodic *et al*., 2011).

Stonebrood is a facultative fungal infection that affects most insects including bees. It is caused by *Aspergillus flavus* or, less often, *Aspergillus fumigatus* fungi and affects the bees in their larval and adult stage (Vojvodic *et al*., 2011; Zijlstra, 2013). This fungi survive in cool temperatures between 7 °C to 40 °C with its optimal growth temperatures being 33 °C to 37 °C, a range similar to the hive temperatures (Research and Extension Unit, 2018). Stonebrood spread horizontally through trophallaxis, a key behavior of honeybees which involves sharing food by passing nectar or the content of the crop from one bee to another or during colony sanitation. The fungi also have the ability to stick on the bee's body and cause harm from outside (Evans & Schwarz, 2011).

The affected larvae appearance is white and fluffy and changes to green brown for *A. fumigatus* affected larvae and yellow for larvae affected by Stonebrood caused by *A. flavus* fungi.

As the infection progress, eventually the larvae becomes hard and mummified. For adult bees, Stone brood causes paralysis making the bee unable to fly out of the hive, weak, agitated, and initiate physical changes like distended abdomen and eventual mortality and mummification (Vojvodic *et al*., 2011; Zijlstra, 2013). By causing death of the brood and adult bees, the diseases affects the colony population and activities.

Nosema disease affects the adult honeybee and the queen causing detrimental effects to the bees' health. It is caused by obligate intracellular spore forming *Microsporidia*, a group of highly specialized fungi (Zijlstra, 2013). At low temperature the spores remain active for years and are responsible for re-infection of the colony. *Nosema Apis* was the first to be discovered in the mid-19th century followed by the recent, more prevalent, infectious and globally distributed *Nosema ceranae*. A third species of the microsporidian associated with Nosema has also been reported in Uganda having been discovered in Ugandan honeybees; *Nosema neumanni* (Higes *et al*., 2006; Research and Extension Unit, 2018; Zijlstra, 2013). The spores of the different strains have similar morphology hence almost indistinguishable using microscopy techniques.

Nosema spread through ingestion of the spores by bees, the spores reside and develop in the bees' mid-gut and are secreted during egestion. Infection occurs when other bees are exposed or interact with spore laden fecal matter from an infected bee (Fries *et al*., 2006; Smith, 2012). Nosema's adverse effects on bee health include; nurse bees' hypopharyngeal infection losing the ability to produce royal jelly, infected queen's eggs have reduced vitality and often cease egg laying and die in severe cases. Additionally, it leads to reduced life expectancy and the young nurse bees abandon their brood rearing task and take up foraging and guarding duties, usually undertaken by older bees. Besides these effects, Nosema infection compromises the bees immunity increasing the effects of other stressors (Chen *et al*., 2009; Forsgren & Fries, 2010). Moreover, exposure to stressors such as pesticide increases susceptibility to Nosema as indicated in (Muli *et al*., 2014; Pettis *et al*., 2013).

2.1.1.4 Honeybee Protozoa

The protozoan responsible for Amebiosis is *Malpighamoeba mellificae*. This adult honeybee disease is prevalent in the temperate northern and southern hemispheres and not commonly observed in the tropics (Research and Extension Unit, 2018; Schäfer *et al*., 2022). It has similar symptoms with Nosemosis and often co-occur in a mixed infection case.

The disease spreads through healthy bees interacting with infectious feces of a diseased bee. Amebiosis affects the malpighian tubules and causes diarrhea in bees, often preceded by a swollen abdomen (Morimoto *et al*., 2013). It's characterized by trembling wing and inability of the infected bees to fly, which reduces the hives activity and workforce leading to neglected brood, which die along with the infected bees lowering the bees population and productivity (Morimoto *et al*., 2013; Research and Extension Unit, 2018; Schäfer *et al*., 2022).

2.1.1.5 Pests

Aethina tumida commonly referred to as the Small Hive Beetle (SHB) is a South African native pest that affects insects of the *Apoidea* family. The SHB is widespread in many parts of the world including North, Central, and South America, Australia and Africa (Hood, 2004; Research and Extension Unit, 2018). It has also been reported as a pest to honeybees in Kenya (Muturi *et al*., 2022) (Figure 7). Most honeybee colonies in Sub-Saharan Africa are reported to have developed mechanisms to cope with SHB infestation when healthy but become susceptible to the pests' damage when weakened and diseased causing weak colonies to die (Cuthbertson *et al*., 2013). The SHB larvae is destructive as it feeds on the honeycomb's content and excrete on the honey triggering fermentation, which ultimately lowers its quality and make it unfit for human consumption (Cuthbertson *et al*., 2013; Hood, 2004; Research and Extension Unit, 2018).

Figure 7: An adult Small Hive Beetle, *Aethina tumida* picture by Muturi *et al*. (2022)

Tropilaelaps is caused by parasitic mites that feed on the bee pupae and larvae causing malformation, mortality and swarming which leads to colony decline (Chantawannakul *et al*., 2018). This pest, although not widespread as others, presents a threat to the growth of honeybee colonies and beekeeping economy. Research has proven *Tropilaelaps* as potential vector for the viral infection, deformed wing virus(DWV), thereby increasing the spread of viral diseases in honeybees (Dainat *et al*., 2009).

Varroa destructor or Varroa mite (Figure 8B) is an ectoparasite which causes a disease called varroatosis, in honeybees. The mites are widespread around the globe and increase infection pressure in infested colonies (Rosenkranz *et al*., 2010). Varroa mites affect the brood and adult bees by feeding on bee fat body tissues, causing immunosuppression which weakens the bee and predisposes it to diseases. The open wounds caused by the mite compromise the bees' defense against pathogens and offer and entry point for pathogenic microbes (Bernardi & Venturino, 2016). Additionally, the mites reproduce in the brood cells allowing their offspring to parasitize developing bee larvae. These mites are a vector for spreading viral diseases, especially DWV, which easily affect the immunocompromised adult bees since it provides an environment for rapid replication of the virus to virulent levels (Bernardi & Venturino, 2016; Grozinger & Flenniken, 2019; Research and Extension Unit, 2018; Tantillo *et al*., 2015; Ullah *et al*., 2021; Yañez *et al*., 2020). The tandem effect of viral diseases and infestation of Varroa mites has been linked to the colony losses observed in the United States (Rosenkranz *et al*., 2010). A heavily infested colony suffers a lifespan reduction of the adult bees by approximately 25% to 50% (Research and Extension Unit, 2018).

Figure 8: A *Varroa* mite attached to an adult bee (Encircled in red) (A) and an adult *Varroa* mite (B) (modified from Research and Extension Unit, 2018)

Honeybees are affected by multiple pests and parasites which weakens their immunity against pathogenic microbes facilitating bacterial, fungal, protozoa and viral infection.

These necessitate the use of chemical therapy in some cases (Doublet *et al*., 2015; Evans & Schwarz, 2011; Fünfhaus *et al*., 2018). Bacterial infections are often treated using antibiotics, which after long-term exposure promotes the accumulation of resistance genes in the bees (Tian *et al*., 2012).

2.2 Effects of Chemical Use in Treating Honeybee Diseases

Agrochemicals exposure to lethal levels and the use of chemical therapy like the administration of antibiotics to cure bee antibacterial diseases have been documented to have grievous effects to the honeybee health and its products. For example, pesticides act as stressors which immunocompromise the bees making them an easy target for pathogens while antibiotics affects the gut microbiome making it incapable of conducting its role in disease prevention (Al-Waili *et al*., 2012; Mullin *et al*., 2010; Raymann *et al*., 2017; Tian *et al*., 2012).

2.2.1 Pesticide Effect on Honeybee Health

Pesticides is a broad term used to refer to harmful biological or chemical agents used to kill, repel or prevent destructive or unwanted pests. They include; herbicide, insecticides (e.g., neonicotinoids, organophosphates, and pyrethroids), fungicides, acaricides, and rodenticides (Collison *et al*., 2016; vanEngelsdorp & Meixner, 2010). They are commonly used in agricultural practices to prevent pests from damaging crops. Honeybees are exposed to pesticides in multiple ways including foraging on sprayed crops and in-hive spraying to manage bee pests or control pathogen infection.
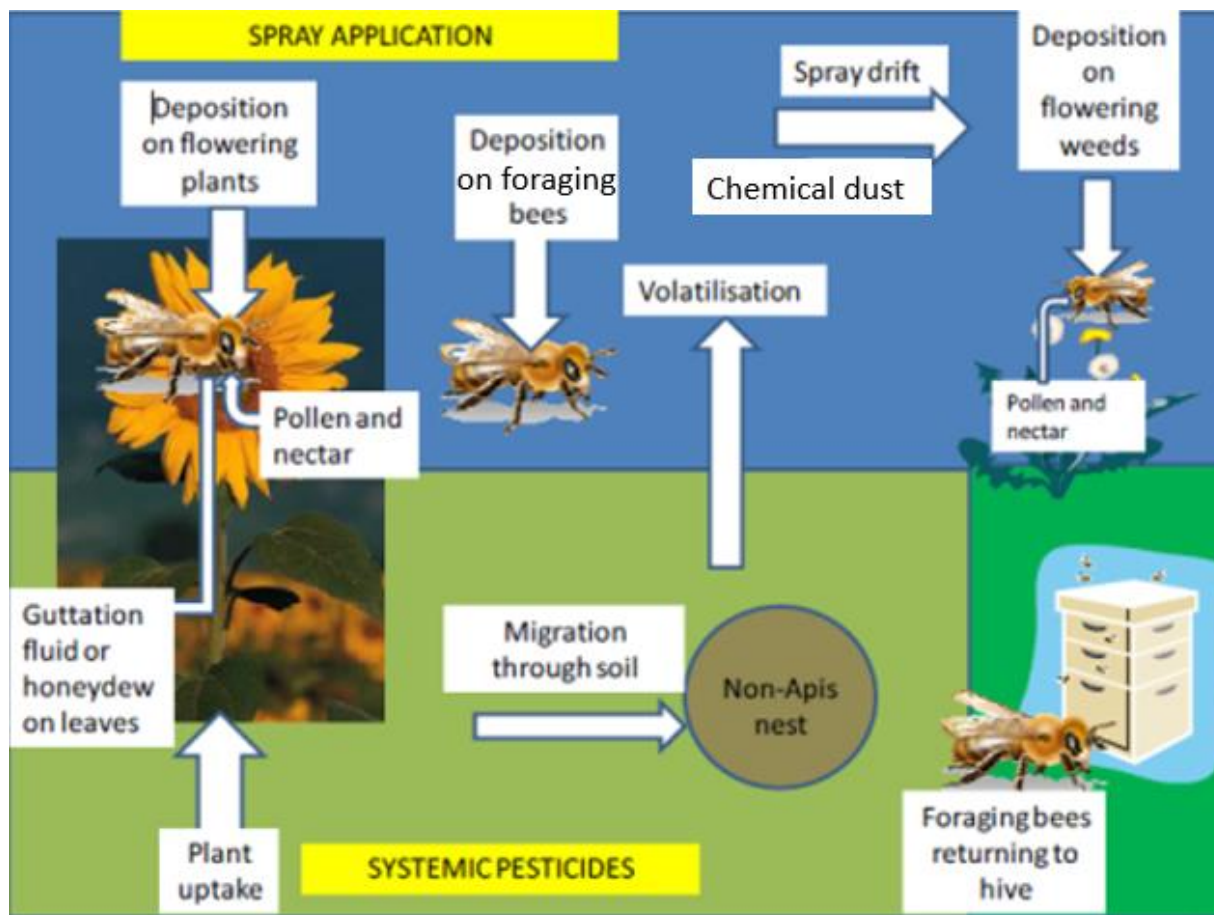
Figure 9: An illustration of the multiple ways bees are exposed to pesticides modified from (Johnson & Lynne, 2015).

Pesticides are composed of the active ingredient and other components designed to optimize their efficiency. They have adjuvants to enhance the pesticide's toxicity and effective delivery of the chemicals to the target pest population (Johnson & Lynne, 2015; Pettis *et al*., 2012). Many of the pesticides developed for agricultural use have been documented to be sub-lethal and have deleterious effects to honeybees when administered above the lethal dose.

Pesticides harm bees in numerous ways; their toxicity reduces bees' survival or kills them, and less severely, they cause immune suppression making bees vulnerable to pests and pathogens (Grassl *et al*., 2018). Studies have recorded increased level of pathogen loads and ultimate mortality when diseased bees are exposed to pesticides (Doublet *et al*., 2015; Evans & Schwarz, 2011; Pettis *et al*., 2012).

In addition, pesticides are also linked to disrupting the core gut microbiota of honeybees responsible for triggering immune response during infection (Collison *et al*., 2016). These studies show that exposure to pesticides exacerbates the effects of pathogens and pests on honeybee health. Honeybees have experienced a more adverse effect during stressor synergies when pest and pathogens co-occur with exposure to pesticides unlike the former occurring individually (Collison *et al*., 2016; Lecocq *et al*., 2016; Pettis *et al*., 2012). Besides acting in tandem with other stressors to impair the bees' immunity, pesticides also have sub-lethal effects to the bees which is often observed in change of individual and social immunity behavior. These include; memory loss, reduced communication, navigation and orientation abilities, disrupted motor activity and foraging behavior, reduced fertility impairing reproduction and development, and impaired sensory detection and learning behavior (Johnson & Lynne, 2015).

2.2.2 Effects of Antibiotics in Treating Honeybee Bacterial Infections

Antibiotics are medicine used to prevent or treat the bacterial infection by impeding their growth (bacteriostatic) or killing the bacterial cells (bactericidal). Honeybees are susceptible to bacterial disease such as AFB and EFB which are contagious, destructive, and prevalent globally (Forsgren, 2010; Genersch, 2010; Hansen & Brødsgaard, 1999; White, 1920). These diseases affect the honeybee brood reducing the colony's population, and reducing its activity and productivity (Hansen & Brødsgaard, 1999).

Broad spectrum antibiotics like tetracycline targeting gram-positive and gram-negative bacteria and others such as tylosin, tartrate lincomycin, and tylosin have been used to prevent and treat honeybee pathogenic bacterial diseases. Despite some success with the treatment, there has been recorded emergence of detrimental effects of antibiotic use to the honeybee health, behavior and survival (Ortiz-Alvarado *et al*., 2020; Raymann *et al*., 2017). In a study evaluating the effect of antibiotic toxicity on the honeybee larvae, Pettis *et al*. (2004) administered oxytetracycline (200 mg in 20 g sugar) which is the concentration used in hives, to a treatment and control group. Compared to the control group, the antibiotic treated brood experienced approximately 80% brood mortality. Exposure to antibiotic for an extended duration has also been recorded to cause undesirable consequences including, reduced colony fitness (Bulson *et al*., 2021).

Regarding behavioral changes, exposure of worker bees to antibiotics during the larval and pupae stages had an influence in their age dependent roles (sanitation to nursing to foraging) with some transitioning earlier and others delayed depending on the onset of antibiotic exposure (Ortiz-Alvarado *et al*., 2020).

The gut microbiome, plays a crucial role in insect health. Honeybees gut microbiome, which spread out through the entire bees' gut from the crop to hindgut, is extensively studied and characterized to have highly specialized community members. It comprises of approximately nine bacterial species (95% of the members), five of which are prevalent in most bee species (Kwong & Moran, 2016). The honeybee gut also harbors potentially harmful but often docile bacteria like the opportunistic *S. marcescens* existing in low relative abundance. Synonymous to the human gut microbiome, the honeybee gut microbiome is acquired socially from the nurse bees during feeding of the larvae (Kešnerová *et al*., 2020; Kwong & Moran, 2016; Raymann & Moran, 2018; Steele *et al*., 2021). Contemporary studies have outlined the roles of a healthy gut microbiome in honeybees which extends to growth and development, immune function, behavior, survival, metabolism regulation, and protection against pathogens (Raymann & Moran, 2018). Administration of antibiotics perturbs the honeybee microbiome disrupting its community size and composition, which causes inhibition of immunity allowing proliferation of bacterial pathogens (Bulson *et al*., 2021; Ortiz-Alvarado *et al*., 2020; Raymann *et al*., 2017).

A recent study has unraveled new evidence showing that the use of antibiotics destroys the gut microbiome making the bees susceptible to *Nosema* infection. Antibiotic treatment co-occurring with stress from the *Nosema* infection significantly reduced honeybee survival (Li *et al*., 2017). Some broad spectrum antibiotics are non-selective and negatively affect key members of the honey bee microbiome inducing dysbiosis and affecting the microbiome's size and composition and its role in immunity and disease prevention. Dysbiosis affects the host-microbiome interaction allowing opportunistic bacteria in the gut microbial community to parasitize the bees (Bulson *et al*., 2021; Li *et al*., 2017; Ortiz-Alvarado *et al*., 2020; Raymann *et al*., 2017; Raymann & Moran, 2018). Emergence of resistance to antibiotics due to prolonged exposure is one of the major effects of antibiotics use in apiculture. Antibiotic resistance occurs when organisms, in this case bacteria, develops counter measures to resist the mode of action of an antibiotic.

That mostly happens through genetic mutations of gene(s) which are passed down the generation (Woolhouse *et al*., 2015). The resistance genes can also be exchanged horizontally or acquired from other bacteria via a non-genomic route. Such is the case for (*tetL*) gene responsible for resistance in *P. larvae* the bacteria responsible for AFB. This gene has an identical sequence to another resistance gene in honeybee gut symbionts indicating the gene's source (Evans, 2003; Raymann *et al*., 2017).

Bacteria often develops resistance to a particular antibiotic after prolonged exposure (Woolhouse *et al*., 2015). Apiculture has been affected by the invasion of bacterial pathogens causing diseases such as AFB and EFB, that necessitated the adoption of antibiotics to prevent or manage infection in bee colonies (Genersch, 2010; White, 1920). In multiple countries globally including the United States (US), bee keepers have been using Oxytetracycline for almost the last five decades and more recently since 2006 they have adopted the use of lincomycin and tylosin (Krongdang *et al.*, 2017; Raymann *et al*., 2017). As a result of such prolonged use, antibiotic resistant strains of *P. larvae* have continually emerged from these countries.

In a study monitoring the emergence of tetracycline resistance genes in the US for the last 25 years compared to countries not using antibiotics, Tian *et al*., (2012) found eight tetracycline resistance genes (*tetY, tetM, tetB, tetW, tetC, tetH,tetL, tetD*) ubiquitous in the American samples and rare in the unexposed honeybee colonies. Antibiotics usage can affect the non-target microbes beneficial to the host enhancing the pool of resistance genes accessible to the pathogenic microbes and alter microbe's community profiles. As a consequence, honeybees' gut microbiome harbor a diverse accumulation of resistance genes compared to bumblebees, which are less exposed to antibiotics (Li *et al*., 2021; Raymann *et al*., 2017; Tian *et al*., 2012).

2.3 Honeybee Diet as an Alternative to Antibiotics Use

Honeybee diet quality is a chief contributor to individual and colony health and fitness. Honeybees are florivorous herbivores and their dietary needs vary along their developmental stages and is role oriented. That means, the larvae, queen and adult bees each are fed on diets enriched with nutrients to assist them in development and perform their roles effectively (Nicolson, 2011). Honey, pollen, royal jelly and water comprise the bees' diet. While pollen contribution to the bees' health has been extensively studied, honey is also gaining popularity as bees' nutraceutical food. Pollen is a source of proteins, which is required by bees during development, multifloral pollen from a diverse

flora is preferred by honeybees compared to a monofloral pollen. Polyfloral pollen has been linked to increased honeybee social and individual immunocompetence by increasing their glucose oxidase activity (Alaux *et al.*, 2010). Effect of variation of pollen diversity and quality in combination with biotic stressors to the honeybee health has shown increased fitness, reduced pathogen load and enhanced survival when the bees were fed on quality multifloral pollen indicating that diet plays a role in infection therapy in honeybees (Rinderer & Elliott, 1977).

Consequently, honey, provides energy to honeybees for the demanding activities like foraging. Adult foragers have a honey rich diet to supply them with the energy required for the foraging flight. Despite its nutritional value, though in a smaller percentage compared to sugars, honey is composed of biologically active exogenous constituents such as secondary plants metabolites, and is also enriched with endogenous substances like antimicrobial peptides which enhance the bees' health (Berenbaum & Calla, 2021; Erler & Moritz, 2016). Honey has extensive medicinal applications due to its antibacterial activity conferred to it by its high sugar saturation which gives it a high osmolarity in addition to the antibacterial secondary plant metabolites such alkaloids, phenolics and flavonoids (Nicolson, 2011). Secondary plant metabolites are a key indicator of honey's prophylactic and therapeutic ability against bacterial pathogens due to the change in foraging patterns and flower preference observed on healthy and diseased bees infected with bacterial pathogens as they seek to self-medicate (Berenbaum & Calla, 2021; Roode & Hunter, 2019; Simone *et al.*, 2009).

2.4 Honey Processing, Composition and Classification

Honey is a natural and nutritious sweetener produced by bees via the processing of nectar obtained from plants (Berenbaum & Calla, 2021; Rao *et al.*, 2016). The nectar is stored in the crop of the bee where it undergoes enzymatic digestion by sucrase, which breaks down the sucrose in nectar into glucose and fructose for enhanced saturation. In the hive, the bees regurgitate the nectar on their proboscis increasing the surface area and enhancing evaporation of water from the nectar to concentrate it. Additionally, other worker bees fan the air in the hive to promote circulation and enhance further evaporation. This process results in a supersaturated solution composed of averagely 80% concentrated sugars – honey, which is stored in the combs and capped for later consumption (Cornara *et al.*, 2017).

During the processing of honey the bees also add endogenous substances like antimicrobial peptides and enzymes such as glucose oxidase, which oxidizes glucose to hydrogen peroxide and gluconic acid, which is among the acids responsible for honey's low acidic pH (Berenbaum & Calla, 2021; Nicolson, 2011). Honey has a high nutraceutical value due to the presence of phytochemicals, hydrogen peroxide, bee secretions and saturated sugars (Pasini *et al*., 2013). It majorly consists of carbohydrates in different forms of sugars and water in abundances of approximately (60-85%) and (12-23%) respectively.

Additionally, it contains other substances in small proportions. These are; vitamins, minerals (potassium, phosphorus, magnesium sodium, calcium, chlorine, and sulfur) hydrogen peroxide, amino acids (proline), proteins, added enzymes (invertase, diastase, sucrase, catalase, acid phosphatase, and glucose oxidase), organic acids (citric, acetic, and formic acids), and volatile compounds (Table 2). Additionally, it contains bioactive substances such as phenolic acids (ferulic, caffeic, gallic, *p*-coumaric, and ellagic acids), alkaloids, glycosides, anthraquinones, terpenoids, flavones and flavonoids (quercetin, chrysin, tectochrysin, pinocembrin, galangin, kaempferol, apigenin, and hesperetin), antioxidants (ascorbic acids, tocopherols, and superoxide dismutase) among others (Berenbaum & Calla, 2021; Erler & Moritz, 2016; Pasini *et al.*, 2013; Rao *et al*., 2016; Samarghandian *et al*., 2017; Santos-Buelga & González-Paramás, 2017).

The constituents of honey have varying abundances depending on the floral origin, climatic condition, and the harvesting and processing seasons and conditions, respectively (Castro-Vázquez *et al*., 2010). Honey is further classified depending on the floral nectar sources. This yields four classes of honey; unifloral honey made from nectar obtained from a single floral source, polyfloral honey made from nectar derived from multiple flowering plants, honeydew honey from excretions of insects that feed on plants or plant exudates, and blossom honey obtained from the nectar of flowers (Olga *et al*., 2012).

Table 2: Average composition of honey (Santos-Buelga & González-Paramás, 2017)

Component	Average abundance by mass in 100g
Carbohydrates	82.4 g
Fructose	38.5 g
Glucose	31 g
Sucrose	1 g
Other sugars	11.7 g
Dietary fiber	0.2 g
Fat	0 g
Protein	0.3 g
Water	17.1 g
Riboflavin (Vit. B$_2$)	0.038 mg
Niacin (Vit. B$_3$)	0.121 mg
Pantothenic acid (Vit. B$_5$)	0.068 mg
Pyridoxine (Vit. B$_6$)	0.024 mg
Folate (Vit. B$_9$)	0.002 mg
Vitamin C	0.5 mg
Calcium	6 mg
Iron	0.42 mg
Magnesium	2 mg
Phosphorus	4 mg
Potassium	52 mg
Sodium	4 mg
Zinc	0.22 mg

2.5 History of Medicinal Property of Honey

Honey has been in use for its nutritional, cosmetic and medicinal properties since 5500 years ago by the Greeks and the Egyptians. It is recorded in the religious history of Islam to have been ordained by God to offer therapeutic services to humanity (Nicolson, 2011; Pasini *et al*., 2013; Samarghandian *et al*., 2017). It has since demonstrated excellent prophylactic and therapeutic measures against multiple animals and human diseases in traditional and modern medicine (Figure 10).
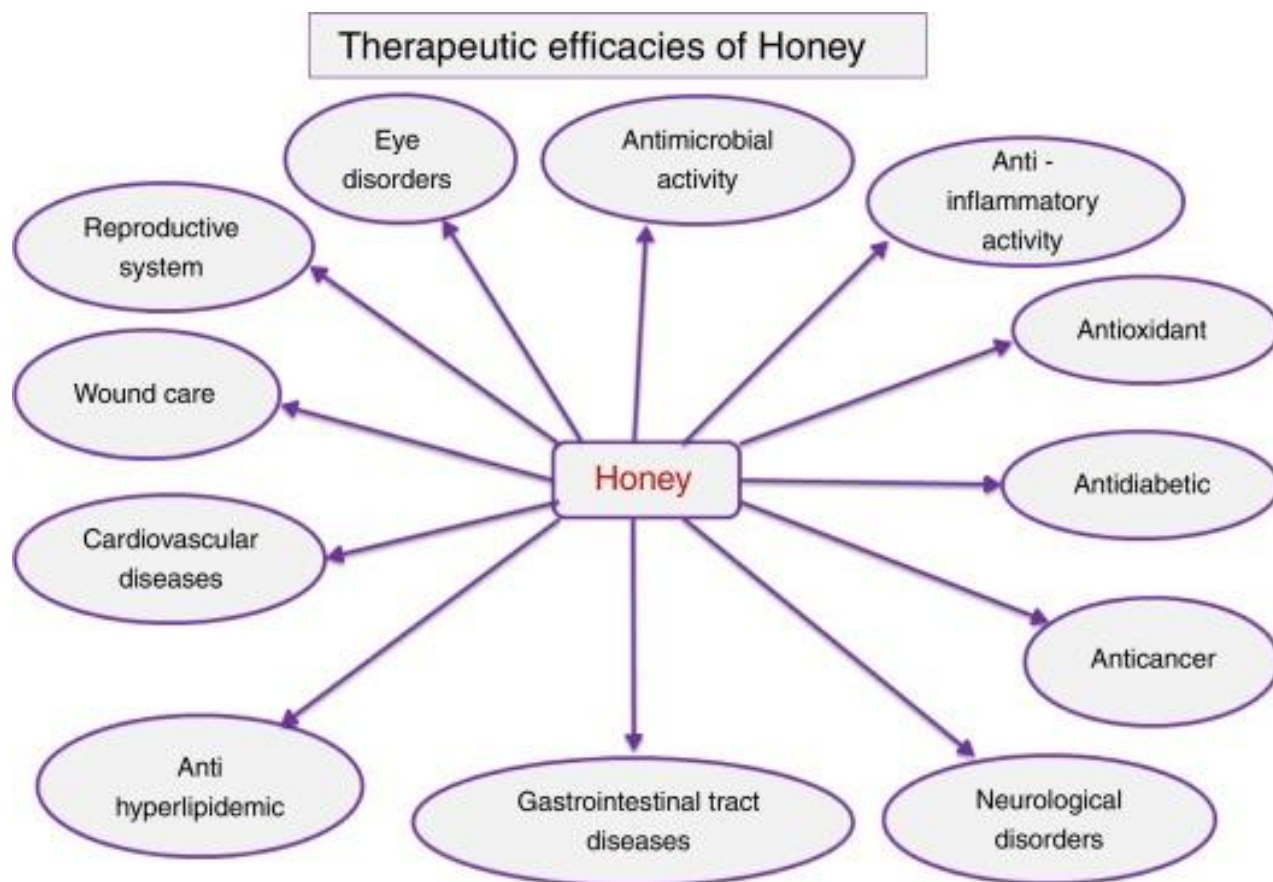
Figure 10: Therapeutic efficacies of honey modified from (Rao *et al*., 2016)

Over time, honey has been extensively studied to determine its bactericidal, virucidal, and fungicidal mechanisms, which gives it dominance over pathogenic microbes. Moreover, these mechanisms prevent the development of resistance against it due to the multiple components in different abundances responsible for its antimicrobial properties (Dixon, 2003; Nicolson, 2011; Santos-Buelga & González-Paramás, 2017).

It has been established that the main components giving honey its medicinal properties include; hydrogen peroxide, high osmolarity, low acidic pH, low water activity, methylglyoxal (MGO) in some honey, defensin-1, and the presence of phytochemicals, and secondary plant metabolites. All these work independently or synergistically to give honey its nutraceutical properties (Johnson & Lynne, 2015; Jończyk-Matysiak *et al*., 2020; Mandal & Mandal, 2011; Nicolson, 2011; Samarghandian *et al*., 2017). These recent scientifically established properties of honey has led to extensive exploration of the medicinal properties of honey and other bee products (Samarghandian *et al*., 2017).

2.6 Antibacterial Property of Honey

Honey has demonstrated excellent antiseptic action against pathogenic microbes; bacteria, viruses and fungi. It contains chemical and bioactive constituents that work via an intricate mechanism to achieve the excellent nutraceutical property (Cornara *et al.*, 2017). Extensive *in vitro* studies have been conducted on multiple types of honey from varied geographical regions to unravel the elements responsible for its antimicrobial (Dixon, 2003; Johnson & Lynne, 2015; Nicolson, 2011; Pasini *et al.*, 2013). The following constituents have a significant contribution to the antiseptic property of honey; hydrogen peroxide produced from the oxidation of glucose is responsible for sterilization. Additionally, honey's osmolarity due to high sugar concentration provides a harsh environment for microbes which are not osmotolerant leading to their death by exosmosis or plasmolysis (Berenbaum & Calla, 2021).

Moreover, honey has a high carbon to nitrogen ratio, low redox potential due to high levels of reducing sugars, high viscosity and low water activity that limits dissolved oxygen for utilization by aerobic microbes. Honey's low acidic pH is due to presence of gluconic and other organic acids which create an unsuitable environment for the residence and growth of most bacteria that are neutrophiles. Phytochemical substituents like the antibacterial methylglyoxal (MGO's) found in Manuka and other honey and the anti-pathogenic bioactive peptide defensin-1 introduced by the bees enhance honey's antibacterial activity (Berenbaum & Calla, 2021; Nicolson, 2011). These components work concertedly against life-threatening and multidrug-resistant (MDR) microbes to ameliorate the bacteriostatic and bactericidal effects of honey.

Honeybee honey has portrayed effective antibacterial action at low minimum inhibitory concentrations (MIC) against pathogens like *E. coli, Salmonella typhimurium, Staphylococcus aureus (S. aureus)* and also against antibiotic-resistant and sensitive species like the methicillin-resistant *S. aureus* (MRSA) and *Klebsiella pneumonia* (Molan, 1992; Rao *et al.*, 2016). Tualang' honey from Malaysia and Manuka honey from New Zealand are among the extensively studied and characterized honeybee honey and their antibacterial activity ascertained and approved for medical use. Stingless bee honey from Costa Rica (Gabriel *et al.*, 2015) has also demonstrated antibacterial activity against clinical isolates of resistant *Pseudomonas aeruginosa* and MRSA, further cementing the antibacterial property of honey against some resistant strains.

Additionally, in-depth investigation into the chemical profile of honey with proven antimicrobial potential has been done and the compounds responsible for the activity identified and characterized. Such compounds include the following isolated from a hexane extract fraction of Goldcrest honey, which recorded the best antibacterial activity against a resistant strain of *Helicobacter pylori*; acetic acid, 5-hydroxymethylfurfural, 2-propanone, butanal, 1,3-benzenediamine, propanenitrile, 2-furanmethanol, propanoic acid, 1,3-butanediol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4Hpyran-4-one, and 1-(1-cyclopentenyl)-1-propanol. The entire chemical profile included compounds from diverse chemical classes; pyran and furan derivatives, aliphatic acids, ketones, alcohols, benzene compounds and hydrocarbons (Manyi-Loh *et al*., 2012). Another study by Samarghandian *et al*. (2017) also identified the antibacterial compounds present in honey which included; pinocembrin, terpenes, benzyl alcohol, 1,4-dihydroxybenzene, syringic acid, 3,4,5-trimethoxybenzoic acid, and methyl-3,5-dimethoxy-4-hydroxybenzoate among others. Honey volatile profiles are equally as important as they infer the volatile antibacterial compounds available in honey.

The multiple sources for the volatiles compounds available in honey include: nectar, environmental or microbial contamination, transformation of plant compounds via the bees' metabolism, or honey processing and storage. Volatile organic compounds (VOCs), especially aromatic compounds, are often linked to honey's aroma and geographical origin determination (Manyi-Loh *et al*., 2011) but the possibility of using the volatile profiles to identify potential antibacterial compounds is often overlooked. During foraging, the bees are guided by the colors of flowers, however, in proximity, odor (volatiles) play a role in the selection of resources to forage on depending on the colony's nutritional needs, i.e. when healthy or diseased. Therefore the volatile profiles of honey are also a pointer toward the bioactive compounds responsible for their antibacterial activity (Manyi-Loh *et al*., 2011, 2012).

Over 600 honey volatile compounds belonging to multiple chemical classes have been reported in the literature to date. The chemical classes include: terpenes, benzene, furan, pyran and their derivatives, alcohols, norisoprenoids, esters, acids, ketones, aldehydes, hydrocarbons and cyclic compounds (Barra *et al*., 2010). some of the compounds identified in honey volatile profiles have antibacterial activity when tested in vitro, indicating that honey volatiles which are majorly derived from nectar are an important indicator of the compounds responsible for the honey's antibacterial activity (Barra *et al*., 2010; Karlıdağ *et al*., 2021; Manyi-Loh *et al*., 2011, 2012).

Honey has introduced a novel, improved, and highly effective approach to the treatment of persistent and resistance bacterial pathogens (Fratellone *et al*., 2016). Though much of the antimicrobial studies have been *in vitro*, there is a need for more scientific inquiry into the *in vivo* mechanisms and antimicrobial efficiency of honey with a clear understanding of the honey – microbiome, honey – host, and honey – pathogen interactions.

2.7 Self-medication in Honeybees

With the continued emergence of antibiotic resistant pathogenic bacterial strains due to extended exposure, natural alternative therapies should be explored to facilitate the discontinuation of antibiotic use. When insects are predisposed to pathogens, structural defense like the cuticle prevent infection. But, when the structural defense is breached, the immune system steps in to clear the infections by encapsulation, melanization, or producing antimicrobial peptides (Abbott, 2014). However, if the immune system is overwhelmed, insects turn to self-medication. Self-medication is commonly described as the use of organic substance(s) either by ingestion, topical application, or absorption to treat infection or reduce the severity of its symptoms (Roode *et al*., 2013; Simone *et al*., 2009). However, for a feeding behavior to be classified as self-medication, a set of four conditions must be satisfied (Figure 11). One, the organism must deliberately contact the bioactive substance. Two, the substance must be deleterious to the pathogen affecting the organism. Three, the harmful effect on the pathogen must lead to enhanced host fitness, and finally the substance must have adverse effects to the organism in absence of the pathogen and if ingested in more quantity than when diseased (Clayton & Wolfe, 1993).
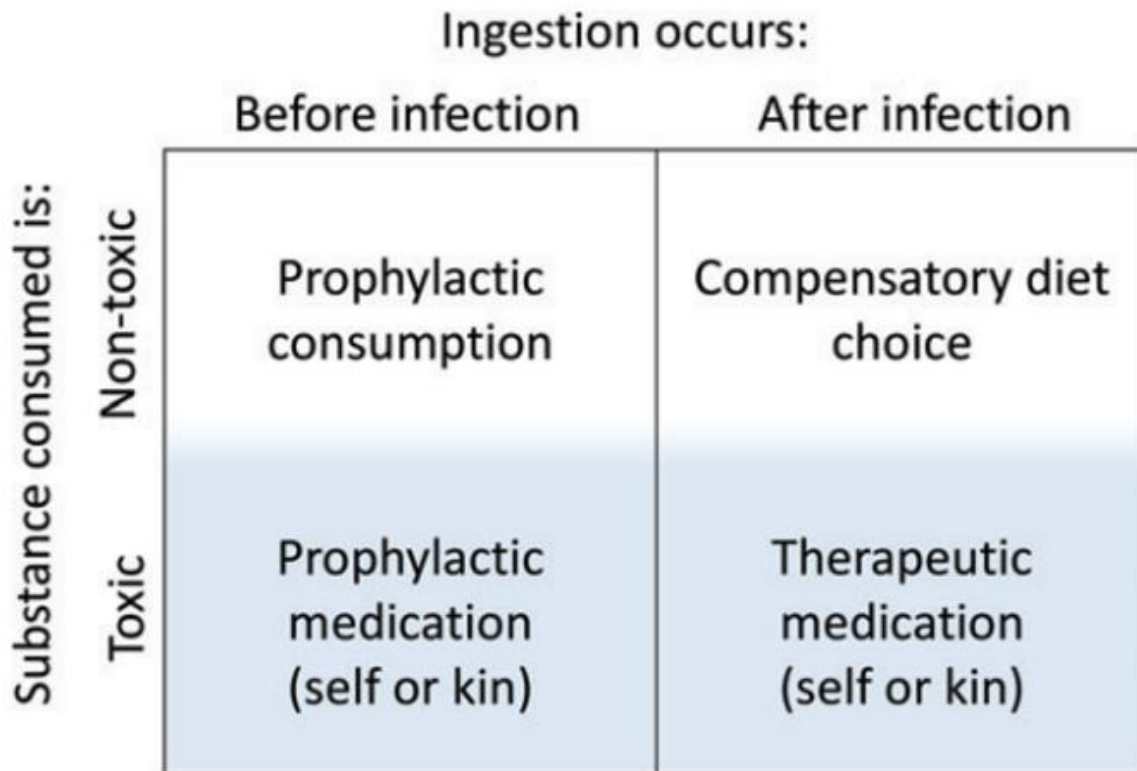
Figure 11: Conditions for self-medication and a criteria of differentiating it from other feeding habits *source* (Abbott, 2014)

Self-medication can also be conducted qualitatively by consuming new dietary substance or quantitatively by increasing the consumption of a substance pre-existing the diet (Abbott, 2014; Simone *et al.*, 2009). In honeybees, self-medication is best demonstrated by the ability of worker bees to contact bioactive components available in various floral nectar in response to an infection or to suppress its symptoms. The intake of secondary plant metabolite compounds can be initiated as a prophylactic measure when the colony is at risk of disease or as a therapeutic measure when the colony is already infected (Simone *et al.*, 2009).

Bees have evolved behaviorally and developed novel ways to complement their innate immunity by selectively altering their dietary preferences and foraging on plants producing nectar containing antimicrobial bioactive components to combat infection and enhance their individual and social immunity. Increased collection of resin when facing a fungal infection is an excellent illustration of self-medication of honeybees with plant resins (Simone-Finstrom & Spivak, 2012).

The plant metabolites exhibiting antimicrobial properties are highly specific to their mother plants and the strain of pathogen they are effective against. Hence, bees have been observed to take advantage of the floral diversity to counteract mutation of the pathogens which often leads to resistance (Pasini *et al*., 2013). This phenomenon is what differentiates the prophylactic and therapeutic abilities of different honey sampled from varied geographical locations since they have diverse bioactive phytochemical compositions due to the biodiversity of floral sources present in the various region.

2.7.1 Honey for Self and Keen Medication in Bees

Flora provides a 'natural pharmacy' to insects, while biodiversity ensures all the needed bioactive components are available in nature for use by the insects in combating diseases. Social insects like bees collect food for themselves, nest mates, and brood. In these highly intricate and organized colonies, division of labor is implemented to deal with the available workload adequately. Foragers bees are obligated to source food with nutritional components and medicinal value for the colony and brood (Poissonnier *et al*., 2018). Nutrition maintains colony fitness, which subsequently promotes colony hygiene, ensuring the emergence of high-quality and healthy adult bees for the colony.

Nutritional geometry indicates that forager bees ingest bioactive components when infected and also alter nutritional intake to alleviate infection in the brood or nest mates (Abbott, 2014; Poissonnier *et al*., 2018; Rao *et al*., 2016). Studies conducted in social insects have shown strong interdependence in the colony and a synergetic working condition against pathogenic infection. Insects self-medication is practiced for self and keen (Abbott, 2014; Roode & Hunter, 2019). Insects trade nutrition for survival by altering their foraging preferences when the brood infected. Bees forage on compounds outside their dietary composition while healthy and increase the intake of a portion of their daily diet. This change in consumption pattern reveals that the new diet helps reduce the virulence of the infection as it contains active antimicrobial components that help to restore the bees' health (Abbott, 2014; Erler & Moritz, 2016; Jończyk-Matysiak *et al*., 2020; Simone *et al*., 2009; Simone-Finstrom & Spivak, 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents, Equipment, and Analytical Instruments

Analytical grade solvents and chemicals were used in this research unless when stated otherwise. The chemicals and solvents included; Sodium nitrate ($NaNO_3$), distilled, deionized and nuclease free water, sodium hydroxide (NaOH), aluminium chloride ($AlCl_3$), Phenol, 5X Hot firepol evagreen, gallic acid, sodium hydroxide (NaOH), Folin-Ciocalteu's phenol reagent, Chloroform, quercetin. Additional chemicals included; Ammonium acetate (NH_4Ac), Ethanol (EtOH), Sodium hypochlorite (bleach), sucrose, sodium carbonate (Na_2CO_3), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Muller-Hinton Agar (MHA), Muller-Hinton Broth (MHB), bacteria, primers, sulphuric acid (H_2SO_4), hydrochloric acid (HCl), phosphate saline buffer (PBS), and dichloromethane (DCM). These reagents and chemicals were sourced from Sigma-Aldrich Kenya and Kobian Kenya Ltd.

The analytical instruments and other equipment used for the study comprised of; an incubator (Thermo Fisher Scientific, USA), incubator shaker (Eppendorf Innova 40, New Brunswick Scientific, Germany), TissueLyzer II (QIAGEN, Germany), vortex (Jenway, UK), centrifuge (5860R, Eppendorf, Germany), UV-Vis spectrophotometer (Jenway 6850, UK), pH meter (Jenway 3540, UK), and Quant studio 3 Real Time PCR 96-Well 0.2 mL Block (Applied Biosystems by Thermo Fisher Scientific, USA). Additionally, the following equipment and instruments were also used; Nanodrop (Thermo Fisher Scientific, USA), 2 mm Zirconia Ceramic Beads (BioSpec Products, USA), Handheld refractometer (Atago, Tokyo, Japan), Air Cadet Vacuum/Pressure Station (Cole-Palmer Instruments, USA), 2 mm Zirconia ceramic beads (BioSpec Products, USA), Flow rate regulator (Gometrics, Barcelona – Spain), Gas chromatograph (GC-((HP-7890, Agilent, USA, coupled with a Mass Spectrometer (MS59-7, Agilent, USA), PoraPak-Q 80-100 mesh 30 mg adsorbents (Sigma-Aldrich Scientific, USA), and Portable volatile extraction pump (Sigma-Aldrich Scientific, USA). Glassware and laboratory consumable were procured from Kobian Kenya Limited.

3.2 Study Area and Sampling

Honey samples were obtained from six geographical locations in Kenya; Arabuko Sokoke, Baringo, Kakamega, Karura, South Kitui and Taita Hills in 2020 (Figure 12; Table 3).

South Kitui and Arabuko Sokoke honey were procured from the local bee keeping community market. The remaining honey were extracted from honeycombs *in situ,* packaged in sterilized labeled glass bottles, kept in a cooler box and transported to the African Reference Laboratory for Bee Health located at the International Centre for Insect Physiology and Ecology (*icipe*), Duduville campus, Kenya. The samples were stored at -4 °C before being analysed and used in subsequent experiments.
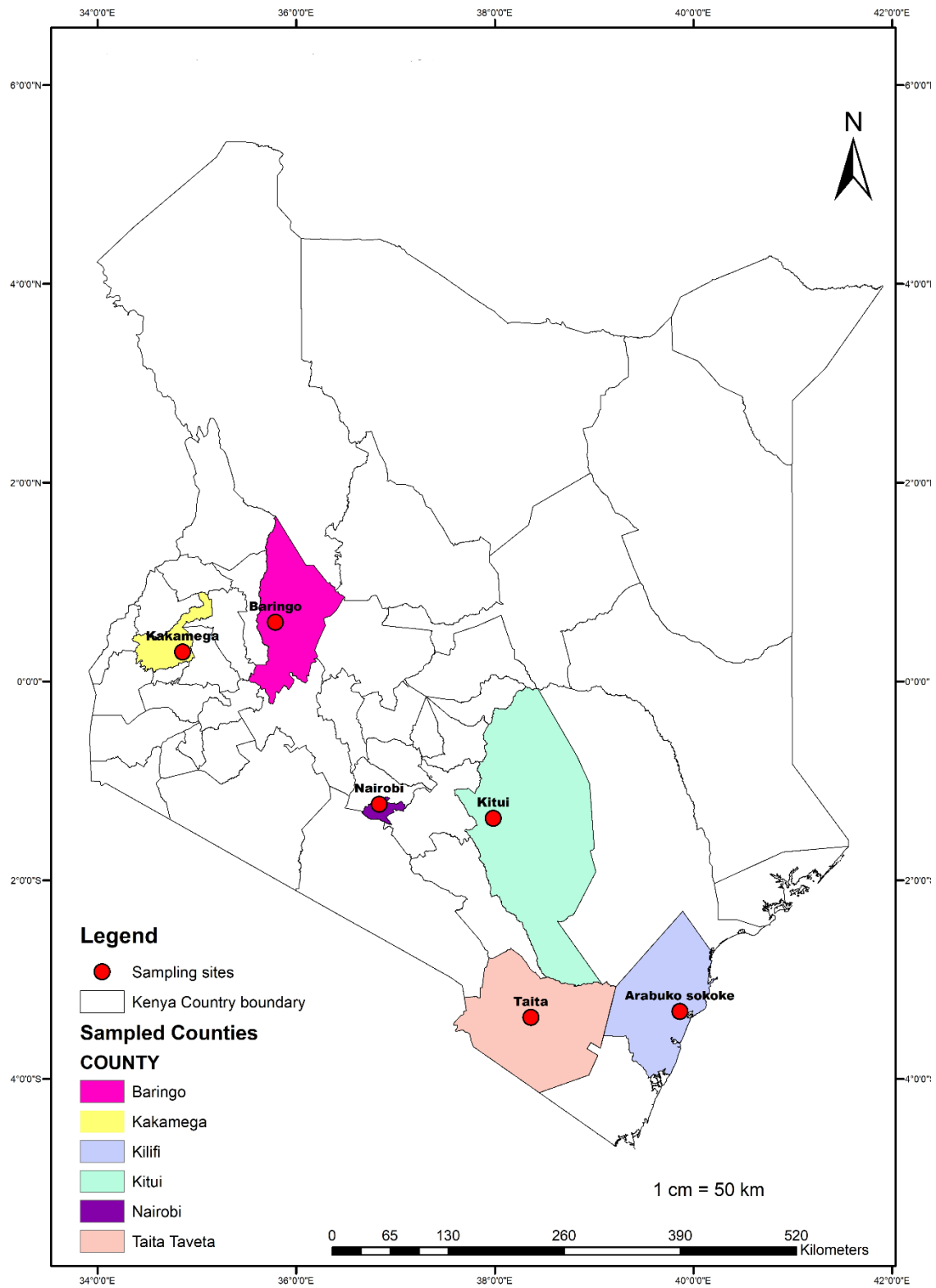
Figure 12: Geographical sampling sites in Kenya.

Table 3: Sampling locations coordinates

Sample Area	Latitude	Longitude
Karura	-1.234420	36.834790
Baringo	0.59514	35.78962
Kakamega	0.2963	34.85531
South Kitui	-1.37588	37.98406
Arabuko Sokoke	-3.32079	39.8635
Taita Hills	-3.38003	38.36174

3.3 Prophylactic and Therapeutic Antibacterial Potential of Honey

The two experimental treatments (prophylactic and therapeutic) involved two bacteria (*Escherichia coli* and *Serratia marcescens*), six sampled honey and Manuka 5+ honey (positive control) and 30% sucrose solution (negative control). Manuka honey was chosen as a positive control as it has been extensively studied and characterized. It has methylglyoxal which is highly antibacterial making it an excellent honey choice for a positive control. Conversely, 30% sucrose solution was selected as the negative control as it supplements the bees' diet by providing them with carbohydrates but, does not contain the antibacterial properties available in honey.

3.3.1 Bacteria Culture and Pre-inoculation Quantification

Non-fastidious and gram-negative bacteria *Escherichia coli* [Escherichia coli ATCC® 25922™] obtained from *icipe*'s Arthropod pathology unit and *Serratia marcescens* extracted from the bee gut and sequenced for accurate identification were cultured on MHA. A single pure colony forming unit (CFU) was obtained and re-cultured aerobically in MHB at 37 °C for 24 hours. Each bacteria-laden broth was put into 2 mL Eppendorf tubes and centrifuged at 3,234 rcf to obtain the bacteria and the supernatant was discarded. The bacteria were diluted to 3 mL with distilled, deionized nuclease-free sterile water and further serially diluted. The bacteria serial dilution was done by measuring the optical density (OD) or absorbance of the dilutions (DF); 5, 10, 15, 20, 25, 30, 35, 40 (from the 3 mL stock) with a double beam UV-Vis equipment at a wavelength of 600nm.

That was done to obtain a turbidity of 0.5 McFarland, which is an approximate equivalent of the recommended bacterial inoculation concentration of 1×10^8 CFU/mL. This concentration has a corresponding absorbance of approximately 0.132 based on the Agilent Genomic bacteria concentration calculator and as described by Kuś *et al*. (2016).

3.3.2 Bee Rearing Conditions

Pre-emergent pupae in a comb attached to a frame (Figure 13A) were obtained from the *icipe's* Environmental Health Theme apiary hive 'CAB 210' at Duduville campus, Nairobi, Kenya. The frame was incubated in the laboratory at 34.5 °C in the dark with 70% relative humidity for 24 h for sterile adult bees to emerge (Thorburn *et al*., 2015). Emerged adult bees were collected and distributed to 5 cm length by 5 cm width by 3 cm height rearing plastic cages (Figure 13B) and kept under standard conditions of 34 °C and 60-70% relative humidity in an incubator for the prophylactic and therapeutic treatments.

Figure 13: (A) A frame with capped brood obtained from the hive for incubation to hatch into young adult bees (B) a sample of the bee rearing cages.

3.3.3 Prophylactic Potential of Honey Treatment

Emerged adult bees were placed in eight different cages each containing 30 bees. In six of the cages, bees were fed on the different sampled honeys. In the positive control cage were fed on Manuka 5+ honey and the negative control cage were fed on 30% sucrose solution for 3 days. On the commence of the 4th day, the bees were starved for one hour then inoculated with bacteria (*E. coli* and *S. marcescens* in the separate setups) through their new diet of 30% sucrose solution. From each of the eight cages three bees were sampled 24 h, 48 h, and 96 h post-inoculation and pooled to make a sample. That was done for both bacteria. The sampled bees' guts were extracted and stored in PBS at -20 °C awaiting DNA extraction and quantification.
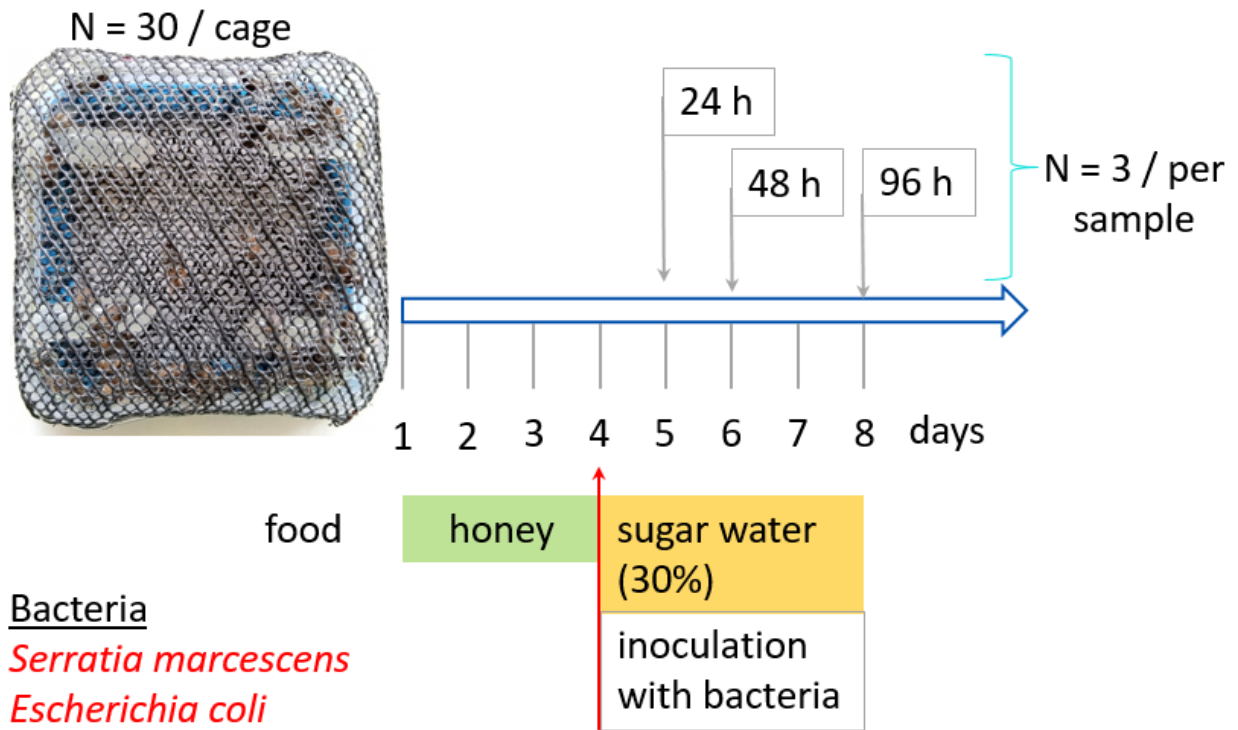
Figure 14: Schematic representation of the prophylactic experiment

3.3.4 Therapeutic Potential of Honey Treatment

Emerged adult bees were placed in eight different cages each containing 30 bees. In each of the cages, bees were fed *ad libitum* on 30% w/v sucrose solution inoculated with bacteria for four days to allow the bacteria to fully colonize the bees' gut as described in (Tesovnik *et al.*, 2020). On the commence of the 5th day, the bees were starved for an hour and their diet changed to the six types of honey in six different cages.

Manuka 5+ honey was used as a positive control and 30% non-inoculated sucrose solution as a negative control. Three individual bees were sampled 24 h, 48 h, and 96 h after the change of diet from the 8 cages and pooled to make a sample. That was done for the two bacterial strains. The sampled bees' gut were extracted and stored in PBS at -20 °C awaiting DNA extraction and quantification.
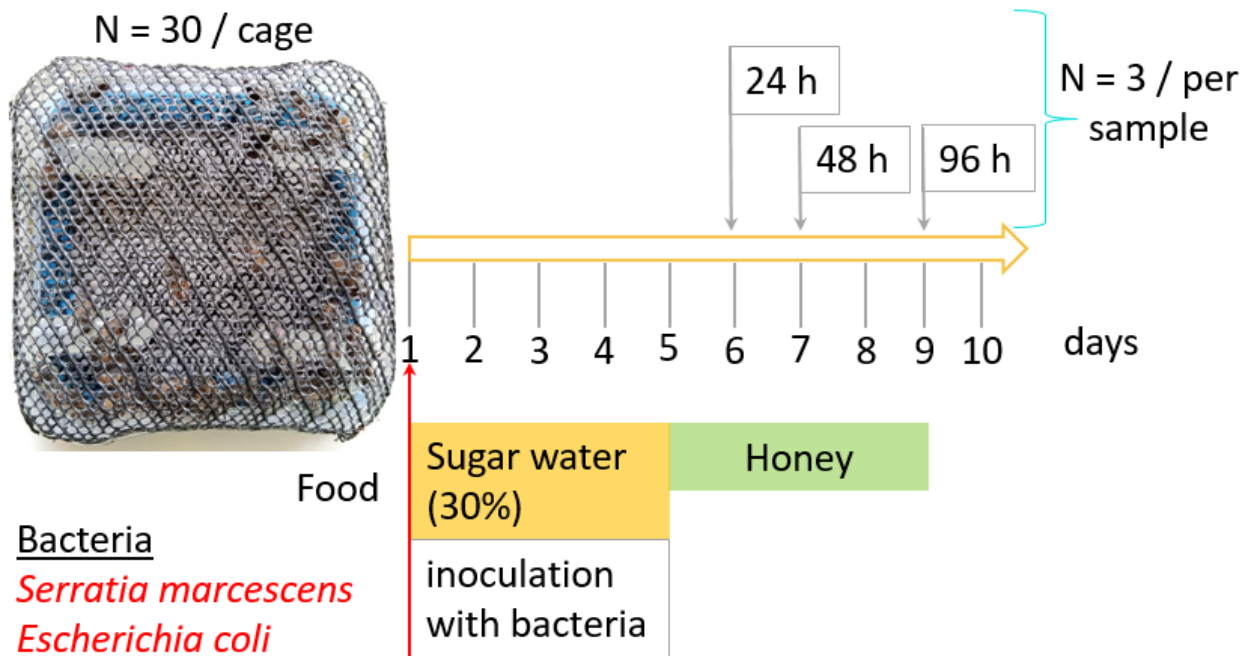
Figure 15: Schematic representation of the therapeutic treatment

3.3.5 Bee Gut Extraction

Each sampled bee was washed in three solutions in successive order 70% Ethanol, 40% Bleach JIK (Sodium hypochlorite), and 1X PBS to sterilize and soften the abdominal tissue for efficient gut extraction. A pair of forceps was used to hold the bee's stinger, gently pulled out the gut and placed it in a clean Eppendorf tube. This was done for each bee and three guts pooled to make one sample. The guts were stored in PBS at -4 °C awaiting DNA extraction.

3.3.6 DNA Extraction from the Extracted Bee Guts

The extracted bee guts were thawed and transferred into labeled 2 mL Eppendorf tubes with approximately four 2 mm Zirconia ceramic beads and crushed using the Tissuelyser II at 6,000 rpm for 2 min to obtain a homogenate. The DNA was then extracted using an organic solvent protocol.

Briefly, 100 μL of the bee gut homogenate was obtained and transferred into a clean 2 mL Eppendorf tube and an equal volume of buffer saturated phenol at pH 8 was added and the tube vortexed at speed 3. The resulting solution was centrifuged at 3,234 rcf for 5 min. The aqueous layer on top was carefully obtained without breaking the interface and transferred into a sterilized 2 mL Eppendorf tube in which the same volume of chloroform was added and the tube inverted gently several times for mixing then centrifuged at 3,234 rcf for 5 min. After centrifuging, the aqueous layer on top was carefully obtained and transferred into another labelled 2 mL Eppendorf tube. To the solution, absolute EtOH 2.5 times its volume and 0.5 of its volume 5M NH_4Ac was added, then carefully shaken horizontally to maximize the surface area between the two liquids. The solution was incubated overnight at -20 °C. After incubation, the solution was centrifuged at 3,234 rcf for 10 min, then the aqueous layer removed using a micropipette and discarded leaving behind a DNA pellet. The DNA pellets were rinsed with 70% EtOH and centrifuged at 3,234 rcf for 30 s, the EtOH was discarded, and the rinsing step repeated under same conditions. The pellets were then dried in a thermostat block at 37 °C for 1 h then re-suspended in 20 μL of autoclaved distilled and deionized nuclease free water and stored at -20 °C awaiting further analysis.

3.3.7 Evaluation of the Extracted DNA Quantity and Quality

A Nanodrop spectrophotometer was used to check the extracted DNA's quality and quantity. Briefly, 2 μL of distilled water was used as a blank to zero the equipment by placing the water sample on the equipment's probe and analyzing it. The same procedure was done for the DNA samples. The data obtained informed the dilution of the DNA to concentrations suitable for qRT-PCR.

3.3.8 Bacteria Quantification Using Real Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Standard curves for quantification of *E. coli* and *S. marcescens* were developed by culturing the bacteria, extracting their DNA using the organic solvent protocol outlined above and measuring the DNA quality and concentration using a Nanodrop equipment. Serial dilution, dilution factor (DF) 2 was done to obtain 6 dilutions for each bacteria in triplicate. The six triplicate dilutions were used as the DNA templates for qRT-PCR to obtain the standard curves for quantification of the bacteria. The standard curves and experimental treatments DNA were analyzed using the same protocols as explained below.

The DNA concentration was determined to be proportional to the amount of bacteria, therefore it was used to evaluate the bacterial concentration (load) in the inoculated bees' guts. Preparation of the extracted DNA for qRT-PCR was done as follows: 6 µL of distilled deionized nuclease free water, 0.5 µL of both forward and reverse primers, 2 µL of Evagreen dye (No ROX) and 1 µL of the DNA template were mixed in an optically clear tube and capped for a10 µL qRT-PCR analysis. Both bacteria were prepared as explained with alterations of their primers. Primers targeting the bacterial 16S rRNA were used for *E. coli* fadD F6 and fadD R3 and *S. marcescens* SMSF and SMFR (Table 4) with annealing temperatures of 54 °C and 60 °C respectively as explained by Iwaya *et al*. (2005).

The qRT-PCR conditions for the 10 µL reaction were set on a Quant studio 3 Real Time PCR 96-Well 0.2 mL Block with the cover temperature set at 105 °C. The qRT-PCR cycling conditions entailed a hold stage for at 95 °C for 15 min then the denaturation stage at 95 °C for 30 s followed by the annealing stage at the primer's annealing temperature for 40 cycles each 30 s, then the extension stage at 72 °C for 2 min, a hold stage at 72 °C for 10 min and finally the melt curve stage at 95 °C for 15 s, 75 °C for 1 min and 95 °C for 15 s.

Table 4: qRT-PCR bacterial primers' sequences

Bacteria	Forward primer	Reverse primer	Sequence
	fadD F6		5'-GCTGCCGCTGTATCACATTT-3'
E. coli		fadD R3	5'-GCGCAGGAATCCTTCTTCAT-3'
	SMSF		5'-GGTGAGCTTAATACGTTCATCAATTG
S. marcescens		SMSR	5'-GCAGTTCCCAGGTTGAGCC-3'

3.4 Evaluation of the Honeybee Choice of Honey

The bees (healthy and diseased) were subjected to an olfactometer dual choice experiment using a triangular glass Y-tube, charcoal filter, flow rate regulators, and an Air Cadet Vacuum/Pressure Station. The six honey were paired against each other for the two experiments, additionally, lemon grass oil was used as positive control and an empty chamber as the negative control. The Y-tube was rotated 180° after every choice to account for any angle bias of the Y-tube arms.

The experiment was conducted in the dark using a direct current red light head torch to eliminate the effect of alternating current white light. The healthy and diseased bees were obtained from *icipe's* apiary and reared in the lab as outlined in the bee rearing conditions in section 3.3.2 above. The diseased bees were inoculated with *S. marcescens* in similar fashion to the therapeutic treatment protocol with minor adjustments; after bacterial inoculation the bees' diet was not changed to honey but they were continued on 30% sucrose solution.

3.4.1 Olfactometer Y-tube Choice Experiment

The healthy and diseased bees' response to different pairs of honey was tested using in a Y-tube olfactometer. Charcoal filtered and humidified air was passed through the flow rate regulators and delivered into the samples chambers then channeled into the two arms of the Y-tube at a flow rate of 350 mL/min. An electric Air Cadet Vacuum/Pressure Station was used to suck air out of the Y-tube at a flow rate of 700 mL/min. In each bioassay, a pair of honey were placed in the separate sample chambers and the extraction pump switched on. A honeybee was then inserted from the bottom of the Y-tube stem and a stopwatch started simultaneously. Each bee was given a maximum of 5 min to make a choice. The experiment was conducted under controlled laboratory conditions (25 °C and 70% relative humidity).

Figure 16: An olfactometer choice experiment setup. Y-tube olfactometer (A), electric Air Cadet Vacuum/Pressure Station (B), Flow Rate regulators (C), air charcoal filter (D), sample chambers (E).

3.5 Honey Quality Analysis

The honey samples were subjected to multiple chemical analysis to understand their quality, chemical composition, and volatile and non-volatile profiles. Physicochemical properties, phytochemicals, antioxidant activity, chemical liquid extract and volatile profiles of the honey were analyzed using various analytical techniques and instruments.

3.5.1 Physicochemical Analysis of Honey

Analysis of all the physicochemical parameters were done according to the Codex Alimentaruis Commission and the International Honey Commission (IHC, 2009; Codex, 1992) guidelines except for carbohydrates and water activity, which were analyzed according to the Association of Official Agricultural Chemists (AOAC) as described in Horwitz & Latimer, (2005).

3.5.1.1 Total Carbohydrates

A handheld refractometer was used to determine the total carbohydrates in the honey samples by measuring their refractive indexes at room temperature (25 °C). Briefly, 1 mL of homogenised honey was obtained and placed on the refractometer's screen and covered with the daylight plate. The honey's carbohydrates level was then observed under light through the refractometer's eyepiece and the value on the Brix scale recorded (Horwitz & Latimer, 2005).

3.5.1.2 Free acidity and pH

A honey solution made by dissolving 10 g of honey in 75 mL of carbon dioxide free water was titrated against 0.1 M NaOH to pH 8.3 to determine the honey's free acidity. The same solution was used to measure the honey's pH using a pH meter.

3.5.2 Phytochemical (Total Phenolic and Flavonoid Content) Analysis of Honey

Phytochemicals analyses were done as described in (Mokaya *et al*., 2020a, 2020b; Mokaya *et al*., 2022) in triplicate with minor alterations.

3.5.2.1 Quantification of Total Flavonoid in Honey

One gram of honey was diluted in 10 mL 50% MeOH. One milliliter of the solution was picked and mixed with 6.4 mL of distilled water then 300 µL, 5% $NaNO_3$ added to it. Five minutes later, 300 µL of 10% $AlCl_3$ was added to the mixture followed by 2 mL of 1 M NaOH after 1 min. The solution's absorbance was measured using UV/Vis spectrophotometer against a blank (a solution containing all reagents with distilled water substituting the honey sample) at 510 nm.

A flavonoid standard calibration curve was developed using Quercetin (Q) at different concentrations of 40, 80, 120, 160 & 200 µg/mL in triplicate and used to quantify the flavonoids content. The flavonoid quantity was expressed as milligrams of quercetin equivalent per 100 grams of honey (mg QE/100 g honey).The flavonoid standard calibration curve was as stated below.

Flavonoid standard (Quercetin) calibration curve equation obtained as $y = 0.0006x + 0.0028$ (1)

3.5.2.2 Quantification of Total Phenolic Content in Honey (TPC)

The quantification of phenols was done in reference to the Folin-Ciocalteu method. One gram of honey was mixed with 10 mL of 50% MeOH. One milliliter of the solution was picked and mixed with 5 mL of 0.2N Folin-Ciocalteu, then 4 mL of 75 g/L Na_2CO_3 added to the mixture 5 min later then incubated in the dark at room temperature (25 °C) for 2 hours. The solution's absorbance was measured at 760nm using a UV/Vis spectrophotometer against a blank (a solution containing all reagents with distilled water substituting the honey sample). A phenolic standard calibration curve was developed using Gallic acid (GA) at different concentrations of 50, 100, 150, 200, & 250 µg/mL in triplicate and used to quantify the total phenolic content of the honey sampled expressed as milligrams Gallic acid equivalent per 100 g of honey (mg GAE/100 g of honey).

Phenolic standard (Gallic acid) calibration curve equation was obtained as $y = 0.0073x + 0.0233$ (2)

3.5.3 Determination of Honey Non-volatile and Volatile Chemical Profiles

The non-volatile and volatile profiles of honey were determined via various extraction techniques then analyzed by GC-MS.

3.5.3.1 Honey Volatile Compounds Collection

The dynamic headspace extraction technique was used to collect honey volatiles. The technique employed the use of PoraPak-Q 80-100 mesh 30 mg adsorbents and a portable volatile extraction pump as described in (Omondi *et al*., 2022). Briefly, the PoraPak-Q was connected to the air extraction tube of the pump and immersed into the headspace of the sample. The pumps air supply tube was also inserted into the airtight sealed jar containing the honey sample to supply filtered air for volatile components equilibration before extraction.

The pumps clean air supply rate was set at 2.5 L/min while the suction rate was set at 2 L/min to slowly draw volatiles through the adsorbent and allow maximum time for adsorption. The experiment ran for 6 hours after which the volatiles trapped on the PoraPak-Q were eluted using 300 µL GC-MS grade DCM and 20 µL of the eluate obtained for GC-MS analysis.

3.5.3.2 Liquid – Liquid Extraction of Honey Non-volatile Compounds

To 5 g of honey solution, 10 mL DCM was added and vortexed for 5 min at speed 5. The mixture was transferred to an incubator shaker maintained at 35 °C for 1 hour at 200 rpm. The mixture was then centrifuged (3,234 rcf, 5 min, 4 °C) and the supernatant transferred to a clean glass test tube and re-centrifuged (3,234 rcf, 10 min, 4 °C), then decanted into a 15 mL glass test tube and concentrated to dryness in the rotary vacuum evaporator (30 min, 45 °C). The residue was reconstituted in 1 mL GC-MS grade DCM, centrifuged (3,234 rcf, 5 min, 4 °C) and 20 µL obtained for GC-MS analysis.

3.5.4 Gas Chromatography coupled with Mass Spectrometry (GC-MS) Analysis

The desorbed honey volatiles and the DCM liquid extract of honey were analyzed by gas chromatography (GC-HP-7890A, Agilent Technologies, USA) coupled with Mass spectrometry. Labeled samples were queued on the GC-MS sample tray where 1 µL of the sample was automatically obtained by an auto sampler and injected into the GC via the injection port at 250 °C in splitless mode. Chromatographic separations were achieved by a HP-5MS capillary column, 30 m X 0.25 mm i.d., 0.25 µm thick immobilized with 5% (phenyl methyl silicone) as the stationary phase. The volatiles were transported by a Helium career gas of 99.99% purity at a flow rate of 1.2 mL/min. The oven temperature programming was set at an isothermal hold at 35 °C for 5 min followed by a gradual constant increase at the rate of 10 °C/min to 280 °C where it was held at an isothermal state for 12 min. The MS detector operated in a scan mode within a mass range (8 – 550) m/z at 1 scan/sec with electron energy of 70 eV. The MS interface line was maintained at 230 °C. The cumulative analysis time was 35 min. The identification of individual compounds was done by comparing their mass spectral data and retention time against the mass selective detector (MSD) library (NIST, 2005, NIST 05a, AND Adams MS HP, USA). The percentage composition of the individual compounds was determined through the integration of their peaks compared to the amount of sample injected and was used as a proxy for their quantity.

3.4 Data Analysis

Statistical data analysis and visual representations were done using three software packages; the R statistical analysis version 4.2.1, Past software version 4.04, and GraphPad Prism Version 9. All the data sets were subjected to the Shapiro-Wilk to test for normality to inform the methods of analysis (parametric or non-parametric). One-way ANOVA together with Tukey test for multiple comparison with the nominal significance level of $p = 0.05$ was used to test for differences and similarities among the tested honey parameters and the bacterial concentration means per sampling time. Mann-Whitney two tailed unpaired t-test was use to compare the differences in time taken and number of choice made by the bees per pair of honey in both treatments. Fischer Exact test was used to highlight the differences in choice of the honey per pair in both treatments (healthy and diseased). Rank sum analysis was done to determine the ranking of the honey in terms of choice in both treatments. The *factoextra* and *ggplot2* packages in R working environment and ChemDraw were used to visualize the data through graphs, chemical structures and chromatograms. The antibacterial activity of the compounds was referenced in published literature and counter checked on Dr Duke's Phytochemical and Ethnobotanical Databases complied by Dr. Jim Duke of Agricultural Research Service/USDA-ARS in 2016

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Antibacterial Activity of Honey

Honey has recorded effective antimicrobial activity across a diverse range of pathogenic microbes such as viruses, fungi and bacteria. Multiple antibacterial assays have been used to test the antimicrobial property of honey originating from diverse geographical sources around the globe against various species and types of bacteria, each confirming the antibacterial potential of honey (Dixon, 2003). However, it's noteworthy to mention that most of the antibacterial assays used to test honey's antibacterial activity were done *in vitro* in controlled setups assessing limited interaction between the honey and the cultured bacteria. As a preliminary to this study, *in vitro* antibacterial activity of the different honey samples was done and the zones of inhibition of the respective honey measured and recorded. The experiment was done to ascertain the antibacterial potential of the sampled honey against a range of bacteria. The results showed that the sampled honey had varying *in vitro* antibacterial potential as they inhibited the growth of bacteria on agar plates (Appendix, Table 1). To assess the antibacterial effect of honey on the producers own health, this study explored an *in vivo* approach by inoculating the bees with the pathogen and studying the bacterial concentration in their gut over time. That was done using prophylactic and therapeutic treatment setups to investigate honey's ability of preventing or treating bacterial infection in bee colonies. The approach simulated colony infection taking into effect the host-pathogen interactions, as bees possess innate physiological and behavioral mechanisms to deal with infection. The effect of the different honey diets on bee fitness were analyzed and quantified.

4.1.1 Prophylactic Antibacterial Effect of Honey

The ability of bees' food, honey, to prevent bacterial infection was investigated by predisposing bees to a honey diet before bacterial inoculation through the food and the bacterial density in their gut monitored over time. Two bacterial candidates (*E. coli and S. marcescens*) were used to assess the prophylactic antibacterial effect of the sampled honey on the bacteria.

In the prophylactic treatment where bees were inoculated with *S. marcescens*, bees fed on the sucrose solution diet recorded the highest bacterial load 24 h post inoculation 42.5 µg/µL while Arabuko Sokoke honey diet had the least 0.3 µg/µL.

Baringo, Kakamega, Karura, Taita honey and the sucrose solution diets had reduced bacterial loads of 1.3 µg/µL, 0.4 µg/µL, 0.6 µg/µL, 0.5 µg/µL, and 39.2 µg/µL, respectively at 48 h after inoculation. However, Arabuko Sokoke and Manuka 5+ honey diets recorded slightly increased bacterial loads at the same time (Table 5). At 96 h post inoculation, bees fed on four of the six honey diets, including Kakamega, Karura, Kitui, and Baringo honey experienced an increase in bacterial loads from the concentration recorded at 48 h (Table 5), indicating a short term prophylactic antibacterial effect of the honey diets. Bees on Taita and Arabuko Sokoke honey diets and the sucrose solution had slightly reduced bacterial loads in the same period. Despite the infinitesimal reduction of the bacterial loads induced by the sucrose solution, it recorded the highest bacterial load compared to the honey diets at all the sampling intervals indicating the honey diets had enhanced antibacterial effect compared to the negative control (Figure 17-A). A comparison of the mean bacterial loads in the bees fed on the different honey diets at 24 h, 48 h, and 96 h respectively, post inoculation had a significant variation at each sampling time (ANOVA, $p < 0.001$) (Table 5).

Table: 5 Variation of the bacterial concentration mean $\pm$ S.D (n = 3) (S.D is the standard deviation) in µg/µL detected in the in the prophylactic treatment group against *S. marcescens*. The p values were determined using One-way ANOVA (p < 0.05)

S. marcescens Prophylactic Experiment (Concentration in µg/µL)			
Samples	**24 h**	**48 h**	**96 h**
Arabuko Sokoke	0.3 ± 0.2	1.5 ± 0.9	1.1 ± 0.7
Baringo	1.4 ± 0.9	1.3 ± 0.9	1.7 ± 1.1
Karura	1.0 ± 0.6	0.6 ± 0.3	1.2 ± 0.5
Kakamega	21.2 ± 6.1	0.4 ± 0.2	2.3 ± 1.4
Kitui	11.2 ± 2.7	1.2 ± 0.7	1.3 ± 0.8
Taita	1.1 ± 0.7	0.5 ± 0.2	0.3 ± 0.2
Manuka	0.5 ± 0.3	1.2 ± 0.7	2.7 ± 1.8
Sucrose	42.5 ± 12.5	39.2 ± 11.0	37.2 ± 9.6
P ($\alpha = 0.05$)	< 0.001	< 0.001	< 0.001
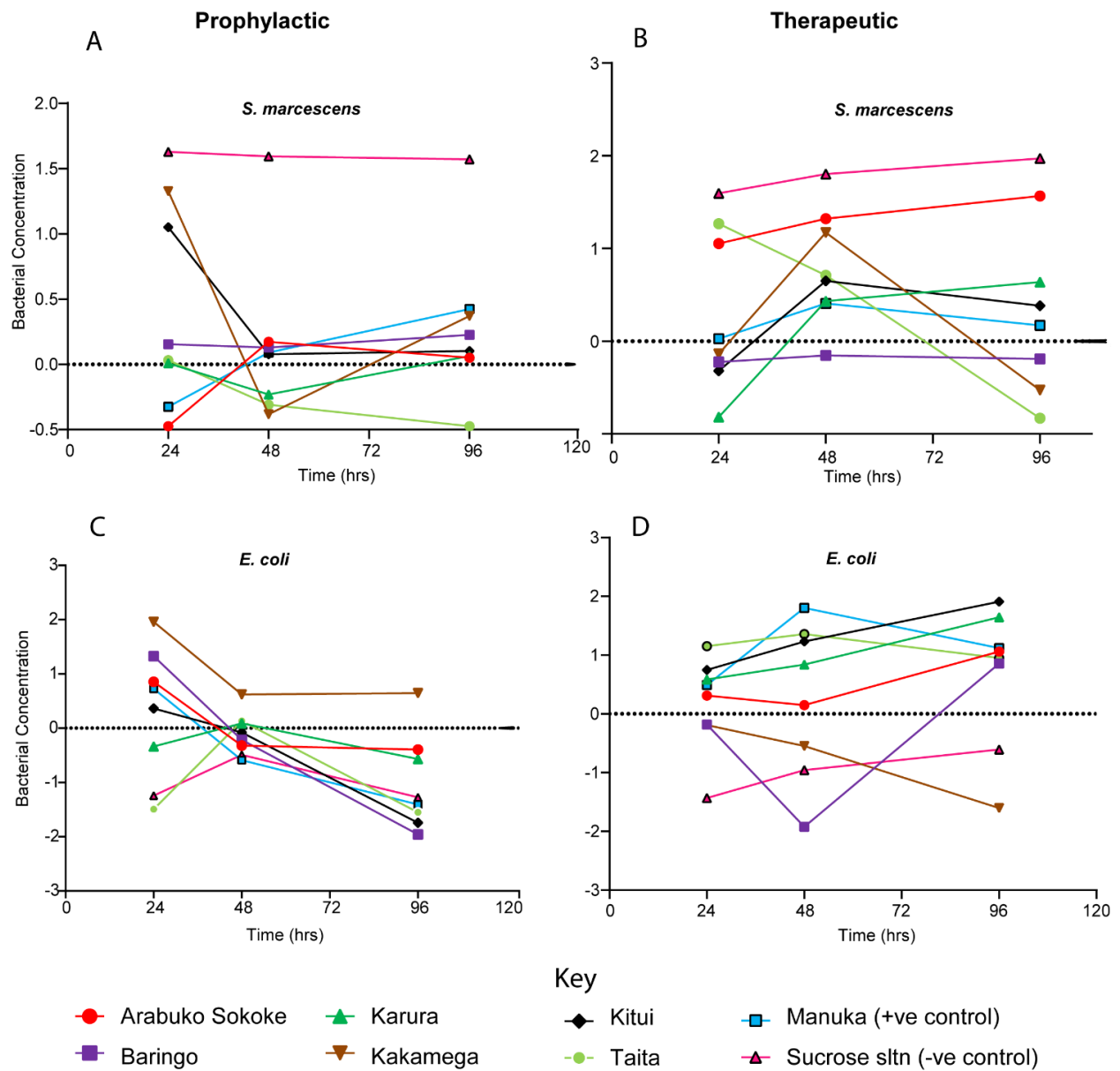
Figure 17: Variation of quantified bacterial concentration (*S. marcescens* and *E. coli*) in the bee gut. (A) Prophylactic treatment with *S. marcescens* (B) Therapeutic treatment with *S. marcescens*, (C) Prophylactic treatment with *E. coli*, and (D) Therapeutic treatment *E. coli*. The y-axis bacterial concentration have been $\log_{10}$ transformed.

The 24 h post inoculation sample from *E. coli* infected bees indicated varied bacterial loads in their guts. The bees fed on Kakamega honey had the highest bacterial load with a concentration of 91.2 µg/µL, while Taita honey recorded the least bacterial concentration 0.03 µg/µL (Table 6).

Bees fed on Manuka 5+ honey had a bacterial load of 5.3 µg/µL while the sucrose solution diet recorded 0.06 µg/µL. Kakamega, Baringo, Arabuko Sokoke, Manuka 5+, and Kitui honey diets recorded reduced bacterial loads 48 h after exposure to the bacteria as illustrated in (Figure 17-C). Bees fed on Taita and Karura honey diets had an increased bacterial loads 48 h post inoculation. All the honey samples reduced the bacterial load in the bees' gut significantly at 96 h post inoculation. Despite the bees on fed Kakamega honey diet having the highest concentration of 4.4 µg/µL at 96 h post inoculation, it was a reduction compared to the bacterial load recorded at 24 h for the same honey. Bees on Baringo honey diet had the least bacterial load at 96 h 0.01 µg/µL having experienced a gradual reduction from the load recorded at 24 h post inoculation. The negative and positive control diets also recorded a reduction of the bacterial load in the bees signified by the diminished concentrations of 0.05 µg/µL and 0.04 µg/µL, respectively. The mean concentrations recorded by the different honey diets at 24 h, 48 h, and 96 h respectively, post-inoculation were significantly different at each sampling time (ANOVA, $p < 0.001$).

The interaction of *E. coli* with the honey diets and sucrose solution produced peculiar results as the bees fed on sucrose solution which is devoid of honey's nutraceutical and antimicrobial properties recorded a reduced and generally low bacterial load than most honey diets at 24 h and 48 h post inoculation (Figure 19-C). That, however, changed in the long term after 96 h as the bees on Baringo, Kitui, Taita and Manuka 5+ honey diets recorded lower bacterial load than the negative control diet. Despite the increase, then eventual decrease in the bacterial load in Taita and Karura honey and the sucrose solution diets, a gradual overall reduction in the bacteria concentration was observed indicating honey's long term prophylactic antibacterial ability against *E. coli* infection with the effect being recorded progressively from 48 h after inoculation.

Table: 6 Variation of the bacterial concentration mean $\pm$ S.D (n = 3) (S.D is the standard deviation) in µg/µL detected in the bees in the prophylactic treatment against *E. coli*. The variation in bacterial concentrations were determined using One-way ANOVA (p < 0.05)

E. coli Prophylactic Experiment (Concentration in µg/µL)			
Samples	**24 h**	**48 h**	**96 h**
Arabuko Sokoke	7.1 ± 1.0	0.5 ± 0.1	0.4 ± 0.05
Baringo	21.1 ± 2.8	0.6 ± 0.3	0.1 ± 0.01
Karura	0.5 ± 0.1	1.2 ± 0.4	0.3 ± 0.06
Kakamega	91.2 ± 13.5	4.2 ± 0.7	4.4 ± 1.0
Kitui	2.3 ± 0.6	0.8 ± 0.3	0.02 ± 0.01
Taita	0.3 ± 0.03	1.4 ± 0.3	0.03 ± 0.01
Manuka 5+	5.3 ± 0.8	0.3 ± 0.1	0.04 ± 0.01
Sucrose	0.06 ± 0.04	0.3 ± 0.1	0.05 ± 0.01
P ($\alpha = 0.05$)	< 0.001	< 0.001	< 0.001

Honey is a functional food to bees and provides energy for their activities such as foraging (Nicolson, 2011). In addition, honey is also considered nutraceutical, a term used to refer to foods or food product(s) taken as part of the diet for nutritional purposes and contains additional benefits, in this case, medicinal (antibacterial) benefits. Nutraceutical foods are also defined as "tool box for prevention of disease" by Berenbaum & Calla, (2021). The quality of food (honey) is important to a colony when anticipating an infection. High quality multifloral honey has prebiotics due to the presence of oligosaccharides hence they have been associated with an extensive and healthy bee gut microbiome which plays an important role in strengthening the honeybee's immune system. The immune system is partly responsible for the prophylactic role of disease prevention by suppressing the replication of pathogenic bacteria in the bee's gut and body (Berenbaum & Calla, 2021; Kwong & Moran, 2016; Mustar & Ibrahim, 2022; Raymann & Moran, 2018).

The prophylactic potential of honey is linked to its immune-stimulating ability to enhance defense against infection (Raymann & Moran, 2018). Additionally, honey is fortified with endogenous bee secretions during its processing, which include the antimicrobial peptide bee defensin-1 that helps in preventing bacterial infections (Kwakman & Zaat, 2012; Mandal & Mandal, 2011; Samarghandian *et al*., 2017). Bees were fed on honey from diverse origins to assess honey's effect in disease prevention.

Against *E. coli,* a progressive reduction of the bacterial load in the honeybee gut was observed in more than 50% of the honey diets, including the positive control with relatively high bacterial load being recorded in the initial sampling time 24 h post inoculation and lower concentration in the last sampling time 96 h. The reduction in bacterial load with time serves as an indication of honey's prophylactic ability to prevent infection in honeybees by curtailing disease progression through bacteriostatic and bactericidal mechanisms.

However, generally, the bees on the negative control diet were observed to have a significantly reduced bacterial load compared to most honey diets. That may be misleading to assume that the negative control had a better prophylactic antibacterial effect than the honey diets, which is not the case as it contained a low concentration of sucrose solution with no antibacterial mechanism. The observation is attributed to *E.coli*'s high affinity and preference for glucose for rapid growth (Bren *et al*., 2016). The absence of glucose and an abundance of sucrose in the negative control is the probable cause of *E.coli*'s diminished and slow growth rate leading to the detection of low bacterial load. In contrast, *S. marcescens* is able to grow in high sucrose concentrations as illustrated in (Begic & Worobec, 2007), it grew in the guts of the bees on the sucrose solution diet without any impediment registering higher bacterial load compared to the bees fed on honey diets across the tested time points in the prophylactic *S. marcescens* treatment.

The negative control unlike the different honey had no antibacterial effect to restrict or prevent the growth of *S. marcescens,* hence the high bacterial load observed. Generally, five of the honey diets; Kakamega, Kitui, Baringo, Karura and Taita honey including the negative control registered a reduction in the bee's bacterial load in the first 24 h post inoculation. However, the effect was short lived as the bacterial load increased through the 48 h to 96 h post inoculation indicating a short term prophylactic effect, which diminishes over time against *S. marcescens*. It is crucial to note that the increase in bacterial load observed from 48 h to 96 h was slow and gradual compared to the decrease experienced between 24h and 48 h. That shows the ability of honey to significantly lower the virulence of *S. marcescens* upon first interaction and slow its growth thereafter. With the low concentration and slowed growth, the bacteria is not able to adversely infect the bee (Forsgren *et al*., 2018).

4.1.1 Therapeutic Antibacterial Effect of Honey

The therapeutic antibacterial potential of honey refers to its ability to cure bacterial diseases upon consumption by an infected organism. In the therapeutic treatment using *S. marcescens* to inoculate the bees, the negative control bees registered the apical bacterial load of 39.3 µg/µL at 24 h post inoculation and the load increased through the 48 h sample and was highest again at 96 h with a bacterial load of 93.6 µg/µL. The positive control 24 h bacterial load was quantified at 1.1 µg/µL, which slightly increased after 48 h registering a concentration of 2.6 µg/µL before decreasing to a final of 1.5 µg/µL after 96 h post inoculation. The treatment group fed on Karura honey had the least bacterial load of 0.2 µg/µL at 24 h post inoculation while Taita honey recorded the highest bacterial load of 18.5 µg/µL among the honey samples (Table 7). A general increase in bacterial load was observed on bees fed on all the honey samples and controls from 24 h to 48 h after exposure to bacteria, except for Taita honey which recorded a decrease (Figure 17-B). After 48 h the bacterial load detected in bees fed on Karura and Arabuko Sokoke honey diets increased slightly while on the other 4 honey diets; Kakamega, Taita, Kitui and Baringo honey, the bacterial loads reduced below the concentrations recorded at 48 h indicating the honey's long term therapeutic effect. Baringo, Kakamega, Kitui and Taita honey registered long term therapeutic effect against *S. marcescens* infection, recording a gradual decrease in the bacterial loads from 48 h to 96 h post inoculation (Figure 17-B). The mean bacterial load at 24 h, 48 h, and 96 h respectively, post inoculation varied significantly (ANOVA, $p < 0.001$) for the different honey diets (Table 7).

Table: 7 Variation of the bacterial concentration mean $\pm$ S.D ($n = 3$) (S.D is the standard deviation) in µg/µL as detected in the bees in the therapeutic treatment against *S. marcescens*. The variation in bacterial concentrations were determined using One-way ANOVA ($p < 0.05$)

S. marcescens Therapeutic Experiment (Concentration in µg/µL)			
Samples	**24 h**	**48 h**	**96 h**
Arabuko Sokoke	11.3 ± 4.7	21.0 ± 13.0	36.9 ± 13.8
Baringo	0.6 ± 0.4	0.7 ± 0.4	0.65 ± 0.2
Karura	0.2 ± 0.08	2.7 ± 1.4	4.3 ± 1.9
Kakamega	0.7 ± 0.3	14.9 ± 4.2	0.3 ± 0.1
Kitui	0.5 ± 0.3	4.5 ± 3.0	2.4 ± 1.2
Taita	18.5 ± 4.3	5.1 ± 2.8	0.1 ± 0.03
Manuka 5+	1.1 ± 0.76	2.6 ± 1.5	1.5 ± 0.2
Sucrose solution	39.3 ± 8.64	63.5 ± 12.4	93.6 ± 15.0
P ($\alpha = 0.05$)	<0.001	<0.001	<0.001

In the therapeutic treatment of *E. coli* infected bees, Taita honey diet recorded the highest bacterial load having 14.2 µg/µL at 24 h post inoculation while the bacterial load in the negative control diet was the least at 0.04 µg/µL (Table 8). Bees fed on Taita, Kitui, and Karura honey diets recorded an increased bacterial load at 48 h post exposure to the bacteria compared to the bacterial loads recorded at 24 h. Kakamega, Baringo and Arabuko Sokoke honey diets experienced a reduced bacterial load at 48 h in comparison to the loads recorded at 24 h for the respective honey (Table 8). The negative and positive control diets also recorded an increased bacterial concentration at 48 h post inoculation (Figure 17-D). Baringo, Kitui, Karura and Arabuko Sokoke honey diets didn't suppress the growth of *E. coli* as it was detected in higher quantities in the bees' gut at 96 h compared to the bacterial load at 24 h post exposure.

Kakamega and Taita honey diets reduced the bacterial load in the bee after 96 h compared to the loads at 48 h. The positive control likewise reduced the bacterial load past the second day post exposure through to the fourth day while the negative control recorded an increase in the same time span. Kakamega and Taita honey portrayed notable therapeutic effect on bees infected with *E. coli* by gradually decreasing the bacterial load and registering lower final bacterial loads at 96 h compared to the initial load at 24 h post inoculation. A variation in the mean bacterial load present in the bee's gut at 24 h, 48 h, and 96 h respectively, post inoculation was recorded (ANOVA, $p < 0.001$) for the different honey diets.

Table: 8 Variation of the bacterial concentration mean ± S.D (n = 3) (S.D is the standard deviation) in µg/µL detected in the bees sustained on the different honey diets the therapeutic treatment with *E. coli*. The statistical significant differences were determined using One-way ANOVA ($p < 0.05$)

E. coli Therapeutic Experiment (Concentration in µg/µL)			
Samples	**24 h**	**48 h**	**96 h**
Arabuko Sokoke	2.05 ± 0.3	1.4 ± 0.2	11.5 ± 1.3
Baringo	0.7 ± 0.07	0.01 ± 0.01	7.2 ± 1.1
Karura	3.8 ± 0.8	6.9 ± 2.0	44.0 ± 9.6
Kakamega	0.64 ± 0.1	0.3 ± 0.1	0.03 ± 0.01
Kitui	5.6 ± 1.4	17.1 ± 2.5	81.1 ± 10.8
Taita	14.2 ± 3.6	22.9 ± 2.3	8.9 ± 5.5
Manuka 5+	3.1 ± 0.5	63.6 ± 8.9	13.2 ± 3.5
Sucrose solution	0.04 ± 0.02	0.11 ± 0.1	0.2 ± 0.1
P (α = 0.05)	p<0.001	p<0.001	p<0.001

The therapeutic effect of honey against bacteria is attributed to its composition since therapy requires the consumption of substances with medicinal effect. Honey has a high sugar content responsible for its high osmolarity, the presence of antiseptic hydrogen peroxide, organic acids responsible for its low pH, and more significantly phytochemicals such as antibacterial phenolic acids, flavanones, flavones, and flavonols (Berenbaum & Calla, 2021; Mandal & Mandal, 2011; Mokaya *et al*., 2020a; Nicolson, 2011; Pasini *et al*., 2013; Samarghandian *et al*., 2017). These components work singularly or in combination to achieve the therapeutic antibacterial activity of honey. Honey are diverse in their chemical composition which is greatly influenced by the floral source hence producing honey of different bioactive activity (Mustar & Ibrahim, 2022).

The sampled honey registered varied prophylactic and therapeutic antibacterial effect against the tested bacteria due to the variation in their composition which affects their interaction with both the bees and bacteria. Much of this variation is observed in (Figure 17-D). Honey therapeutic effect is widely documented, as an example (Berenbaum & Calla, 2021), demonstrates that when bees are parasitized with *N. caranae* and fed on different types of honey (black locust, liden, honeydew and sunflower), sunflower honey reduced the prevalence of the bacteria faster, was consumed more, and was observed to have the highest antibacterial activity when tested against the bacteria *in vitro*. Additional analysis revealed sunflower honey's richness in flavonoids which the authors speculate could be one of the reasons for its exemplary antibacterial activity compared to the other honey samples tested in the study.

4.2 Honeybee Choice of Honey Depending on Disease Status

Healthy and diseased honeybees (parasitized with *S. marcescens*) were subjected to a choice experiment in a Y-tube olfactometer. The six honey were paired each against the others to generate 15 test pairs. To eliminate any side preference bais due to irregularities in the Y-tube angle, 10 healthy and diseased bees were tested without any sample and they each had a 50% selection rate indicating no bias in the angles of the Y-tube. Also, the positive control, lemon grass oil was tested against an empty Y-tube arm and 90% of the healthy and 100% diseased bees chose the positive control further proving that the choices were made due to bees' preference and were not affected by the experimental setup such as disparity in the Y-tube angle.

4.2.2 Choice of Honey by Healthy Honeybees

For all the 15 honey pairs tested using healthy honeybees, no honey was significantly preferentially chosen over the other (Fisher exact test, $p > 0.05$). The mean time of selection for each pair was almost similar indicating the healthy bees had no preferred honey in the choice experiment and regarded all honey sample similarly attractive despite their differences in chemical composition. However, the bees were observed to choose some honey within a shorter duration compared to others.

4.3.3 Choice of Honey Diseased Honeybees

Similar to the healthy ones, the diseased bees inoculated with *S. marcescens* showed no statistical significant preference for a particular type of honey for the 15 pairs tested (Fisher exact test, $p > 0.05$). However, diseased bees took averagely less time 23.57 s to make a choice compared to the healthy ones 43.37s (Figure 18). When compared, the overall time taken by the healthy and diseased bees to make a choice were significantly different (Mann Whitney test, $p < 0.001$). Parasitized bees were quicker to find their preferred source of food indicating a role of diet as a therapeutic option to alleviate disease. Also important to note is that despite no records of significant differences in the choice of diseased bees, compared to the healthy choices, the bees almost dominantly chose a specific honey against the other by higher margins (Table 9).
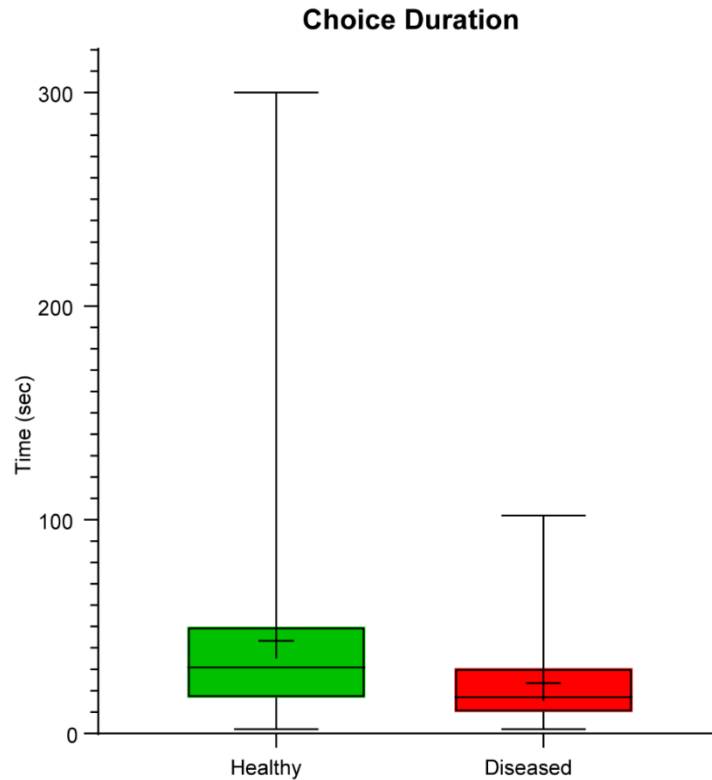
Figure 18: Box plot showing the overall choice duration for healthy and diseased bees (+) represents the mean value while the line across the box plots represent the median value.

4.3.4 Healthy Vs Diseased Bees Choice

The choices made by bees when healthy and diseased for the same pair of honey were compared to establish any change in the frequency of selection of a particular honey in the two health status. As shown in (Table 9) and illustrated in (Figure 19), there was a significant preferential change (Fisher exact test, $p < 0.05$) in choice between two pairs of honey by diseased and healthy bees. Healthy honeybees chose Kitui honey 50% as many times as they did Taita honey 50% of the total choices for that pair of honey. When diseased, the bees preferred Kitui honey 87.5% compared to Taita 12.5% (Table 9). Also important to note, for the same pair of honey both the healthy and diseased honeybees spent averagely less time to select Kitui honey compared to Taita honey (Figure 19 A-B).

In the second pair of the honey, Baringo honey was selected 52.2% of the total choices made, therefore being preferred to Kitui honey (47.8%) by the healthy bees. When inoculated with bacteria, the diseased bees overwhelmingly chose Kitui honey 81.3% compared to Baringo honey 18.7 % (Table 9).

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In terms of time of choice, averagely, both the healthy and diseased bees chose Kitui honey within a shorter time compared to Taita and Baringo honey (Figure 19), indicating strong attraction by Kitui honey to influence the quick choice. The change in choice by the healthy and diseased bees was significant (Fischer exact test, $p < 0.05$). Kitui honey was preferred by the diseased bee in both honey comparison suggesting the presence of therapeutic compounds in the honey volatile profile as the bees were guided to the choices they made by perceiving the volatile compounds transported from the honey chamber by the air with their antennae.

Table 9: A comparison of the choice made by healthy and diseased bees for the same pair of honey

Sample	Selection		p value
	Healthy	**Diseased**	
Baringo	11	13	0.2701
Arabuko	7	3	
Kakamega	12	12	0.7146
Arabuko	6	4	
Baringo	21	4	0.0792
Kakamega	19	12	
Arabuko	17	7	1
Karura	19	9	
Karura	13	10	0.7471
Baringo	11	6	
Karura	11	11	0.2026
Kakamega	13	5	
Arabuko	11	8	1
Kitui	15	9	
Kitui	**15**	**14**	**0.023**
Taita	**15**	**2**	
Kakamega	14	9	0.7425
Kitui	8	7	
Kitui	19	5	0.3727
Karura	21	11	
Kitui	**11**	**13**	**0.0485**
Baringo	**12**	**3**	
Baringo	17	10	0.498
Taita	6	6	
Kakamega	16	15	1
Taita	5	5	
Taita	6	4	0.7225
Karura	16	16	
Taita	15	10	0.271
Arabuko	30	10	
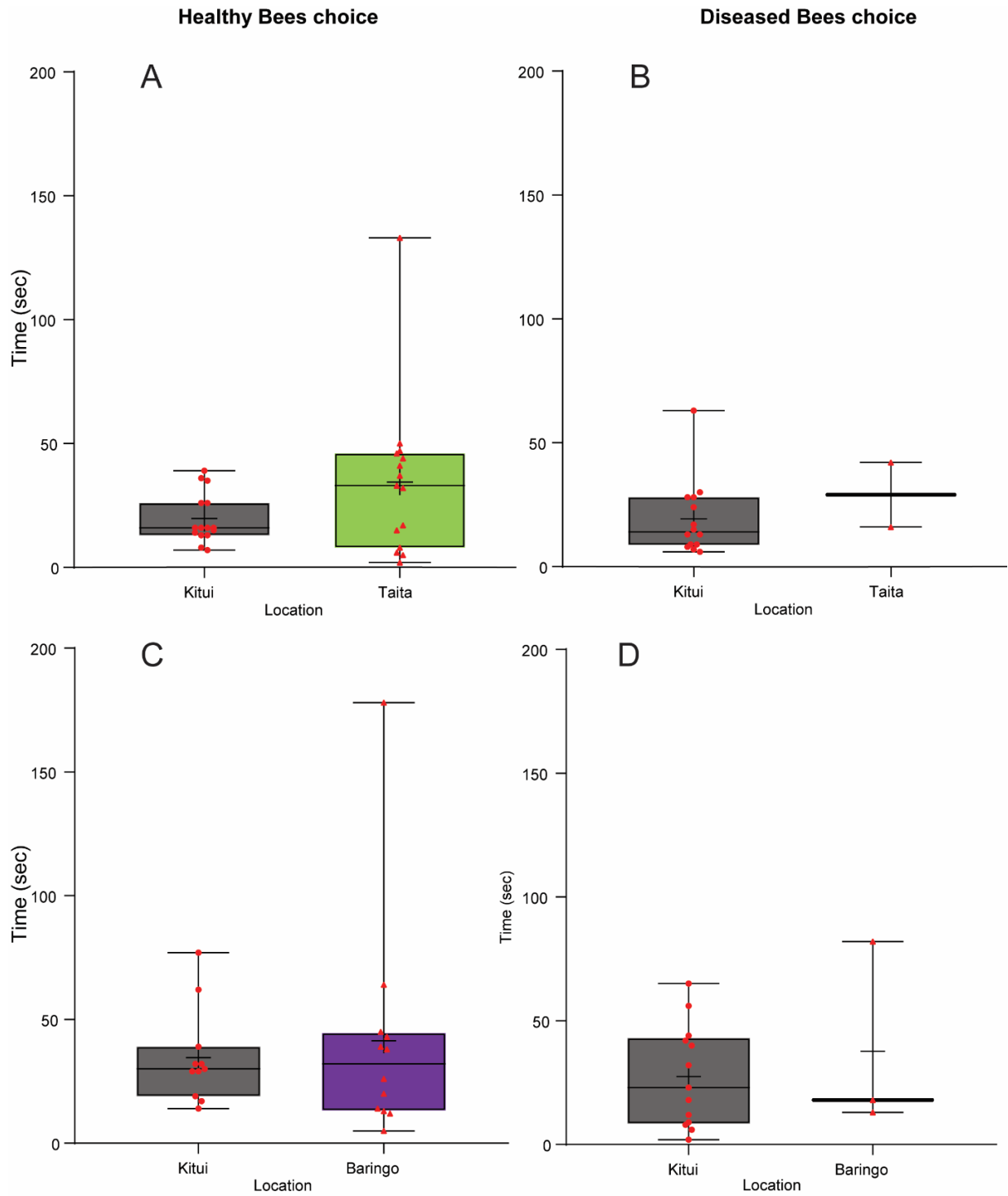
Figure 19: Box plots showing change in preference by healthy and diseased bees for the same pair of honey. The horizontal line through the boxes represent the median while (+) represents the mean value. The red dots represent the frequency of choice by bees.

In a rank sum analysis according to the cumulative number of times a honey was selected in all its pairing in the two treatments, Kitui honey recorded an increased affinity by diseased bees, ranking third most preferred and chosen by diseased bees compared to fifth by healthy honeybees as shown in (Figure 20).
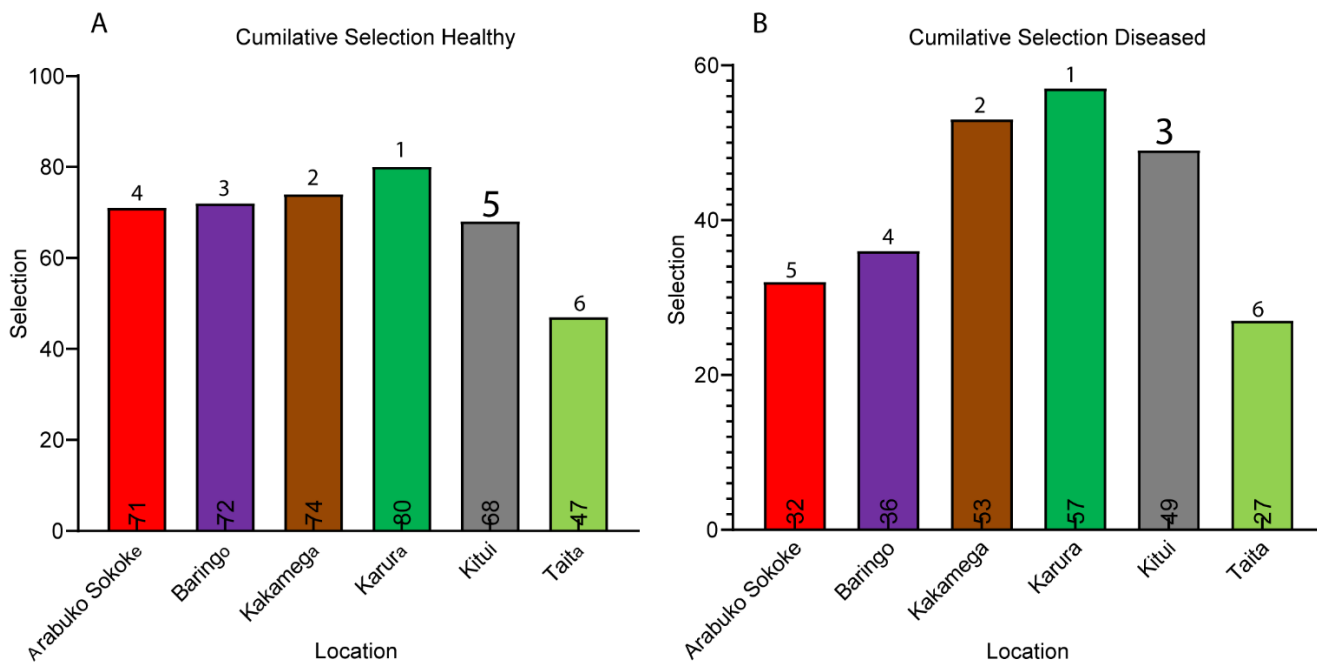
Figure 20: Rank sum analysis showing the different honey choice cumulative frequency in each treatment, (A) healthy bees, (B) diseased bees.

The observed improvement in ranking of Kitui honey from 5th in the healthy bees experiment to 3rd in the diseased bees experiment was due to the increased overall preference for Kitui honey by the diseased bees. Bees contact different floral sources to obtain nectar depending on the floral biodiversity available and is benefits.

In a regression analysis to assess the relationship between the healthy and diseased bees' choice of honey with the antibacterial activity of the respective honey, a distinct pattern was observed (Figure 21). The strongest choice changes in honey preference as observed for healthy and diseased bees occurred among the honey with high antibacterial activity against the 5 bacteria they were tested against (Appendix, Table 1). The results indicate that diseased bees' preferential choice was influenced by the antibacterial activity of the honey in that honey with enhanced antibacterial activity were preferred by parasitized honeybees.
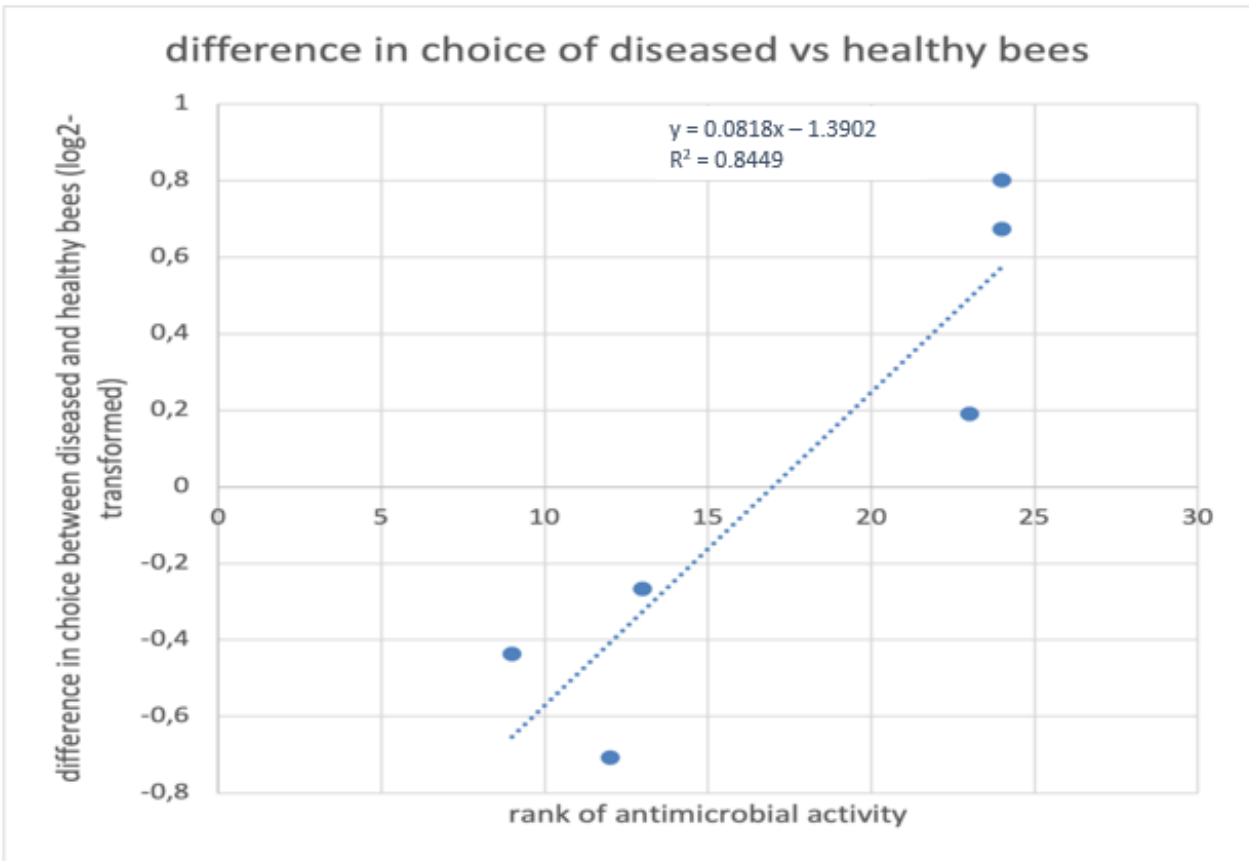
Figure 21: Regression analysis showing the difference in choice between healthy and diseased bees with respect to the antibacterial activity of the honey

Studies have shown higher cognitive function in bees that enables them to locate, remember and even share the location of high quality nectar sources with nest mates to enhance the collection of quality resources (Hoshiba & Sasaki, 2008). When foraging, honeybees prefer high sucrose floral nectar which mostly serves as a guiding factor in floral choice in addition to the flower appearance and volatile chemicals it emits. When the colony, brood or individual bee is infected, honeybees are observed to practice self-medication by foraging on new floral sources with antimicrobial properties or increase the intake of substances with antimicrobial properties in their existing diet (Abbott, 2014; Roode & Hunter, 2019; Simone *et al.*, 2009). Self-medication is practiced by honeybees to enhance brood, colony and individual health by preferentially foraging on floral resources with nutritional and medicinal benefits either as a prophylactic measure when anticipating infection or as a therapeutic measure in response to infection (Abbott, 2014); this behavior is widely observed in insects.

Preferential intake for therapeutic purposes is also observed in other studies where infected armyworms *Spodoptera littoralis* preferred protein rich diets compared to healthy ones in the control group; the authors reported that the protein rich diet promoted increased resistance to nucleopolyhedro virus in the infected worms. Another example is on *Drosophila melanogaster* exposed to *Leptopilina heterotoma* and *Leptopilina boulardi* parasitoid wasps, where the larvae of *D. melanogaster* portrayed a preference for ethanol containing foods compared to ethanol free food. The consumption of the new ethanol containing food enhanced survival in the parasitized group compared to the control which was maintained on ethanol free food (Abbott, 2014; Roode & Hunter, 2019). These examples further cement the preferential change in diet to achieve a therapeutic effect and also provides evidence on diet serving more purpose than nutrition in animals, hence the ability to achieve therapeutic effect against bee bacterial diseases through their diet, honey.

4.4.4 Influence of Honeys Antibacterial Activity on Honeybee Choice

4.4 Chemical Composition of Honey

Phytochemical, physicochemical, volatile and non-volatile chemical profiles of honey were analyzed to determine their chemical composition and assess for variability.

4.4.1 Physicochemical Composition of Honey Samples

4.4.4.1 Carbohydrates Content (°Bx)

The carbohydrates content varied significantly among the honey samples (ANOVA, p < 0.001). Arabuko Sokoke honey had the highest concentration of carbohydrates at 81.5% while honey from Taita recorded the least content with 74% (Figure 22-A). The values were within the recommended range of 70 to 88 °Bx (Codex, 2001) for mature honey and similar to the carbohydrates content reported in (Boateng & Diunase, 2015; Oroian *et al.*, 2017) for Romania, New Zealand and Cameroon honey where they recorded ranges of 76.3 to 85.3 °Bx, 70 to 88 °Bx, and 82.00 ± 0.58 °Bx, respectively. Carbohydrates constitute approximately 95% of honey's dry weight and are composed of 95% sugars of which 75% are monosaccharides (glucose and Fructose) and approximately 5% disaccharides (sucrose) (Codex, 2001). Other sugars often detected in honey in minor quantities include; maltose, trehalose, and palatinose among others. The high sugar content is responsible for honey's high viscosity, hygroscopicity and energy value. In addition, it creates a high osmotic pressure that prevents microbial survival and growth through dehydration,

contributing to honey's antibacterial activity and prolonged shelf life. Additionally, by occupying a significant composition volume of honey, carbohydrates reduce the available moisture content for utilization of bacteria as most thrive in high moisture environments (Dixon, 2003; Nicolson, 2011; Santos-Buelga & González-Paramás, 2017).

4.4.4.2 pH

A low pH value is indicative of an acidic solution which shifts to basic as the pH value increases. The honey samples' pH ranged from 4.2 recorded by Arabuko Sokoke honey to 4.8 for Kakamega honey (Figure 22-B). The recorded range was similar with pH values of honey reported in other studies; honey from Kenya and New Zealand reported mean pH values of 4.8, and 4.5, while the pH of Romanian and Cuban honey ranged from 3.88 to 6.39 and 3.2 to 4.8, respectively (Morroni *et al*., 2018; Oroian *et al*., 2017). A comparison of the mean pH values among the sampled honey showed variation among the samples (ANOVA, $p < 0.001$). Generally, honey has a low pH due to the presence of phenolics and organic acids such as gluconic acid produced through the oxidation of glucose by glucose oxidase (Berenbaum & Calla, 2021; Nicolson, 2011). Additionally, honey's pH is influenced by the harvesting and storage methods. The low pH creates an unconducive environment for bacterial survival. Most bacterial are neutrophiles which survive in neutral pH 7, therefore the low acidic pH curtails their survival contributing to the antibacterial effects of honey (Zarei *et al*., 2019).
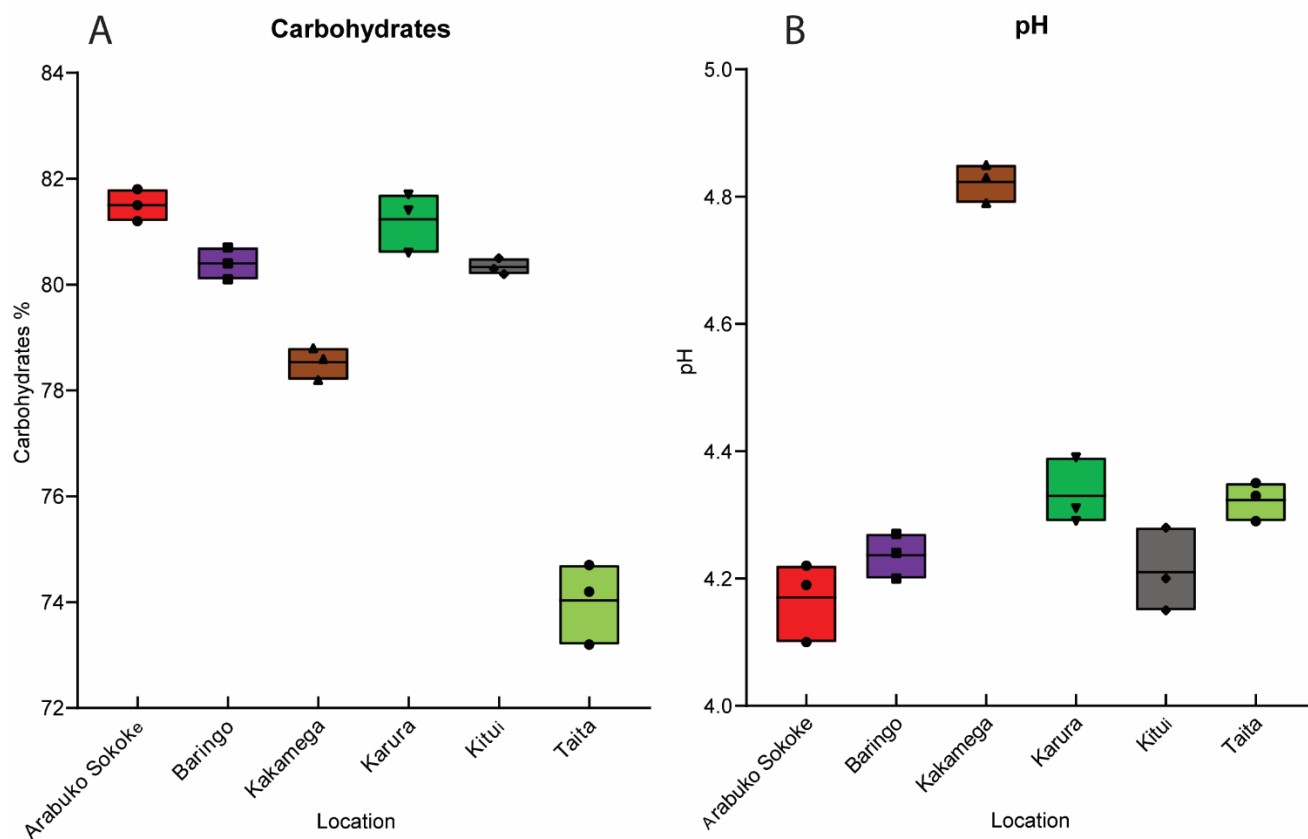
Figure 22: Boxplots showing the physicochemical composition of the honey. (A) Carbohydrates and (B) pH. The horizontal line in the boxes shows the mean while the black dots represent the data points.

4.4.2 Phytochemicals present in Honey

The phenolic concentration recorded had an extensive variability among honey samples (ANOVA, $p < 0.001$). Arabuko Sokoke honey had the highest concentration of 186 mg GAE/mg, while Karura recorded the lowest concentration of 42.5 mg GAE/100 g (Figure 23-A). The results were similar to other studies by (Kolayli *et al.*, 2016; Morroni *et al.*, 2018), which reported TPC ranges of 70 to 105 mg GAE/100 g and 43.16 to 126.84 mg GAE/100 g, respectively. The volatile chemical profile revealed the presence of phenolic compounds such as p-cresol and butylated hydroxytoluene in Manuka 5+ honey which was used as a positive in assessing the antibacterial potential of the honey and 2,4-bis(1,1-dimethylethyl)-phenol in Kitui honey. Specifically, phenols in honey are responsible for hydrogen atom donation which scavenges for free radicals enhancing honey antioxidant activity.

Moreover, studies on phenolic compounds such as chrysin, cinnamic acid, *p*-hydroxibenzoic acid, naringenin, and pinocembrin have shown they possess antibacterial activity against a wide range of bacteria. This activity is majorly influenced by the length of the alkyl chain and number of hydroxyl and methoxy groups they possess (Estevinho *et al*., 2008; Kumar & Goel, 2019; Morroni *et al*., 2018).

The mean flavonoid concentration recorded for the honey samples varied significantly across the different honey (ANOVA, $p < 0.001$). The concentration ranged from 129.2 mg QE/100 g, which was the highest and recorded by Kitui honey to 42.0 mg QE/100 g the lowest, recorded for Karura honey (Figure 23-B). In comparison, the recorded flavonoid concentrations were within the same range and even higher than concentrations reported in other research. For instance, (Mokaya, *et al*., 2020a) recorded a range of 13.47 and 73.02 mg QE/100 g for the honey sample used in the study, while (Velásquez *et al*., 2019) recorded a range of 43 to 90 mg QE/100 g. The flavonoids are part of the polyphenols present in honey which are mostly responsible for honey's antioxidant activity. However, studies have also shown that the phytochemicals present in honey like flavonoids (chrysin, hesperetin, pinocembrin and hesperidin) enhance its bioactive property and quality as they are associated with honey's anti-cancer, anti-inflammatory, anti-mutagenic, neuro-protective, antidiabetic and antibacterial activity (Kumar & Goel, 2019; Kuś *et al*., 2016). Flavonoids antibacterial effect is a result of inhibition of DNA gyrase, bacteria energy metabolism and cytoplasmic membrane function (Mieles *et al*., 2022)
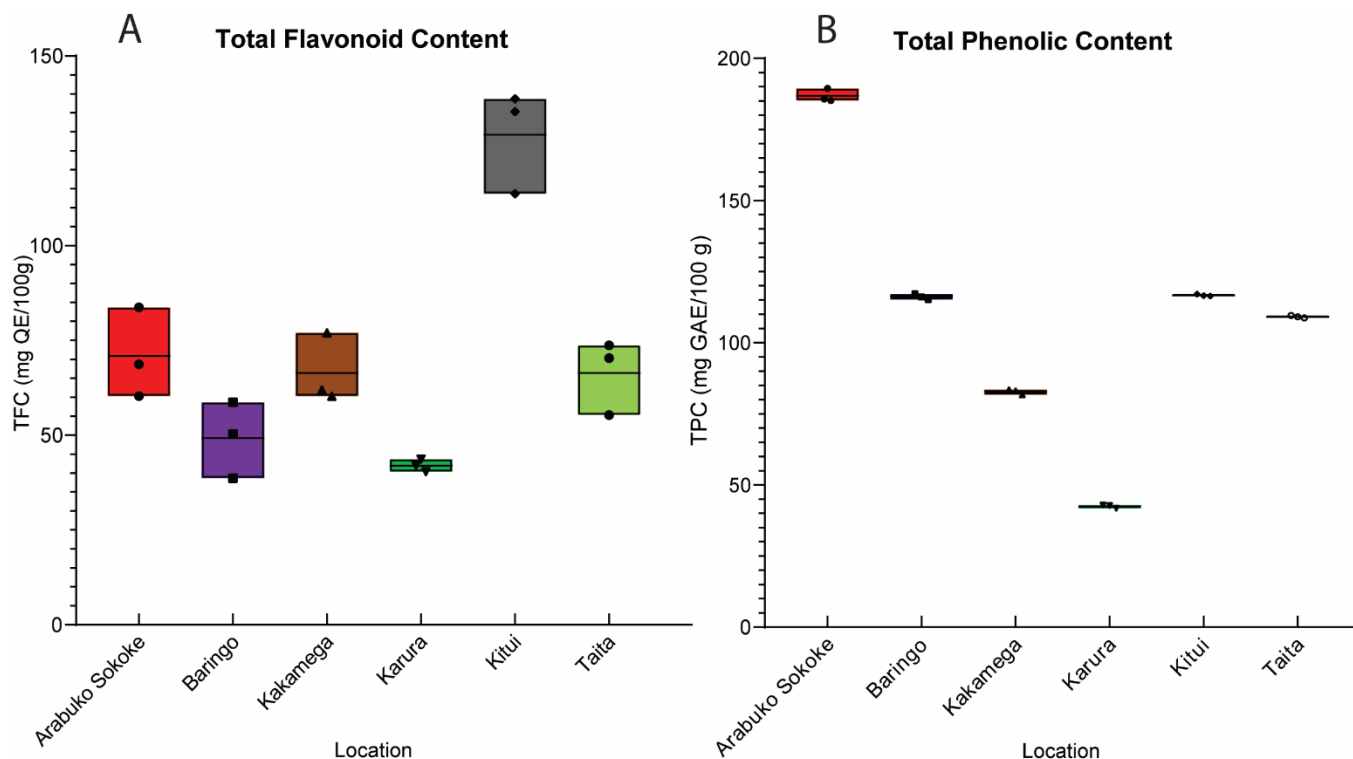
Figure 23: Boxplot representation of the total phenol and flavonoid content honey. The horizontal line across the boxes represent the mean value while the dots represent individual data points.

4.5 Chemical Profile of Honey

Headspace extraction of volatiles in honey was conducted and the trapped volatiles analyzed and identified using GC-MS. Additionally, the chemical profile of honey was examined through GC-MS analysis of honey's solvent extract using DCM to assess the composition of non-volatile chemical species. The honeys were found to possess a diverse range of compounds belonging to different chemicals classes.

4.5.1 Honey Volatile and Non-volatile Chemical Profiles

Honey's volatile chemical profiles were analyzed to understand their chemical diversity and abundances of the available compounds. Moreover, the analysis enabled the identification of possible potential compounds influencing the preferential choice of honey by the diseased bees and the honey's antibacterial activity. The analysis of the trapped volatiles yielded a total of 108 compounds across all honey samples of which 2 alkanes were shared among all the six honey samples: 4-methyloctane and 2,4-dimethylheptane.

The compounds were classified into various chemical classes including; aliphatic, cyclic and aromatic hydrocarbons, halocarbons, esters, ketones, alkaloids, aldehydes, acids, terpenoids, phenols, terpenes, amides, alcohols, nitrogen containing compounds, oxolanes, benzene derivatives, phenothiazines, chromenes. acridones, adipates and acyloins (Table 20). Arabuko Sokoke, Baringo, Kakamega, Karura, Kitui and Taita honey samples recorded 40, 27, 62, 22, 70 and 13 volatile compounds, of which, 5, 5, 14, 4, and 20 were specific to each honey, respectively. Kitui honey had the highest number of volatile compounds of which more than 18% of the total volatiles were unique to it.

Analysis of the major chemical classes regarding their abundance, variation in occurrence among the honey samples, and their influence on the observed change in honey preference by the healthy and diseased honeybees was done and presented in a principal component analysis (PCA) biplot. Principal component (PC) 1 and PC 2 accounted for 84.4% of the observed variation among the chemical classes. Most of the compound belonging to the major chemical classes were detected in Kitui and Kakamega honey samples as shown by the clustering of the chemical classes on the right side of PC 1 and distributed on the top and bottom quadrant around Kitui and Kakamega honey, respectively (Figure 24-A). A majority of the compounds belonging to the recorded chemical classes were found in abundance in Kitui honey (Table 10). Among the major chemical families found in Kitui honey included: terpenes, terpenoids, alkanes, aldehydes, alcohols, and phenols.

Additionally, PC analysis showed the distribution of the identified volatile compounds among the honey samples and their influence on the observed change in honey preference by the diseased bees (Figure 24-B). PC 1 and PC 2 accounted for 75.5% of the variation observed in the distribution of compounds among the honey samples. Similar to the chemical classes, a majority of the volatile compounds were present in Kitui and Kakamega honey as shown by the clustering patterns on the right side of PC 1 in the top and bottom quadrants, respectively (Figure 24-B). Some compounds were also found in Arabuko Sokoke honey and a few shared among Baringo, Karura and Taita honey. The distribution of the compounds was based on their relative abundance and occurrence in honey with respect to their influence in the choice of honey by the bees. The preference for a particular honey by the honeybees in the pair was influenced bythe compounds it posseses. The biplot shows β-pinene, γ-amorphene, sibinene, 2,4-dimethyl-1-heptene, and 2,5-bis(1,1-dimethyl)-phenol having a strong influence on the choice of Kakamega honey.

Similarly, some of the compounds recorded in Kitui honey, including: cedrol, α- and β-cedrene, p-cymene, camphene and trans-2-dodecen-1-ol influenced its choice by the bees (Figure 24-B). When compared to the other honey, Kitui honey possessed a majority of the major volatile compounds indicating the possible reason for its strong preference by diseased bees.
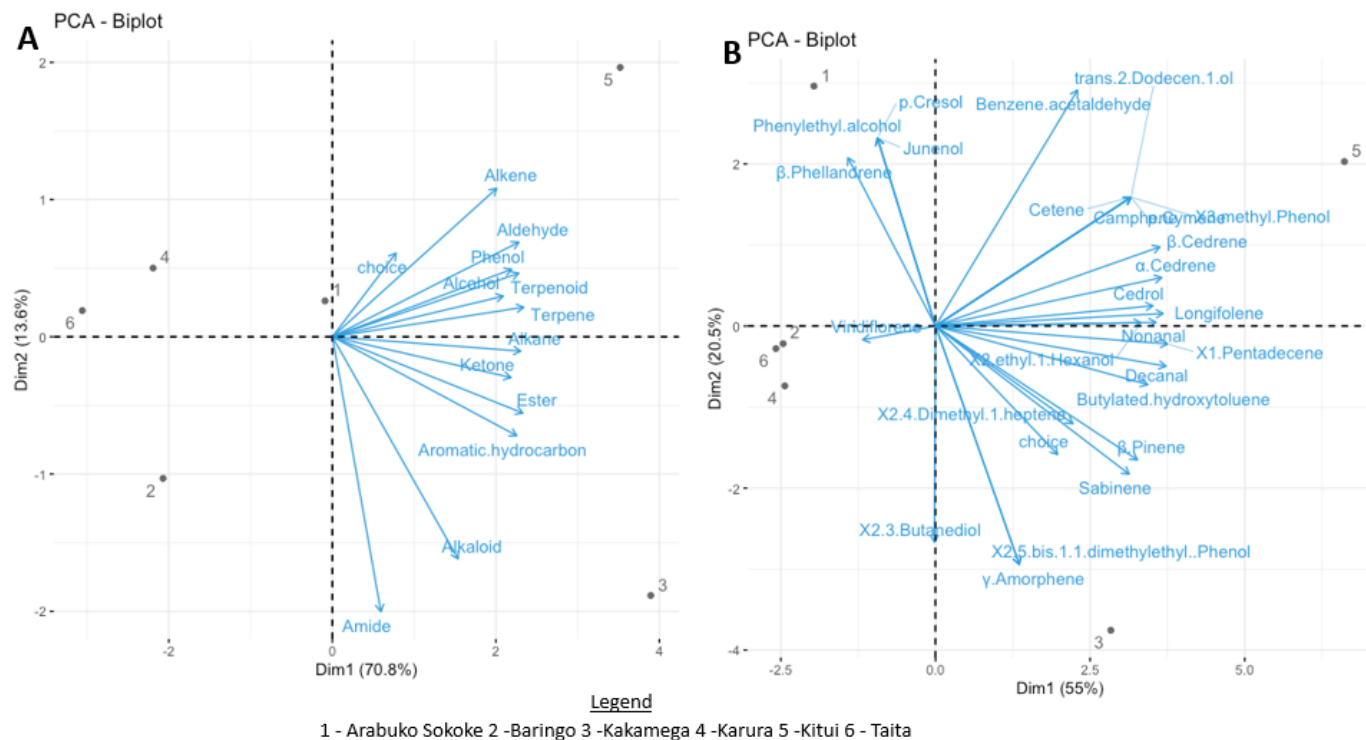
Figure 24: PCA bi-plots showing (A) distribution of major chemical classes among the honey samples and (B) the distribution of major volatile chemical compounds among the honey samples.

Table 10: Chemical profile of the volatile antibacterial compounds in honey color coded to match their source honey. ***Bold and italicized*** compounds solely occur in one honey, black and straight compounds are present in multiple honey while underlined compounds possess antibacterial activity. R_T is the retention time of compounds while ABA represents the antibacterial activity.

R_T	Compounds	Arabuko Sokoke	Baringo	Kakamega	Karura	Kitui	Taita	Classification	ABA
				Relative abundance 10^7					
4	2-Butenoic acid, methyl ester				0.16	0.03		Ester	Yes
6.3	2,3-Butanediol	0.29		1.74	1.41	0.03	0.19	Alcohol	Yes
8	*5,15-dimethylnonadecane*				*0.02*			Alkane	Yes
8	*2,3-dimethylheptane*					*0.07*		Alkane	Yes
8.2	4-methyloctane	0.33	0.07	1.44	0.15	0.63	0.11	Alkane	Yes
8.3	*o-Xylene*			*0.1*				Aromatic hydrocarbon	Yes
8.3	p-Xylene	0.05			0.03	0.09		Aromatic hydrocarbon	Yes
8.3	*1,3-dimethylbenzene*		*0.03*					Aromatic hydrocarbon	Yes
8.9	Pyridine, 3-(1-methyl-2-pyrrolidinyl)			0.09			0.01	Alkaloid	Yes
10.1	*Camphene*					*0.05*		Terpene	Yes
10.6	Sabinene		0.02	0.31		0.19		Terpene	Yes
10.7	β-Phellandrene	0.1			0.04			Terpene	Yes
10.7	β-Pinene		0.02	0.27		0.19		Terpene	Yes
11.5	2,5-dimethylnonane			0.58	0.01			Alkane	Yes
11.7	o-Cymene	0.11		0.47				Aromatic hydrocarbon	Yes
11.7	*p-Cymene*					*0.28*		Aromatic hydrocarbon	Yes

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11.9	*Pantolactone*					0.77		Butyrolactone	Yes
12	Benzene acetaldehyde	0.4				0.63		Aldehyde	Yes
12.5	Acetophenone	0.04				0.17		Ketone	Yes
12.5	Linalool oxide	0.2	0.22		0.08	0.54		Oxolane	Yes
12.6	*p-Cresol*	2.24						Phenol	Yes
12.6	*3-methyl-phenol*					0.31		Phenol	Yes
13	Nonanal	0.21		0.52		0.67		Aldehyde	Yes
13.2	*Phenylethyl alcohol*	0.37						Alcohol	Yes
14.5	Dodecane			1.93		0.79		Alkane	Yes
14.7	Decanal			0.81		1.26		Aldehyde	Yes
15.2	*Untriacontane*					0.44		Alkane	Yes
15.9	Pentadecane	1.18	0.14	0.23		1.02		Alkane	Yes
15.9	*Hexadecane*				0.04			Alkane	Yes
16.6	Nonadecane	0.32	0.57		0.18	1.12	0.07	Alkane	Yes
16.7	*3-hydroxy-4-phenyl-2-butanone*			1.14				Ketone	Yes
17.3	Tetradecane	0.6		1.33	0.15	2.56	0.12	Alkane	Yes
17.5	*trans-2-dodecen-1-ol*					0.81		Alcohol	Yes
17.6	*γ-Amorphene*			0.32				Cadinene	Yes
17.6	Longifolene	0.15		0.44		0.69		Terpene	Yes
17.7	α-Cedrene	0.46		1.08		2.63	0.12	Terpene	Yes
17.8	β-Cedrene	0.22		0.41		1.33		Terpene	Yes
18	*Diisopropyl adipate*					0.64		Ester	Yes
18.3	2,5-di-tert-butyl-1,4-benzoquinone	0.66	0.43	*1.03*				Quinone	Yes
18.7	Docosane	0.77	0.24	*4.67*	0.08	1.86		Alkane	Yes
18.9	Butylated hydroxytoluene		1.25	2.71	0.55	5.62	0.18	Phenol	Yes
19.8	Hexadecane	1.2	0.6	0.66		1.59		Alkane	Yes
20.1	Cedrol	0.52	0.31	1.2		1.83		Alcohol	Yes
20.3	*Junenol*	0.42						Alcohol	Yes

No.	Compound							Class	
21	Heptadecane	0.42		3.16		1.64		Alkane	Yes
21.1	Tricosane	0.68		1.09	0.16			Alkane	Yes
21.6	Heneicosane	0.1				1.12		Alkane	Yes
21.6	Tetracosane	0.29	0.52	0.54		0.67		Alkane	Yes
22	Octadecane	0.68	0.31	1.16		1.05		Alkane	Yes
22.9	Phthalic acid, isobutyl nonyl ester	*0.14*		*4.75*				Ester	Yes
23.9	Dibutyl phthalate	0.23				1.08		Ester	Yes
25	Eicosane	0.93	0.43	0.82	0.27	0.21		Alkane	Yes
25.5	Corydaldine			0.21		0.01		Alkaloid	Yes
26.5	Acetyl tributyl citrate			0.03		0.15		Ester	Yes
31	2-Trifluoromethyl-7-phenothiazone			0.21		0.01		Phenothiazine	Yes
31.3	1,2-Dimethoxy-4-(1-methoxy-1-propenyl)benzene			0.06			0	Phenyl propanoid	Yes
36.1	3,4,5-trimethoxy- N-cyano-Benzenamide			0.22		0.01		Amide	Yes
36.8	5-Methyl-2-N-methylaminobenzophenone semicarbazone			0.22		0.01		Amide	Yes

Additional compounds found in the volatile profiles of the honey tested are recorded in (Appendix 6).

For the non-volatile chemical analysis, a total of 61 compounds from the six honey were detected and identified (Table 11). The total number of compounds in each honey were 22, 21, 51, 24, 24 and 22 recorded for Arabuko Sokoke, Baringo, Kakamega, Karura, Kitui, and Taita, respectively. Variability was observed among the chemical profiles of the different honey. However, some compounds co-occurred in two or more honey while others occurred exclusively in a particular type of honey. The compounds shared among all the honey were majorly alkanes, these included; 2,4-dimethyl-heptane, tetratriacontane, nonacosane, octadecane, hexacosane, heneicosane, heptacosane, tricosane, tetracosane, pentacosane, nonadecane, dotriacontane, and eicosane. Few unique compounds were observed for each honey. Arabuko Sokoke had octadecanal, Baringo had tridecane and Kitui honey had cedrol, each having one unique compound while Karura and Taita honey had none. Kakamega honey had a majority of compounds, 29 solely occurring in its chemical profile, some of these include: (Z)-9-hexadecen-1-ol, 10-methyl-eicosane, acetic acid, [4-(1,1-dimethylethyl) phenoxy]-methyl ester, 1-methyl-pyrrolidine and 1H,1H,2H,2H-perfluorooctan-1-ol among others.

Different chemical classes were detected in the honey samples. Ketones, hydrocarbons, alcohols, terpenes, aromatic hydrocarbons, phenols, aldehydes, esters, terpenoids, amines, acids, ethers, pyrollidines, halocarbons, pyrazzines, pyranones, amides, and anthracenes were among the chemical classes present in the honey chemical profiles. The variability of the major chemical classes among the honey samples were assessed using PCA and visualized on a biplot. Both PC 1 and PC 2 cumulatively accounted for 71.5% of the variability observed among the major chemical classes. Aldehydes and terpenes were abundant in honey from Arabuko Sokoke and Kitui, respectively with the Taita honey also having terpenes and phenols. Honey from Kakamega had multiple non-volatile classes including, amines, esters, terpenoids and ketones. Karura honey was abundant in phenols (Figure 25-A). The biplot also shows that phenols, alcohols, and alkanes had a significant contribution to the antibacterial activity of the honey.

A PCA of the major non-volatile chemical compounds showed variation among honey with PC 1 and PC 2 accounting for 77.7% of the observed variability (Figure 25-B). The compounds were related to the antibacterial activity of honey. Cedrol and 2,5-bis(dimethylethyl)-phenol had strong contribution to the antibacterial potential of the honey.

Cedrol occurred in Kitui honey while Kakamega honey had a majority of the compounds including, 5-methyl-2-hexanol, 1-heptacosanol, hexadcen-1-ol, and 2,3-butanediol.
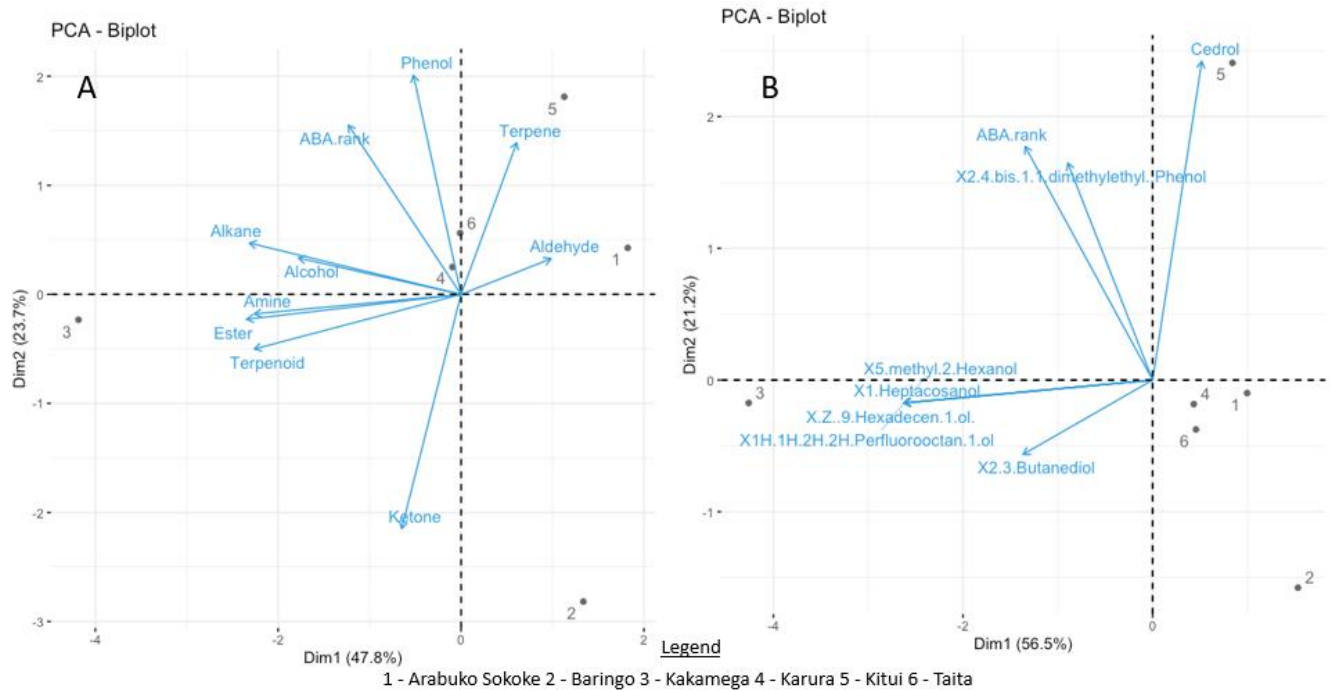
Figure 25: PCA bi-plots showing (A) distribution of major non-volatile chemical classes among the honey samples and (B) the distribution of major non-volatile chemical compounds among the honey samples, ABA.rank represents the antibacterial activity of honey.

Table 11: Chemical profile of non-volatile antibacterial compounds in honey color coded to match their source honey. ***Bold and italicized*** compounds solely occur in one honey, black and straight compounds are present in multiple honey while <u>underlined</u> compounds possess antibacterial activity. R_T is the retention time of compounds while ABA represents the antibacterial activity.

R_T	Compound	Arabuko Sokoke	Baringo	Kakamega	Karura	Kitui	Taita	Classification	ABA
3.9	***<u>3-Hydroxy-2-butanone</u>***			***5.13***				Ketone	Yes
6.3	<u>2,3-Butanediol</u>			3.04	2.92		4.81	Alcohol	Yes
7.5	<u>2-methyl-3-heptanone</u>			0.76		0.1		Ketone	Yes
8.2	***<u>5-methyl-2-hexanol</u>***			***0.44***				Alcohol	Yes
10.1	***<u>Sulfurous acid, cyclohexylmethyl hexadecyl ester</u>***			***0.28***				Ester	Yes
16.7	***<u>3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy</u>***			***0.05***				Amine	Yes
18	***<u>1-methyl-pyrrolidine</u>***			***0.01***				Amine	Yes
18.7	***<u>10-methyl-eicosane</u>***			***1.06***				Alkane	Yes
18.8	2,4-bis(1,1-dimethylethyl)-phenol	1.1		0.98	0.38	1.07	1.06	Phenol	Yes
19.6	***S-(Propoxythiocarbonyl)thiohydroxylamine***			***0.02***				Amine	Yes
19.8	***<u>1H,1H,2H,2H-Perfluorooctan-1-ol</u>***			***0.01***				Alcohol	Yes
19.9	***N-(5-Aminopentyl)-oxalamic acid***			***0.01***				Acid	Yes
20	Hexadecane	0.12		0.94	2.49	0.08		Alkane	Yes
20.1	*Cedrol*					17.25		Alcohol	Yes
20.3	***2,6,10,14-tetramethyl-hexadecae***			***0.2***				Alkane	Yes
20.6	***<u>Menthofuran</u>***			***1.54***				Terpenoid	Yes
20.9	Heptadecane	0.48	0.23	1.06	0.37		1.56	Alkane	Yes
21.7	***2-Methyl-3-isobutoxy-5-propargylcyclopent-2-en-1-one***			***0.09***				Ketone	Yes

RT	Compound							Class	
21.8	*Tricosyl pentafluoropropionate*			0.11				Ester	Yes
21.8	Tetratriacontane	0.05	0.17	0.11	0.21	1.01	0.59	Alkane	Yes
21.9	*4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-butanone*			0.44				Ketone	Yes
22	*1-(ethenyloxy)-octadecane*			0.06				Ether	Yes
22	*5,6,6-trimethyl-undeca-3,4-diene-2,10-dione*			0.05				Ketone	Yes
22.4	*2-methoxy-3-(2-methylpropyl-)-pyrazine*			0.08				Pyrazine	Yes
22.4	*3,5-dimethyl-4H-pyran-4-one*			0.29				Pyrone	Yes
22.5	*6-Chlorobicyclo[2.2.1]hept-2-ene-2-carboxylic acid, methyl ester*			0.15				Ester	Yes
22.8	*Sulfurous acid, butyl hexadecyl ester*			0.06				Ester	Yes
24.1	*2-methyl-octadecane*			0.07				Alkane	Yes
24.3	*(Z)-9-Hexadecen-1-ol*			0.3				Alcohol	Yes
24.4	2,6,10-trimethyl-Tetradecane		0.16	0.07	0.19			Alkane	Yes
24.7	Octadecane	0.24	1.3	24.99	3.48	2.36	1.71	Alkane	Yes
24.8	Hexacosane	0.81	0.11	1.16	1.3	1.76	0.67	Alkane	Yes
25	Heneicosane	4.62	2.13	1.18	4.42	1.51	3.83	Alkane	Yes
26.7	Tricosane	1.15	1.44	9.87	1.05	1.38	1.01	Alkane	Yes
26.8	Untriacontane	0.47	0.18		0.26	0.39	0.03	Alkane	Yes
27.6	Tetracosane	1.68	1.79	1.56	2	1.8	1.88	Alkane	Yes
28.4	Pentacosane	4.05	3.47	8	5.49	3.99	2.95	Alkane	Yes
28.9	Nonadecane	3.08	2.39	2.57	2.07	1.36	3.22	Alkane	Yes
29.7	*N-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxamide*			0.3				Amide	Yes
29.8	*1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline*			0.35				Alkaloid	Yes
29.9	Docosane	3.56	2.39		5.93	4.72	3.47	Alkane	Yes
30.1	Tritriacontane	1.09	0.74	1.62		2.69	1.7	Alkane	Yes

30.3	Dotriacontane	1.62	1.55	1.88	1.22	1.11	1.36	Alkane	Yes
31.5	*1-Heptacosanol*			0.53				Alcohol	Yes
31.7	Eicosane	6.51	7.63	16.24	9.08	8.02	21.67	Alkane	Yes
33.5	*9,10-diethyl-9,10-dihydro-anthracene*			0.03				Anthracene	Yes
33.7	*4-Hydroxyphenyl pyrrolidinyl thione*			0.06				Ketone	Yes
35.8	*Acetic acid, [4-(1,1-dimethylethyl)phenoxy]-, methyl ester*			0.34				Ester	Yes

Additional compounds found in the non-volatile profiles of the honey tested are recorded in (Appendix 7).

The major volatile and non-volatile compounds, the antibacterial activity of the honey and the change in choice of honey by the healthy and diseased bees were further analyzed using PCA to identify the compounds strongly contributing to these factors and their source honey. The PC 1 and PC 2 explained 76.5% of the variability in the volatile and non-volatile compounds responsible for the antibacterial activity and influencing the choice of honey by the bees.

The volatile compounds sabinene and β-pinene classified as terpenes, 2,4-dimethyl-1-heptene, an alkene and 2,5-bis(1,1-dimethylethyl)-phenol, a phenol influenced the change in choice of honey by the healthy and diseased bees (Figure 26). Healthy honeybees had no significant preference for any of the honey tested in the choice experiment. However, when infected with the opportunistic bacteria *S. marcescens*, a significant preference for Kitui honey was observed (Figure 19). Additionally they spent averagely less time to contact a chosen honey when diseased in comparison to when healthy (Figure 18). The change in choice when diseased favored Kitui honey which was predominantly and significantly chosen in two pairings compared to the same pairs by healthy bees. Insects including honeybees have been observed to changed their foraging patterns to contact food with nutraceutical property as a therapeutic measure (Abbott, 2014; Roode & Hunter, 2019; Simone *et al*., 2009).
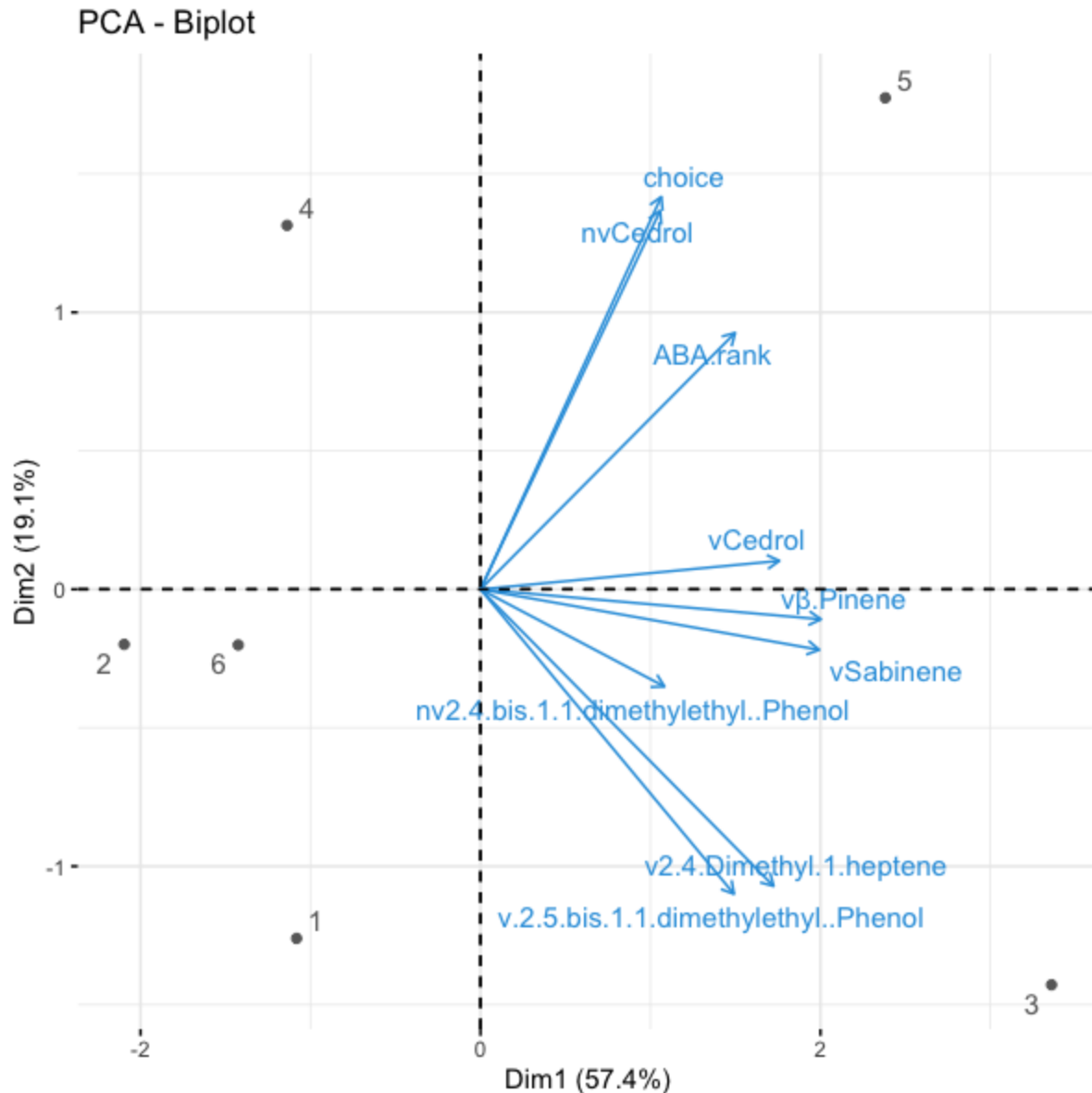
Figure 26: PCA bi-plots showing the interaction among volatile and non-volatile compounds, the antibacterial activity of the honey (ABA.rank) and the change in choice of honey by the healthy and diseased bees (choice). The prefix v are volatile and nv – non-volatile.

In this study, the probable compounds influencing the change in choice of honey were identified. Terpenes are commonly found in essential oils from plants and are widely documented in literature to have antimicrobial property (Trombetta *et al*., 2005). In a study analyzing the essential oils of Aguaribay, fractions containing a mixture of β-pinene and sabinene and another containing sabinene alone were assayed for their antibacterial activity against *Staphylococcus aureas*,

Pseudomonas aeruginosa and *E. coli*. Both fraction registered antibacterial activity against all the tested bacteria. The mixed fraction had an additional effect registering an enhanced antibacterial activity against the bacteria compared to the sabinene alone (Rocha *et al*., 2012). Additional evidence of the antibacterial activity of β-pinene and sabinene is registered in a study analyzing the antibacterial effect of essential oil from various parts of *Ferula ovina* against Methicillin-resistant *Staphylococcus aureus* (MRSA). The major components of the essential oils were α- and β-pinene and sabinene, which were independently tested for their antibacterial activity against MRSA. All the terpenes recorded antibacterial activity against MRSA with the best activity recorded by sabinene (Utegenova *et al*., 2018). The alkene 2,4-dimethyl-1-heptene obtained from brown algea has recorded antibacterial activity against a range of bacteria *B. subtilis, S. aureus*, and MRSA (Demirel *et al*., 2009). The alkene 2,5-bis(1,1-dimethylethyl)-phenol has been recorded in the chemical volatile profile of *Actinobacteria* from an unexplored forest in India. When tested for its biological activity, it was found to have antibacterial activity (Das *et al*., 2018). The volatile chemical compounds influencing the change in choice of honey by the healthy and diseased bees were all found to have antibacterial activity recorded in multiple studies. The bees changed their choice to contact food with antibacterial activity to alleviate the infection as a therapeutic measure.

Non-volatile compounds including cedrol found in high abundance in Kitui honey and 2,4-bis(1,-dimethyl) phenol majorly found in Kakamega honey (Figure 26), but both also occurring in smaller abundances in the other honey (Table 11), were identified as the major compounds responsible for the honey's antibacterial activity. Cedrol is a sesquiterpene alcohol commonly found in cedar wood and the essential oil obtained from conifers of the genus *Cupressus* and *Juniperus* but it has also been found in the chemical profile food of crop plants such as sorghum (Khwatenge, 1999). Additionally, cedrol has also been found as a major constituent in chemical volatile profiles of three Anatolian propolis whose extracts were bactericidal in low concentrations against 10 bacterial food contaminants (Esin *et al*., 2013). In another study testing the antimicrobial activity of bamboo leaf essential oil, cedrol was identified as one of major alcohol present in the leaf oil. It was further tested for antimicrobial activity against eight microbes including *E. coli* and *Bacillus subtilis*. Cedrol inhibited the growth of all the bacteria at low concentrations of 2.5 mg/mL, showing the significant contribution of cedrol to the bamboo leaf essential oil antimicrobial activity (Tao *et al*., 2018).

Additionally, Wang *et al*., (2020) extracted, characterized and positively identified cedrol from the stems and roots of *Thuja sutchuenensis* and tested its antibacterial activity against *Bacillus cereus* and *Staphylococcus epidermids*. Cedrol registered antibacterial effect against the two bacteria at low concentrations (Wang *et al*., 2020). Finally, Lindh *et al*. (2015) reported that cedrol attracts mosquitos to oviposition due to its microbial activity. These studies among many other have proven cedrol's antibacterial activity and occurrence in plant extracts and products giving it a high possibility to be consumed and end up in honey as bees forage or even introduced into the honey from the propolis as Kocabas *et al*. (2013) has shown its presence in propolis. Phenols have been widely characterized in honey from diverse geographical location and floral sources (Santos-Buelga & González-Paramás, 2017). Although different types of phenolic compounds have been registered in honey as they highly depend on the floral sources, generally, most of the phenolic compounds found in honey have registered antibacterial activity (Esin *et al*., 2013; Kumar & Goel, 2019; Velásquez *et al*., 2019; Mieles *et al*., 2022).

In this study, 2,4-bis(1,-dimethyl) phenol, a phenolic compound was detected in the non-volatile chemical profile of the honey and was found to strongly associated with the antibacterial activity of the honey (Figure 25B; Figure 26). The compound 2,4-bis(1,-dimethyl) phenol is widely documented as an antifungal agent (Rangel-Sánchez *et al*., 2014). However, its antibacterial activity has also been proven. In recent research, 2,4-bis(1,-dimethyl) phenol was isolated from *Streptomyces* during fermentation and its antibacterial activity tested against a wide range of gram positive and gram negative bacteria producing the best antibacterial activity compared to the rest of the isolates (Saravana Kumar *et al*., 2014). The antibacterial activity of 2,4-bis(1,-dimethyl) phenol in addition to cedrol majorly contributed to the antibacterial activity of the honey against the pathogenic bacteria. The varied prophylactic and therapeutic antibacterial activity of the honeys observed on the honeybees parasitized by the two bacteria (*E. coli and S. marcescens*) is partly as a result of the presence of antibacterial cedrol and 2,4-bis(1,1-dimethyl)-phenol in the honey, which have the ability to alleviate bacterial infection.

CHAPTER FIVE

5.1 Conclusion

The different honey exhibited excellent and promising both short and long term prophylactic and therapeutic effects against the two bacteria (*E. coli* an ubiquitous pathogenic bacterium that bees interact with as they forage and *S. marcescens,* a gut microbiome opportunistic resident) used in this study. The honeys' non-volatile and volatile chemical profiles were diverse but harbored compounds with antibacterial activity further cementing the benefit of honey in colony health. The chemical components with major effect on the antibacterial activity of the honey were identified as cedrol and 2,4-bis(1,1 dimethyl) phenol.

The choice experiment assay showed that Kitui honey's volatile richness with compounds possessing antibacterial activity was the probable cause for the preferential choice of honeybees depending on disease status proving that their diet is a go-to natural 'pharmacy' when infected. The volatile chemical compounds influencing the change in choice of the honey were also identified: sabinene, β-pinene, 2,4-dimethyl-1-heptene and 2,5-bis(1,1-dimethylethyl)-phenol.

The phytochemical and physicochemical analysis of honey ascertained their quality due to the presence of beneficial antibacterial phytochemicals such as phenols and flavonoids. The honey tested portrayed variable physicochemical content which were within the honey quality parameters set by the International Honey Commission (IHC, 2009). These parameters, such as the high sugar content and low acidity also contribute to the antibacterial potential of honey.

Generally, the ability of honey to be used as an alternative natural antibiotic in prevention and treatment of bees' bacterial diseases was demonstrated. This will create a steady pathway to the discontinuation of usage of antibiotics on bee colonies enhancing their health and quality of their products and most importantly curb the emergence and spread of pathogenic antibiotic restraint bacteria.

5.2 Recommendations

Based on the finding of the study, the following are recommended;

1. Additional research with more honey samples and honeybee pathogenic bacterial candidates should be done to further assess the prophylactic and therapeutic antibacterial potential of honey against multiple bee bacterial pathogens.

2. Sensitive analytical methods like gas chromatography coupled with electro electroantennographic detector (GC-EAD) should be used to test the response of healthy and diseased honey to the compounds influencing the choice of honey.

3. Florals source of the honey should be identified and the nectar analyzed in comparison with the honey. The floral source(s) from which honey with excellent prophylactic and therapeutic ability are sourced could be a relief to apiculture and a pointer towards more beneficial plants for bee keeping.

4. Investigation into the bee-pathogen, microbiota-pathogen, and honey-pathogen interaction and the bees' *in vivo* physiological process when healthy and diseased should be done to help understand the interaction among the bee, its diet and the pathogen in both healthy and diseased status to better explain the role of diet in disease prevention and cure.

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Appendix 1

Table 1. *In vitro* antibacterial analysis of the honey showing inhibition zones for every honey against the bacteria tested in mm^2

Antibacterial Mean ± SD					
Samples	*E. coli*	*E. aphidicola*	*B. apis*	*B. thuringiensis*	*L. apis*
Arabuko Sokoke	937.5 ± 18.7 **b**	856.2 ± 18.9 **b**	878.0 ± 25.4 **bc**	975.9 ± 60.5 **b**	255.3 ± 27.6 **b**
Baringo	854.8 ± 38.0 **cd**	856.7 ± 35.3 **b**	850.3 ± 14.7 **c**	976.7 ± 34.4 **b**	176.0 ± 12.4 **c**
Kakamega	916.1 ± 17.6 **bcd**	1,189 ± 16.5 **a**	919.0 ± 23.3 **ab**	981.9 ± 6.7 **b**	323.1 ± 15.2 **a**
Karura	925.7 ± 13.6 **bc**	875.7 ± 12.7 **b**	955.4 ± 19.0 **a**	1,126 ± 25.6 **a**	296.9 ± 7.5 **ab**
Kitui	1,056 ± 47.9 **a**	881.4 ± 11.0 **b**	942.2 ± 32.3 **ab**	1,001 ± 35.6 **b**	272.6 ± 22.6 **b**
Taita	841.5 ± 24.5 **d**	887.3 ± 21.7 **b**	879.0 ± 27.7 **bc**	940.7 ± 35.7 **b**	272.13 ± 4.5 **b**
P (α= 0.05)	<0.001	<0.001	<0.01	<0.001	<0.001

The data are expressed as mean ± S.D (n = 3); ± refers to the standard deviation, the different letters within the rows indicate statistically significant differences determined using Tukey's test (p < 0.05)

Appendix 2

Table 2: Choice experiment for healthy bees

No	Left	None	Right	1st decision	Time (s)			
**Choice Experiment - Healthy Bees**								
No	**Choice**			**1st decision**	**Time (s)**			
	Left	**None**	**Right**					
	Empty		**Empty**			**Median**		
1	■			0.33.87	34	**14.5**		
2			■	0.14.64	15			
3	■			0.36.57	37			
4			■	0.09.45	9			
5			■	0.09.56	10		**Mean**	
6	■			0.14.09	14		**Empty Left**	**Empty Right**
7			■	0.23.23	23		**24.4**	**13.4**
8	■			0.12.98	13			
9	■			0.24.45	24			
10			■	0.09.55	10			
	Lemon grass oil		**Empty**			**Median**		
1		■		5.00.00	300	**68.5**		
2	■			0.19.57	20			
3	■			02.74.46	194			
4	■			01.18.87	79			
5	■			01.05.97	66		**Mean**	
6	■			01.10.52	71		**Lemon grass**	**Empty**
7	■			0.58.34	58		**72.33**	**0**
8	■			01.23.65	84			
9	■			0.45.49	46			
10	■			0.33.24	33			
	Baringo		**Arabuko**			**Median**		
1	■			0.22.57	23	**31**		
2	■			0.39.08	39			
3	■			0.49.29	49			
4	■			0.41.02	41			
5			■	0.12.27	12		**Mean**	
6			■	0.30.01	30		**Baringo**	**Arabuko**
7	■			0.28.86	29		**31.45**	**35**

#	Kakamega		Arabuko	Value	Count	Median	Kakamega	Arabuko
8			■	0.49.98	50			
9			■	0.31.77	32			
10	■			0.09.21	9			
11	■			0.48.97	49			
12			■	0.09.26	9			
13			■	0.53.69	54			
14	■			0.21.67	22			
15	■			0.21.27	21			
16	■			0.19.27	19			
17			■	0.57.87	58			
18	■			0.44.76	45			
	Kakamega		**Arabuko**			**Median**		
1			■	0.31.77	32	**33.5**		
2	■			0.31.10	31			
3	■			0.36.69	37			
4			■	0.21.69	22			
5			■	0.49.71	50		**Mean**	
6			■	0.10.67	11		**Kakamega**	**Arabuko**
7	■			0.35.03	35		**42.67**	**34.33**
8	■			0.10.52	11			
9	■			0.11.84	12			
10			■	0.30.19	30			
11	■			0.46.50	47			
12	■			01.05.16	65			
13			■	01.00.94	61			
14	■			0.53.46	54			
15	■			0.07.40	7			
16	■			1.05.87	66			
17	■			2.01.07	121			
18	■			0.26.27	26			
	Kakamega		**Baringo**			**Median**		
1	■			0.19.00	19	**33**		
2	■			0.32.73	33			
3	■			01.53.65	114			
4	■			0.14.93	15			
5	■			0.22.41	22		**Mean**	
6		■		5.00.00	300		**Kakamega**	**Baringo**
7			■	0.50.83	51		**41.37**	**40.85**
8		■		5.00.00	300			

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#	Karura		Arabuko		Time	Count	Median		
9			■		2.49.99	170			
10	■				0.15.52	16			
11	■				0.12.16	12			
12			■		0.09.38	9			
13			■		0.56.42	56			
14			■		0.10.68	11			
15	■				1.07.48	67			
16	■				0.31.85	31			
17			■		0.19.33	19			
18			■		0.21.03	21			
19			■		0.34.24	34			
20			■		0.22.87	23			
21			■		0.09.53	10			
22	■				0.56.40	56			
23	■				0.25.36	25			
24	■				02.10.47	130			
25			■		0.53.40	53			
26	■				01.29.71	90			
27			■		0.48.69	49			
28			■		0.52.12	52			
29	■				0.18.18	18			
30	■				0.32.21	32			
31	■				0.08.92	8			
32			■		0.09.51	10			
33			■		01.16.66	77			
34			■		0.50.55	51			
35			■		0.29.84	30			
36			■		0.32.68	33			
37			■		0.53.98	54			
38			■		0.03.64	4			
39	■				0.09.79	10			
40	■				0.21.72	22			
41			■		0.40.77	41			
42		■			5.00.00	300			
43	■				1.06.13	66			
44		■			5.00.00	300			
	Karura		**Arabuko**				**Median**		
1	■				0.08.10	8	**31**		
2	■				0.56.42	56			
3	■				01.59.08	119			

#	Karura			Value	Sec			
4			■	0.27.56	28			
5	■			0.37.51	38		**Mean**	
6			■	0.47.22	47		**Karura**	**Arabuko**
7			■	0.30.74	31		**38.47**	**38.81**
8	■			0.29.84	30			
9	■			0.05.56	6			
10			■	01.12.40	72			
11			■	0.40.57	41			
12	■			01.10.92	71			
13	■			0.12.90	13			
14	■			0.11.19	11			
15	■			0.06.89	7			
16			■	0.38.93	39			
17	■			0.11.65	12			
18	■			0.26.68	27			
19		■		5.00.00	300			
20			■	1.07.49	67			
21			■	0.43.53	44			
22	■			0.25.00	25			
23			■	0.44.94	45			
24	■			0.28.61	29			
25	■			01.53.55	114			
26			■	0.18.12	18			
27			■	0.17.41	17			
28	■			0.59.92	60			
29	■			0.08.33	8			
30			■	0.30.83	31			
31			■	0.25.32	25			
32	■			01.24.71	85			
33			■	0.13.57	14			
34			■	0.44.97	45			
35	■			0.12.32	12			
36			■	0.57.37	57			
	Karura		**Baringo**			**Median**		
1	■			01.06.18	66	**29**		
2	■			0.23.49	23			
3	■			0.27.86	28			
4			■	01.00.40	60			
5			■	0.29.67	30			
6	■			0.39.33	39		**Mean**	
							Karura	**Baringo**

#	Karura	Kakamega	Time	Value		
7		■	0.18.25	18	**40**	**32**
8		■	0.43.35	43		
9		■	0.35.63	36		
10		■	0.26.43	26		
11		■	0.19.36	19		
12	■		0.27.49	27		
13	■		0.42.47	42		
14		■	01.24.90	85		
15		■	0.19.70	20		
16		■	0.07.27	7		
17	■		0.35.79	36		
18	■		0.34.21	34		
19	■		01.06.56	67		
20	■		0.15.67	16		
21	■		0.12.00	12		
22		■	0.08.21	8		
23	■		01.49.23	109		
24	■		0.21.44	21		
	Karura	**Kakamega**			**Median**	
1		■	0.19.44	19	**27**	
2	■		0.27.23	27		
3		■	0.56.13	56		
4		■	0.15.06	15		
5		■	0.28.90	29	**Mean**	
6	■		0.11.01	11	**Karura**	**Kakamega**
7		■	0.32.18	32	**32.36**	**29.78**
8		■	0.15.53	16		
9	■		0.34.46	34		
10	■		0.15.06	15		
11		■	0.36.97	37		
12		■	0.04.67	5		
13	■		1.23.46	83		
14		■	0.29.94	30		
15	■		0.12.19	12		
16		■	0.56.68	57		
17	■		01.04.70	65		
18	■		0.24.76	25		
19		■	0.59.31	59		
20		■	0.12.82	13		
21	■		0.27.02	27		

22			■	0.19.41	19			
23	■			0.40.71	41			
24	■			0.16.27	16			
	Kitui		**Arabuko**			**Median**		
1			■	0.17.41	17	**20**		
2			■	0.26.19	26			
3	■			0.15.60	16			
4			■	0.18.43	18			
5			■	0.11.48	11		**Mean**	
6			■	0.46.99	47		**Kitui**	**Arabuko**
7	■			0.36.43	36		**23**	**28.36**
8	■			0.43.77	44			
9			■	0.16.81	17			
10			■	0.51.09	51			
11	■			0.16.04	16			
12	■			0.37.22	37			
13	■			0.09.42	9			
14	■			0.06.71	7			
15	■			0.06.25	6			
16	■			0.19.31	19			
17	■			0.23.30	23			
18			■	0.04.18	4			
19	■			0.20.88	21			
20	■			0.23.36	23			
21			■	01.15.28	75			
22	■			0.12.71	12			
23	■			0.41.97	42			
24			■	0.41.37	41			
25			■	0.05.07	5			
26	■			0.33.76	34			
	Kitui		**Baringo**			**Median**		
1			■	0.37.81	38	**31**		
2			■	0.38.63	39			
3			■	1.03.75	64			
4			■	0.43.43	43			
5			■	0.25.94	26		**Mean**	
6			■	02.58.18	178		**Kitui**	**Baringo**
7	■			0.29.11	29		**34.54**	**41.42**
8			■	0.19.95	20			

No.	Kitui		Kakamega		Value	No.	Median	Mean Kitui	Mean Kakamega
9	■				0.19.15	19			
10	■				0.31.53	32			
11			■		0.12.89	13			
12	■				1.01.83	62			
13			■		0.05.22	5			
14		■			5.00.00	300			
15	■				0.17.25	17			
16	■				0.39.07	39			
17			■		0.12.17	12			
18			■		0.44.91	45			
19	■				0.13.57	14			
20	■				0.28.89	29			
21	■				0.30.04	30			
22	■				1.16.58	77			
23	■				0.32.14	32			
24			■		0.14.43	14			
	Kitui		**Kakamega**				**Median**		
1	■				0.24.35	24	**31.5**		
2			■		0.11.40	11			
3			■		0.10.93	11			
4			■		0.14.05	14			
5			■		0.21.26	21			**Mean**
6	■				0.47.19	47		**Kitui**	**Kakamega**
7			■		0.24.73	24		**44.75**	**25.93**
8		■			5.00.00	300			
9	■				0.56.90	57			
10	■				0.14.87	15			
11	■				01.34.87	95			
12			■		0.45.66	46			
13	■				0.59.50	60			
14			■		0.43.32	43			
15			■		0.19.73	19			
16			■		0.33.47	34			
17	■				0.16.74	17			
18			■		0.34.77	35			
19			■		0.29.03	29			
20			■		0.53.39	53			
21	■				0.43.12	43			
22		■			5.00.00	300			
23			■		0.13.56	14			

#	Kitui	Karura			Median	Mean	
24		■	0.09.16	9			
	Kitui	**Karura**			**Median**		
1	■		0.57.18	57	**31.5**		
2	■		0.14.75	15			
3	■		0.50.07	50			
4	■		0.30.23	30			
5		■	0.17.25	17		**Mean**	
6	■		0.52.57	53		**Kitui**	**Karura**
7		■	0.19.86	20		**50.15**	**39.24**
8		■	02.37.07	157			
9		■	0.08.91	9			
10	■		01.50.14	110			
11	■		0.23.63	24			
12	■		0.07.28	7			
13		■	0.09.37	9			
14		■	01.23.86	84			
15	■		01.13.31	73			
16	■		02.34.37	154			
17	■		0.15.61	16			
18		■	0.08.50	9			
19		■	0.33.32	33			
20	■		0.22.54	23			
21		■	0.24.63	25			
22	■		0.16.35	16			
23		■	1.24.44	84			
24		■	0.39.57	40			
25		■	0.23.49	23			
26	■		0.06.30	6			
27		■	0.54.32	54			
28	■		01.04.43	64			
29		■	01.26.79	87			
30	■		0.09.22	9			
31	■		0.46.97	47			
32		■	0.34.72	35			
33	■		01.22.43	82			
34		■	0.08.38	8			
35		■	0.16.52	17			
36		■	0.18.54	19			
37		■	0.10.77	11			
38	■		01.57.27	117			

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	Kitui		Taita						
39			■		0.36.68	37			
40			■		0.45.76	46			
	Kitui		**Taita**				**Median**		
1	■				0.14.40	14	**16.5**		
2			■		02.12.77	133			
3			■		0.45.68	46			
4			■		0.06.44	6			
5	■				0.39.34	39		**Mean**	
6			■		0.14.76	15		**Kitui**	**Taita**
7	■				0.13.03	13		**19.73**	**34.4**
8			■		0.21.80	2			
9	■				0.13.07	13			
10			■		0.47.23	47			
11	■				0.14.99	15			
12	■				0.25.95	26			
13	■				0.15.75	16			
14			■		0.27.88	8			
15	■				0.07.37	7			
16	■				0.07.93	8			
17	■				0.34.94	35			
18	■				0.36.48	36			
19			■		0.32.06	32			
20	■				0.25.53	26			
21			■		0.43.59	44			
22			■		0.32.60	33			
23			■		0.36.59	37			
24	■				0.15.59	16			
25			■		0.17.23	17			
26			■		0.41.48	41			
27	■				0.16.20	16			
28			■		0.49.78	50			
29			■		0.04.94	5			
30	■				0.15.50	16			
	Taita		**Baringo**				**Median**		
1			■		0.17.92	18	**34**		
2			■		0.25.61	26			
3			■		0.32.30	32			
4			■		1.02.03	62			
5	■				0.45.01	45		**Mean**	

#	Taita		Kakamega	Value	Number		Taita	Baringo
6			█	0.06.93	7		**Taita**	**Baringo**
7	█			0.56.72	57		**40.83**	**43.47**
8			█	0.21.88	22			
9			█	0.47.12	47			
10			█	01.45.99	106			
11			█	0.34.55	35			
12			█	0.17.95	18			
13		█		0.35.84	36			
14	█			0.23.65	24			
15			█	0.26.43	26			
16	█			0.56.83	57			
17			█	0.25.67	26			
18	█			0.45.76	46			
19			█	01.56.45	117			
20			█	0.17.23	17			
21	█			0.15.75	16			
22			█	0.34.83	35			
23			█	01.52.24	112			
24			█	0.32.56	33			
	Taita		**Kakamega**			**Median**		
1	█			01.18.43	78	**46**		
2	█			0.41.62	42			
3			█	1.16.12	66			
4			█	0.35.30	35			
5		█		5.00.00	300		**Mean**	
6			█	1.35.19	95		**Taita**	**Kakamega**
7			█	0.50.64	51		**50.6**	**49.68**
8			█	1.35.19	95			
9			█	1.25.43	85			
10			█	0.44.44	44			
11			█	0.22.19	22			
12	█			0.07.82	8			
13			█	0.12.34	12			
14	█			1.39.62	100			
15			█	0.19.17	19			
16	█			0.25.24	25			
17			█	0.29.20	29			
18			█	1.49.33	109			
19			█	0.48.10	48			
20			█	0.59.63	60			

	Taita		Karura			Median		
21			█	0.17.08	17			
22			█	0.08.48	8			
	Taita		**Karura**			**Median**		
1	█			0.21.91	22	**21**		
2			█	0.37.79	38			
3	█			0.20.07	20			
4			█	0.39.23	39			
5			█	1.09.53	69		**Mean**	
6			█	0.17.31	17		**Taita**	**Karura**
7			█	0.14.69	15		**24.5**	**37.375**
8			█	0.34.99	35			
9	█			0.14.57	15			
10			█	0.28.14	28			
11			█	0.17.47	17			
12			█	0.09.40	9			
13			█	0.43.93	44			
14			█	0.53.91	54			
15	█			0.14.62	14			
16			█	0.19.95	20			
17			█	2.12.00	132			
18	█			1.03.74	64			
19			█	0.10.09	10			
20			█	0.14.53	15			
21	█			0.11.49	12			
22			█	0.55.78	56			
	Taita		**Arabuko**			**Median**		
1			█	0.26.32	26	**33**		
2	█			0.15.17	15			
3	█			0.16.27	16			
4			█	0.37.43	37			
5			█	0.22.31	22		**Mean**	
6	█			1.29.37	89		**Taita**	**Arabuko**
7	█			0.43.93	44		**41.6**	**36.7**
8			█	0.15.37	15			
9	█			0.46.97	47			
10			█	1.06.92	67			
11	█			0.32.88	33			
12			█	0.37.22	37			
13	█			0.12.26	12			

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14	■				0.59.50	60			
15	■				0.54.49	54			
16	■				0.31.22	31			
17				■	0.44.44	44			
18				■	0.33.07	33			
19				■	0.17.22	17			
20				■	0.04.29	4			
21				■	0.30.36	30			
22	■				0.38.14	38			
23	■				0.13.84	14			
24				■	0.27.96	28			
25				■	0.42.83	43			
26			■		5.00.00	300			
27				■	1.42.48	102			
28				■	1.01.49	61			
29				■	0.50.13	50			
30				■	1.26.46	86			
31				■	0.32.82	33			
32				■	0.31.35	31			
33				■	0.17.16	17			
34	■				1.40.59	101			
35				■	0.16.77	17			
36				■	0.37.59	38			
37				■	0.10.57	11			
38				■	0.40.66	41			
39				■	1.17.37	77			
40				■	0.31.91	32			
41				■	0.12.40	12			
42				■	0.34.98	35			
43				■	0.27.37	27			
44	■				0.13.74	14			
45				■	0.27.50	28			
46	■				0.56.31	56			

Appendix 3

Table 3: Significance test for honey choice by healthy bees

	Sample	Selection	*Total*	*p value*	Significance
	Empty	5			
	Empty	5	10	1	**FALSE**
	Lemon grass	9			
	Empty	0	10	0.0325	**SIGNIFICANT**
	Baringo	11			
1	Arabuko	7	18	0.738	**FALSE**
	Kakemega	12			
2	Arabuko	6	18	0.4998	**FALSE**
	Kakamega	19			
3	Baringo	21	44	0.8308	**FALSE**
	Karura	19			
4	Arabuko	17	36	0.8136	**FALSE**
	Karura	13			
5	Baringo	11	24	1	**FALSE**
	Karura	11			
6	Kakamega	13	24	1	**FALSE**
	Kitui	15			
7	Arabuko	11	26	0.7813	**FALSE**
	Kitui	11			
8	Baringo	12	24	1	**FALSE**
	Kitui	8			
9	Kakamega	14	24	0.388	**FALSE**
	Kitui	19			
10	Karura	21	40	1	**FALSE**
	Kitui	15			
11	Taita	15	30	1	**FALSE**
	Taita	6			
12	Baringo	17	24	0.1351	**FALSE**
	Taita	5			
13	Kakamega	16	22	0.1159	**FALSE**
	Taita	6			
14	Karura	16	22	0.2152	**FALSE**
	Taita	15			
15	Arabuko	30	46	0.1378	**FALSE**
	Total Entries		**422**		

Appendix 4

Table 4: Choice experiment for diseased bees

No	Choice Left	None	Right	1st decision (s)	Time (s)			
	Empty		Empty			Median		
1	■			0.33.87	34	**14.5**		
2			■	0.14.64	15			
3	■			0.36.57	37			
4			■	0.09.45	9			
5			■	0.09.56	10		Mean	
6	■			0.14.09	14		Empty Left	Empty Right
7			■	0.15.97	16		**25.2**	**12.6**
8	■			0.06.56	7			
9	■			0.34.33	34			
10			■	0.13.15	13			
	Lemon grass oil		Empty			Median		
1	■			0.45.78	46	**51.5**		
2	■			0.19.57	20			
3	■			02.74.46	194			
4	■			01.18.87	79			
5	■			01.05.97	66		Mean	
6	■			01.10.52	71		Lemon grass	Empty
7	■			0.56.78	57		**62.6**	**0**
8	■			0.13.67	14			
9	■			0.34.65	35			
10	■			0.44.32	44			
	Baringo		Arabuko			Median		
1			■	1.04.69	65	**10**		
2	■			1.41.56	102			
3			■	0.21.32	21			
4	■			0.04.45	4			
5	■			0.07.63	8		Mean	
6			■	0.28.34	28		Baringo	Arabuko
7	■			0.13.15	13		**17.31**	**38**

	Kakamega		Arabuko					
8	■			0.05.46	5			
9	■			0.11.24	11			
10	■			0.07.67	8			
11	■			0.05.53	6			
12	■			0.11.52	12			
13	■			0.04.33	4			
14	■			0.09.16	9			
15	■			0.36.73	37			
16	■			0.06.17	6			
	Kakamega		**Arabuko**			**Median**		
1	■			1.20.07	80	**19.5**		
2			■	0.16.88	17			
3			■	0.29.63	30			
4			■	0.12.76	13			
5	■			0.18.10	18		**Mean**	
6	■			0.18.34	18		**Kakamega**	**Arabuko**
7	■			1.07.70	68		**31.33**	**20.25**
8	■			0.22.80	23			
9	■			1.09.27	69			
10	■			0.08.84	9			
11	■			0.09.14	9			
12	■			0.30.96	31			
13	■			0.31.36	31			
14	■			0.07.20	7			
15	■			0.13.13	13			
16			■	0.20.62	21			
	Baringo		**Kakamega**			**Median**		
1			■	0.10.44	10	**15**		
2			■	0.35.29	35			
3			■	0.15.18	15			
4	■			1.14.57	15			
5			■	0.58.42	58		**Mean**	
6			■	0.19.38	19		**Baringo**	**Kakamega**
7	■			0.22.77	23		**18.25**	**18.92**
8	■			0.05.62	6			
9			■	0.10.13	10			
10			■	0.21.95	22			
11			■	0.07.88	8			
12			■	0.06.69	7			

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13			■		0.10.65	11			
14	■				0.29.27	29			
15			■		0.25.84	26			
16			■		0.06.07	6			
	Arabuko		**Karura**				**Median**		
1			■		0.18.13	18	**19**		
2			■		0.05.34	5			
3			■		0.12.99	13			
4			■		0.13.99	14			
5	■				0.16.27	16		**Mean**	
6	■				0.12.33	12		**Arabuko**	**Karura**
7	■				0.42.79	43		**23**	**23**
8			■		0.49.53	50			
9	■				0.30.10	30			
10	■				0.19.00	19			
11			■		0.17.33	17			
12			■		0.18.60	19			
13	■				0.21.47	21			
14			■		0.39.42	39			
15	■				0.20.36	20			
16			■		0.31.63	32			
	Karura		**Baringo**				**Median**		
1			■		0.12.23	12	**13**		
2	■				0.39.34	39			
3	■				0.03.79	4			
4	■				0.15.99	16			
5	■				0.03.18	3		**Mean**	
6	■				0.14.63	15		**Karura**	**Baringo**
7			■		0.20.13	20		**12.7**	**28.17**
8	■				0.21.67	22			
9			■		0.13.42	13			
10			■		1.04.37	64			
11			■		0.12.73	13			
12	■				0.09.20	9			
13	■				0.05.47	5			
14	■				0.07.83	8			
15			■		0.46.50	47			
16	■				0.06.48	6			

	Karura		Kakamega			Median		
1			■	0.43.24	43	**22**		
2	■			0.20.10	20			
3	■			0.31.49	31			
4	■			0.12.27	12			
5			■	0.43.27	43		Mean	
6			■	0.13.62	14		Karura	Kakamega
7			■	0.05.27	5		**24.18**	**22.6**
8	■			0.27.49	27			
9	■			0.28.30	28			
10	■			0.12.37	12			
11	■			0.23.37	23			
12	■			0.44.93	45			
13			■	0.07.87	8			
14	■			0.14.51	15			
15	■			0.32.02	32			
16	■			0.20.91	21			
	Arabuko		Kitui			Median		
1			■	1.26.67	87	**19**		
2			■	0.12.83	13			
3	■			0.08.06	8			
4			■	0.19.13	19			
5	■			0.32.10	32		Mean	
6			■	0.10.44	10		Arabuko	Kitui
7	■			0.27.99	28		**34.88**	**23.56**
8			■	0.09.20	9			
9	■			0.15.46	15			
10	■			0.27.87	28			
11	■			1.14.18	74			
12			■	0.09.12	9			
13	■			0.42.71	43			
14	■			0.51.09	51			
15			■	0.09.97	10			
16			■	0.45.20	45			
17			■	0.10.36	10			
	Kitui		Baringo			Median		
1			■	0.12.91	13	**20.5**		
2			■	0.18.12	18			
3	■			0.32.10	32			

#				Value	Count			
4	■			0.05.96	6			
5	■			0.42.40	42		Mean	
6	■			0.39.66	40		Kitui	Baringo
7	■			0.56.17	56		**27.47**	**37.67**
8			■	1.21.57	82			
9	■			0.23.14	23			
10	■			0.11.54	12			
11	■			0.09.24	9			
12	■			0.18.25	18			
13	■			0.02.90	2			
14	■			0.44.48	44			
15	■			1.05.33	65			
16	■			0.07.99	8			
	Kakamega		**Kitui**			**Median**		
1			■	0.08.18	8	**22**		
2	■			0.14.56	15			
3			■	0.26.13	26			
4	■			0.15.21	15			
5			■	0.15.89	16		Mean	
6	■			0.46.03	46		Kakamega	Kitui
7			■	0.25.37	25		**27.75**	**22.5**
8			■	0.19.13	19			
9			■	0.38.78	39			
10	■			0.47.42	47			
11	■			0.24.72	25			
12			■	0.35.63	36			
13	■			0.17.36	17			
14	■			0.07.82	8			
15			■	0.11.43	11			
16	■			0.49.09	49			
	Kitui		**Karura**			**Median**		
1			■	0.10.63	11	**15**		
2			■	0.14.29	14			
3	■			0.07.82	8			
4			■	0.28.97	29			
5			■	0.43.13	43		Mean	
6	■			0.37.92	38		Kitui	Karura
7	■			0.22.19	22		**20.2**	**17.91**
8			■	0.32.51	33			

9			■		0.15.17	15			
10	■				0.20.26	20			
11			■		0.05.18	5			
12			■		0.11.12	11			
13			■		0.15.19	15			
14			■		0.22.83	23			
15			■		0.03.18	3			
16	■				0.13.36	13			
	Taita		**Kitui**				**Median**		
1	■				0.41.90	42	**15.5**		
2			■		0.23.95	24			
3			■		0.29.90	30			
4			■		0.27.23	28			
5			■		0.28.69	28		**Mean**	
6			■		0.15.38	15		**Taita**	**Kitui**
7			■		0.07.13	7		**29**	**19.28**
8			■		0.12.73	13			
9			■		0.07.93	8			
10			■		0.13.34	13			
11			■		0.06.30	6			
12			■		1.03.27	63			
13			■		0.16.82	17			
14	■				0.15.93	16			
15			■		0.08.52	9			
16			■		0.09.35	9			
	Baringo		**Taita**				**Median**		
1	■				1.13.18	73	**13.5**		
2	■				0.43.07	43			
3	■				0.21.24	21			
4			■		0.07.66	8			
5	■				0.14.29	14		**Mean**	
6			■		0.09.63	10		**Baringo**	**Taita**
7	■				0.07.47	7		**27**	**14.5**
8			■		0.12.66	13			
9	■				0.05.78	6			
10	■				0.29.04	29			
11			■		0.11.90	12			
12	■				0.11.79	12			
13			■		0.20.15	20			

#	Kakamega	Taita	Value	Number		
14		■	0.24.33	24		
15	■		0.09.50	10		
16	■		0.54.57	55		
	Kakamega	**Taita**			**Median**	
1		■	0.22.31	22	**23**	
2		■	1.11.19	71		
3	■		0.22.36	22		
4		■	0.36.29	36		
5		■	0.48.62	49	**Mean**	
6	■		0.54.34	54	**Kakamega**	**Taita**
7	■		0.26.29	26	**24**	**38**
8	■		0.34.29	34		
9	■		0.08.48	8		
10	■		0.09.54	10		
11	■		0.49.26	49		
12	■		0.23.52	24		
13	■		0.14.22	14		
14	■		0.27.08	27		
15		■	0.11.91	12		
16	■		0.13.69	14		
17	■		0.19.22	19		
18	■		0.13.86	14		
19	■		0.15.23	15		
20	■		0.29.52	30		
	Taita	**Karura**			**Median**	
1		■	0.07.24	7	**17.5**	
2		■	0.33.36	33		
3		■	0.24.37	24		
4		■	0.06.42	6		
5	■		0.10.42	10	**Mean**	
6		■	0.28.41	28	**Taita**	**Karura**
7		■	0.15.40	15	**15.75**	**25.81**
8		■	0.06.85	7		
9		■	0.17.98	18		
10		■	0.50.47	50		
11	■		0.42.36	42		
12		■	0.11.75	12		
13		■	0.16.40	16		
14		■	0.25.42	25		

	Taita		Arabuko			Median		
15	■			0.07.03	7			
16			■	0.35.39	35			
17			■	0.18.07	18			
18			■	0.17.18	17			
19			■	1.42.37	102			
20	■			0.03.67	4			
	Taita		**Arabuko**			**Median**		
1	■			1.39.49	99	**15.5**		
2			■	0.16.95	17			
3			■	0.13.26	13			
4	■			0.02.95	3			
5			■	0.05.53	6		**Mean**	
6	■			0.08.67	9		**Taita**	**Arabuko**
7	■			0.50.93	51		**30.5**	**14.3**
8			■	0.16.99	17			
9			■	0.10.19	10			
10	■			0.07.53	8			
11			■	0.22.78	23			
12			■	0.08.09	8			
13	■			0.13.48	13			
14	■			0.32.32	32			
15	■			0.57.46	57			
16	■			0.18.19	18			
17	■			0.15.26	15			
18			■	0.17.57	18			
19			■	0.16.21	16			
20			■	0.14.76	15			

Appendix 5

Table 5: Significance test for choice of honey by diseased bees

	Sample	Selection	*Total*	*p value*	Significance
	Empty	5			
	Empty	5	10	1	**FALSE**
	Lemon grass	10			
	Empty	0	0	0.0325	**SIGNIFICANT**
	Baringo	13			
1	Arabuko	3	16	0.1351	**FALSE**
	Kakamega	12			
2	Arabuko	4	16	0.2734	**FALSE**
	Baringo	4			
3	Kakamega	12	16	0.2734	**FALSE**
	Arabuko	7			
4	Karura	9	16	1	**FALSE**
	Karura	10			
5	Baringo	6	16	0.7224	**FALSE**
	Karura	11			
6	Kakamega	5	16	0.4725	**FALSE**
	Arabuko	8			
7	Kitui	9	17	1	**FALSE**
	Kitui	13			
8	Baringo	3	16	0.1351	**FALSE**
	Kakamega	9			
9	Kitui	8	17	1	**FALSE**
	Kitui	5			
10	Karura	11	16	0.4725	**FALSE**
	Taita	2			
11	Kitui	14	16	0.0538	**FALSE**
	Baringo	10			
12	Taita	6	16	0.7224	**FALSE**
	Kakamega	15			
13	Taita	5	20	0.1908	**FALSE**
	Taita	4			
14	Karura	16	20	0.0958	**FALSE**
	Taita	10			
15	Arabuko	10	20	1	**FALSE**
	Total Entries		**254**		

Appendix 6

Table 6: Chemical profile of volatile compounds in honey color coded to match their source honey. ***Bold and italicized*** compounds solely occur in one honey, black and straight compounds are present in multiple honey, while underlined compounds possess antibacterial activity. R_T is the retention time of compounds while ABA represents the antibacterial activity.

R_T	Compounds	Arabuko Sokoke	Baringo	Kakamega	Karura	Kitui	Taita	Manuka 5+	Classification	ABA
		Relative abundance 10^7								
3.6	Heptane		0.10		0.24				Alkane	
3.6	*Trichloroethylene*					0.04			Halocarbon	
3.9	***Acetoin***			0.67					Ketone	
4.0	2-Butenoic acid, methyl ester				0.16	0.03			Ester	Yes
4.7	2,4-dimethyl-3-pentanone			0.06			0.00		Ketone	
6.1	*3-methyleneheptane*					0.04			Alkane	
6.3	2,3-Butanediol	0.29		1.74	1.41	0.03	0.19		Alcohol	Yes
6.8	2,3,5-trimethylhexane	0.09		0.20		0.14	0.01	0.02	Alkane	
7.1	2,4-dimethylheptane	1.17	0.10	2.76	0.18	1.93	0.20	0.14	Alkane	
7.5	***Acetylvaleryl***			0.71					Ketone	
7.6	2,4-dimethyl-1-heptene	0.09		0.19		0.08	0.03		Alkene	
7.7	Chlorobenzene		0.03		0.02	0.03			Halobenzene	
8.0	4,5-diethyloctane	0.03	0.01	0.11					Alkane	
8.0	***5,15-dimethylnonadecane***				0.02				Alkane	Yes
8.0	*2,3-dimethylheptane*					0.07			Alkane	Yes
8.2	4-methyloctane	0.33	0.07	1.44	0.15	0.63	0.11	0.05	Alkane	Yes
8.3	***o-Xylene***			0.10					Aromatic hydrocarbon	Yes
8.3	p-Xylene	0.05			0.03	0.09		0.02	Aromatic hydrocarbon	Yes

8.3	*1,3-dimethylbenzene*		*0.03*					Aromatic hydrocarbon	Yes
8.8	*Styrene*					*0.04*		Aromatic hydrocarbon	
8.9	Pyridine, 3-(1-methyl-2-pyrrolidinyl)			0.09			0.01	Alkaloid	Yes
9.1	Nonane			0.09		0.03		Alkane	
10.1	*Camphene*					*0.05*		Terpene	Yes
10.5	Mesitylene			0.26		0.45		Aromatic hydrocarbon	
10.5	1,2,4-trimethylbenzene	0.14	0.02	0.50	0.02			Aromatic hydrocarbon	
10.6	Sabinene		0.02	0.31		0.19		Terpene	Yes
10.7	β-Phellandrene	0.10			0.04			Terpene	Yes
10.7	β-Pinene		0.02	0.27		0.19		Terpene	Yes
11.5	2,5-dimethylnonane			0.58	0.01			Alkane	Yes
11.6	*4-methyldecane*					*0.18*		Alkane	
11.7	o-Cymene	0.11		0.47				Aromatic hydrocarbon	Yes
11.7	*p-Cymene*					*0.28*		Aromatic hydrocarbon	Yes
11.7	2-ethyl-1-hexanol	1.01	0.11	2.09	0.27	2.32		Alcohol	
11.9	*Pantolactone*					*0.77*		Butyrolactone	Yes
12.0	*Chloromethyl thiocyanate*		*0.01*					Thiocyanate	
12.0	Benzene acetaldehyde	0.40				0.63		Aldehyde	Yes
12.5	Acetophenone	0.04				0.17		Ketone	Yes
12.5	Linalool oxide	0.20	0.22		0.08	0.54		Oxolane	Yes
12.6	*p-Cresol*	*2.24*						Phenol	Yes
12.6	*3-methyl-phenol*					*0.31*		Phenol	Yes
12.9	*6-ethyl-2-methyloctane*			*0.45*				Alkane	
13.0	Nonanal	0.21		0.52		0.67		Aldehyde	Yes

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								Class	
13.1	*5-ethyl-2-methyloctane*				0.08			Alkane	
13.2	**Phenylethyl alcohol**	0.37						Alcohol	Yes
13.4	2-Ethylhexanoic acid			1.26		2.79		Acid	
13.7	**4-ketoisophorone**			0.27				Ketone	
14.5	Dodecane			1.93		0.79		Alkane	Yes
14.7	Decanal			0.81		1.26		Aldehyde	Yes
14.8	**2,5-dimethylundecane**			0.39				Alkane	
14.9	**4-methyldodecane**			0.22				Alkane	
15.1	*isothiocyanato-cyclohexane*					0.34		Nitrogen containing compound	
15.2	*Untriacontane*					0.44		Alkane	Yes
15.4	*3-methylundecane*					0.38		Alkane	
15.9	Pentadecane	1.18	0.14	0.23		1.02		Alkane	Yes
15.9	**Hexadecane**				0.04			Alkane	Yes
16.0	Tridecane	0.22	0.51	0.50		0.90		Alkane	
16.1	*Oxalic acid, butyl 6-ethyloct-3-yl ester*					1.32		Ester	
16.6	3,3-dimethylhexane			0.44		0.02		Alkane	
16.6	Nonadecane	0.32	0.57		0.18	1.12	0.07	Alkane	Yes
16.7	**3-hydroxy-4-phenyl-2-Butanone**			1.14				Ketone	Yes
17.0	*3,5-Dimethyldodecane*					0.49		Alkane	
17.1	Butylbutanoate	0.65		2.85				Ester	
17.1	**Butanoic acid, butyl ester**		0.43					Ester	
17.3	1-Pentadecene			0.40		0.72		Alkene	
17.3	Tetradecane	0.60		1.33	0.15	2.56	0.12	Alkane	Yes
17.5	*trans-2-Dodecen-1-ol*					0.81		Alcohol	Yes
17.6	**γ-Amorphene**			0.32				Cadinene	Yes
17.6	Longifolene	0.15		0.44		0.69		Terpene	Yes
17.6	**Viridiflorene**		0.16					Terpene	

RT	Compound								Category	Detected
17.7	α-Cedrene	0.46		1.08		2.63	0.12		Terpene	Yes
17.7	*Italicene*		0.22						Terpene	
17.7	*α-2-epi-Funebrene*				0.09				Other	
17.8	β-Cedrene	0.22		0.41		1.33			Terpene	Yes
18.0	*Diisopropyl adipate*					0.64			Ester	Yes
18.1	Geranyl acetone			0.63		1.48			Ketone	
18.3	*3-methyl-tetradecane*					0.73			Alkane	
18.3	2,5-di-tert-Butyl-1,4-benzoquinone	0.66	0.43	*1.03*					Quinone	Yes
18.7	Docosane	0.77	0.24	*4.67*	0.08	1.86			Alkane	Yes
18.8	Methyl p-tert-butylphenyl acetate	0.35	0.12			1.18			Ester	
18.8	*2,5-bis(1,1-dimethylethyl)-phenol*			*0.34*					Phenol	
18.9	*5-Cedranone*	*1.92*							Ketone	
18.9	Butylated hydroxytoluene		1.25	2.71	0.55	5.62	0.18		Phenol	Yes
19.4	2-methylpentadecane			0.68		1.40			Alkane	
19.7	*Cetene*					*1.21*			Alkene	
19.8	Hexadecane	1.20	0.60	0.66		1.59			Alkane	Yes
19.9	*Diethyl phthalate*			*1.7*					Ester	
20.1	Cedrol	0.52	0.31	1.20		1.83			Alcohol	Yes
20.3	*Junenol*	*0.42*							Alcohol	Yes
20.5	*2-methylhexadecane*					*0.55*			Alkane	
21.0	Heptadecane	0.42		3.16		1.64			Alkane	Yes
21.1	Tricosane	0.68		1.09	0.16				Alkane	Yes
21.3	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-			0.81		1.06			Cyclic hydrocarbon	
21.6	Heneicosane	0.10				1.12			Alkane	Yes
21.6	Tetracosane	0.29	0.52	0.54		0.67			Alkane	Yes
22.0	Octadecane	0.68	0.31	1.16		1.05			Alkane	Yes
22.9	Phthalic acid, isobutyl nonyl ester	*0.14*		*4.75*					Ester	Yes

RT	Compound								Class	Confirmed
23.6	*α-Irone*					*0.16*			Ketone	
23.9	Dibutyl phthalate	0.23				1.08			Ester	Yes
25.0	Eicosane	0.93	0.43	0.82	0.27	0.21			Alkane	Yes
25.5	Corydaldine			0.21		0.01			Alkaloid	Yes
26.5	Acetyl tributyl citrate			0.03		0.15			Ester	Yes
31.0	2-Trifluoromethyl-7-phenothiazone			0.21		0.01			Phenothiazine	Yes
31.3	1,2-Dimethoxy-4-(1-methoxy-1-propenyl)benzene			0.06			0.00		Phenyl propanoid	Yes
36.1	3,4,5-trimethoxy- N-cyano-Benzenamide			0.22		0.01			Amide	Yes
36.8	5-Methyl-2-N-methylaminobenzophenone semicarbazone			0.22		0.01			Amide	Yes
37.7	N-(4-bromo-2-chlorophenyl)-Acetamide			0.14		0.01			Amide	Yes
38.0	3-Phenyl-2H-chromene			0.38		0.04	0.02		Chromene	Yes

Appendix 7

Table 7: Chemical profile of non-volatile compounds in honey color coded to match their source honey. ***Bold and italicized*** compounds solely occur in one honey, black and straight compounds are present in multiple honey, while <u>underlined</u> compounds possess antibacterial activity. R_T is the retention time of compounds while ABA represents the antibacterial activity.

R_T	Compound	Arabuko Sokoke	Baringo	Kakamega	Karura	Kitui	Taita	Manuka 5+	Classification	ABA
				Relative abundance 10^7						
3.9	*<u>3-Hydroxy-2-butanone</u>*			*5.13*					Ketone	Yes
4.3	*Heptane*							*13.36*	Alkane	
6.3	<u>2,3-Butanediol</u>			3.04	2.92		4.81		Alcohol	Yes
7.3	2,4-dimethyl-heptane	0.70	0.81	0.48	0.69	0.66	0.62	22.37	Alkane	
7.5	<u>2-methyl-3-heptanone</u>			0.76		0.10			Ketone	Yes
7.8	*2,4-Dimethyl-1-heptene*							*2.14*	Alkene	
7.8	*chlorobenzene*							*0.31*	Halobenzene	
8.2	*<u>5-methyl-2-hexanol</u>*			*0.44*					Alcohol	Yes
8.3	*4-methyl-octane*							*9.33*	Alkane	
9.0	*p-Xylene*							*2.58*	Aromatic hydrocarbon	Yes
9.1	*Nonane*							*1.57*	Alkane	
9.8	*2,7-dimethyl-octane*							*0.62*	Alkane	
10.1	*<u>Sulfurous acid, cyclohexylmethyl hexadecyl ester</u>*			*0.28*					Ester	Yes
10.1	*Camphene*							*1.36*	Terpene	Yes
10.6	*1,2,4-Trimethyl benzene*							*3.07*	Aromatic hydrocarbon	
10.7	*Sabinene*							*6.41*	Terpene	Yes
10.8	*Arsenous acid, tris(trimethylsilyl) ester*							*8.15*	Ester	
11.7	*2-ethyl-1-hexanol*							*35.55*	Alcohol	

RT	Compound								Class	
12.6	*p-Cresol*							*19.14*	Phenol	Yes
13.7	*4-keto-isophorone*							*4.01*	Ketone	Yes
14.5	*Dodecane*							*7.10*	Alkane	Yes
14.6	*Decanal*							*6.39*	Aldehyde	Yes
14.7	*2-octyl-1-Ddodecanol*							*4.20*	Alcohol	Yes
15.3	*3,8-dimethyl-decane*							*8.94*	Alkane	
16.0	*o-Acetanisole*							*25.88*	Ketone	
16.7	*3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy*			*0.05*					Amine	Yes
17.2	*Tetradecene*							*3.13*	Alkene	Yes
17.3	*Tetradecane*							*14.16*	Alkane	Yes
17.7	*α-Cedrene*							*13.46*	Terpene	
17.9	*Trichloromethane*			*0.01*					Halocarbon	
18.0	*1-methyl-pyrrolidine*			*0.01*					Amine	Yes
18.3	*2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione*							*15.08*	Ketone	
18.7	*10-methyl-eicosane*			*1.06*					Alkane	Yes
18.8	2,4-bis(1,1-dimethylethyl)-phenol	1.10		0.98	0.38	1.07	1.06		Phenol	Yes
18.8	*Butylated hydroxytoluene*							*44.30*	Phenol	Yes
19.2	Octacosane	0.74	2.14			0.29	0.60		Alkane	
19.3	*1,6-Dioxacyclododecane-7,12-dione*							*19.19*	Ketone	
19.6	*S-(Propoxythiocarbonyl)thiohydroxylamine*			*0.02*					Amine	Yes
19.8	*1H,1H,2H,2H-Perfluorooctan-1-ol*			*0.01*					Alcohol	Yes
19.9	*N-(5-Aminopentyl)-oxalamic acid*			*0.01*					Acid	Yes
20.0	Hexadecane	0.12		0.94	2.49	0.08			Alkane	Yes
20.0	*Tridecane*		*0.28*						Alkane	
20.1	*Cedrol*					*17.25*			Alcohol	Yes
20.3	*2,6,10,14-tetramethyl-Hexadecane*			*0.20*					Alkane	Yes
20.6	*Menthofuran*			*1.54*					Terpenoid	Yes

Time	Compound								Type	Present
20.9	Heptadecane	0.48	0.23	1.06	0.37		1.56	18.19	Alkane	Yes
21.6	*3-Methyl-2-(3-methylpentyl)-3-buten-1-ol*							*10.11*	Alcohol	
21.7	*2-Methyl-3-isobutoxy-5-propargylcyclopent-2-en-1-one*			*0.09*					Ketone	Yes
21.8	*Tricosyl pentafluoropropionate*			*0.11*					Ester	Yes
21.8	Tetratriacontane	0.05	0.17	0.11	0.21	1.01	0.59		Alkane	Yes
21.9	*4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-Butanone*			*0.44*					Ketone	Yes
22.0	*1-(ethenyloxy)-octadecane*			*0.06*					Ether	Yes
22.0	*5,6,6-trimethyl-undeca-3,4-diene-2,10-dione*			*0.05*					Ketone	Yes
22.0	*Pentadecane*							*4.04*	Alkane	Yes
22.1	4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-2-Cyclohexen-1-one			4.71	3.75				Ketone	
22.4	*2-methoxy-3-(2-methylpropyl-)-Pyrazine*			*0.08*					Pyrazine	Yes
22.4	*3,5-dimethyl-4H-pyran-4-one*			*0.29*					Pyrone	Yes
22.5	*6-Chlorobicyclo[2.2.1]hept-2-ene-2-carboxylic acid, methyl ester*			*0.15*					Ester	Yes
22.8	*Sulfurous acid, butyl hexadecyl ester*			*0.06*					Ester	Yes
22.8	*1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester*							*10.35*	Ester	
23.5	*Di-sec-butyl phthalate*							*3.40*	Ester	Yes
24.1	*2-methyl-octadecane*			*0.07*					Alkane	Yes
24.3	*(Z)-9-Hexadecen-1-ol,*			*0.30*					Alcohol	Yes
24.3	Hexadecyl-oxirane				0.34	0.35			Epoxide	
24.4	*Octadecanal*	*0.54*							Aldehyde	
24.4	2,6,10-trimethyl-tetradecane		0.16	0.07	0.19				Alkane	Yes
24.6	Nonacosane	0.24	0.35	0.17	0.92	0.38	0.42		Alkane	
24.7	Octadecane	0.24	1.30	24.99	3.48	2.36	1.71	24.57	Alkane	Yes

RT	Name								Type	
24.8	Hexacosane	0.81	0.11	1.16	1.30	1.76	0.67		Alkane	Yes
25.0	Heneicosane	4.62	2.13	1.18	4.42	1.51	3.83	20.78	Alkane	Yes
25.4	Heptacosane	0.89	0.13	1.54	0.27	0.74	0.81	8.08	Alkane	
25.6	Triacontane				0.52	0.76	0.17		Alkane	
26.7	Tricosane	1.15	1.44	9.87	1.05	1.38	1.01		Alkane	Yes
26.8	Untriacontane	0.47	0.18		0.26	0.39	0.03		Alkane	Yes
27.6	Tetracosane	1.68	1.79	1.56	2.00	1.80	1.88		Alkane	Yes
27.6	Hexanedioic acid, bis(2-ethylhexyl) ester							1.91	Ester	
28.4	Pentacosane	4.05	3.47	8.00	5.49	3.99	2.95		Alkane	Yes
28.9	Nonadecane	3.08	2.39	2.57	2.07	1.36	3.22	7.59	Alkane	Yes
29.7	*N-[2-(trifluoromethyl)phenyl]-Pyridine-3-carboxamide*			*0.30*					Amide	Yes
29.8	*1,2,3,4-Tetrahydro-3-(phenylacetamido)quinoline*			*0.35*					Alkaloid	Yes
29.9	Docosane	3.56	2.39		5.93	4.72	3.47		Alkane	Yes
30.1	Tritriacontane	1.09	0.74	1.62		2.69	1.70		Alkane	Yes
30.3	Dotriacontane	1.62	1.55	1.88	1.22	1.11	1.36		Alkane	Yes
31.5	*1-Heptacosanol*			*0.53*					Alcohol	Yes
31.7	Eicosane	6.51	7.63	16.24	9.08	8.02	21.67	8.27	Alkane	Yes
33.5	*9,10-diethyl-9,10-dihydro-Anthracene*			*0.03*					Anthracene	Yes
33.7	*4-Hydroxyphenyl pyrrolidinyl thione*			*0.06*					Ketone	Yes
35.8	*Acetic acid, [4-(1,1-dimethylethyl)phenoxy]-, methyl ester*			*0.34*					Ester	Yes
39.5	Silicic acid, diethyl bis(trimethylsilyl) ester	0.17	0.22	0.16	0.26	0.19	0.26		Ester	

Appendix 8
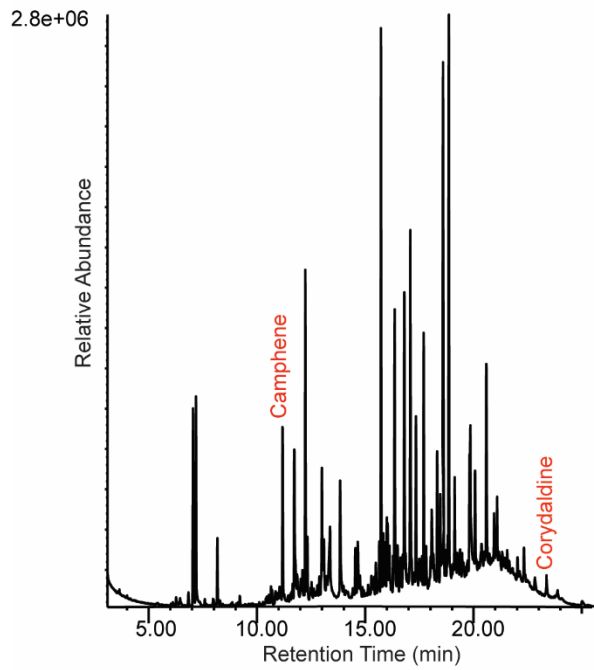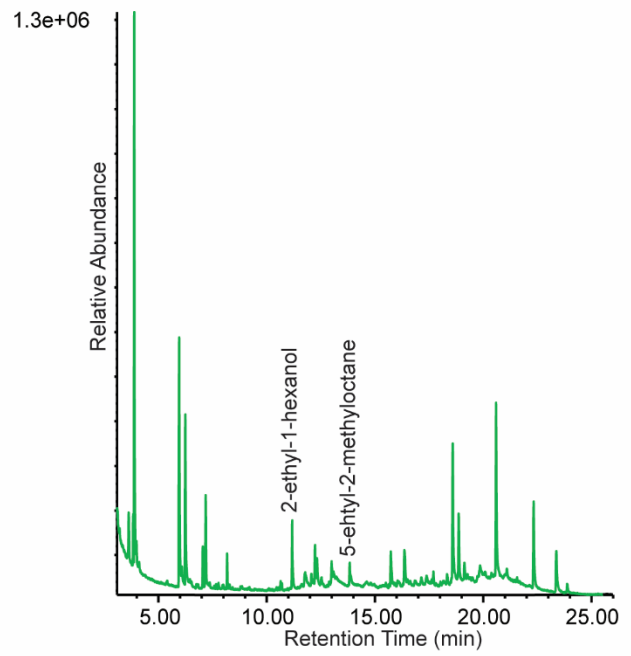
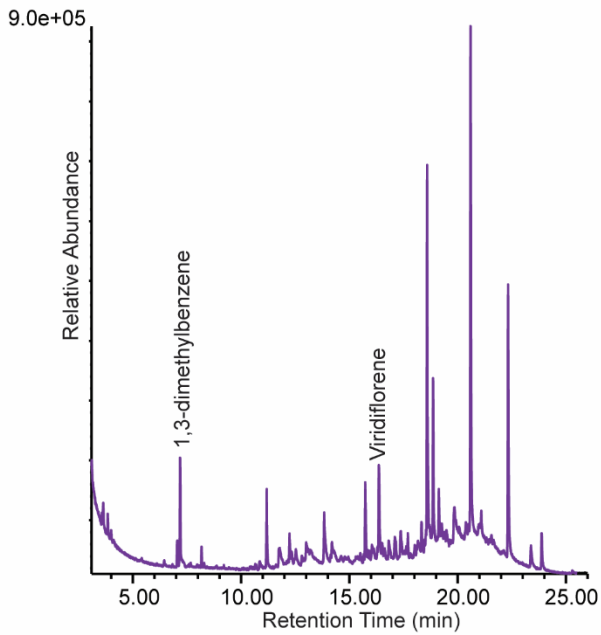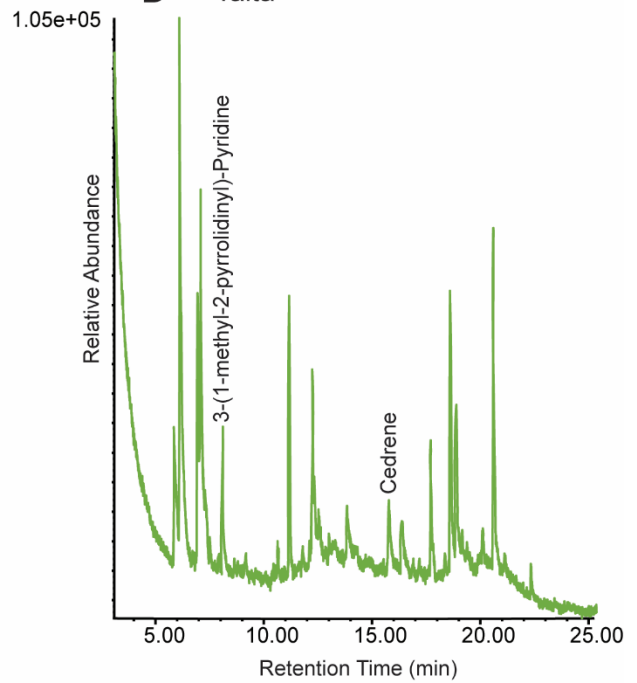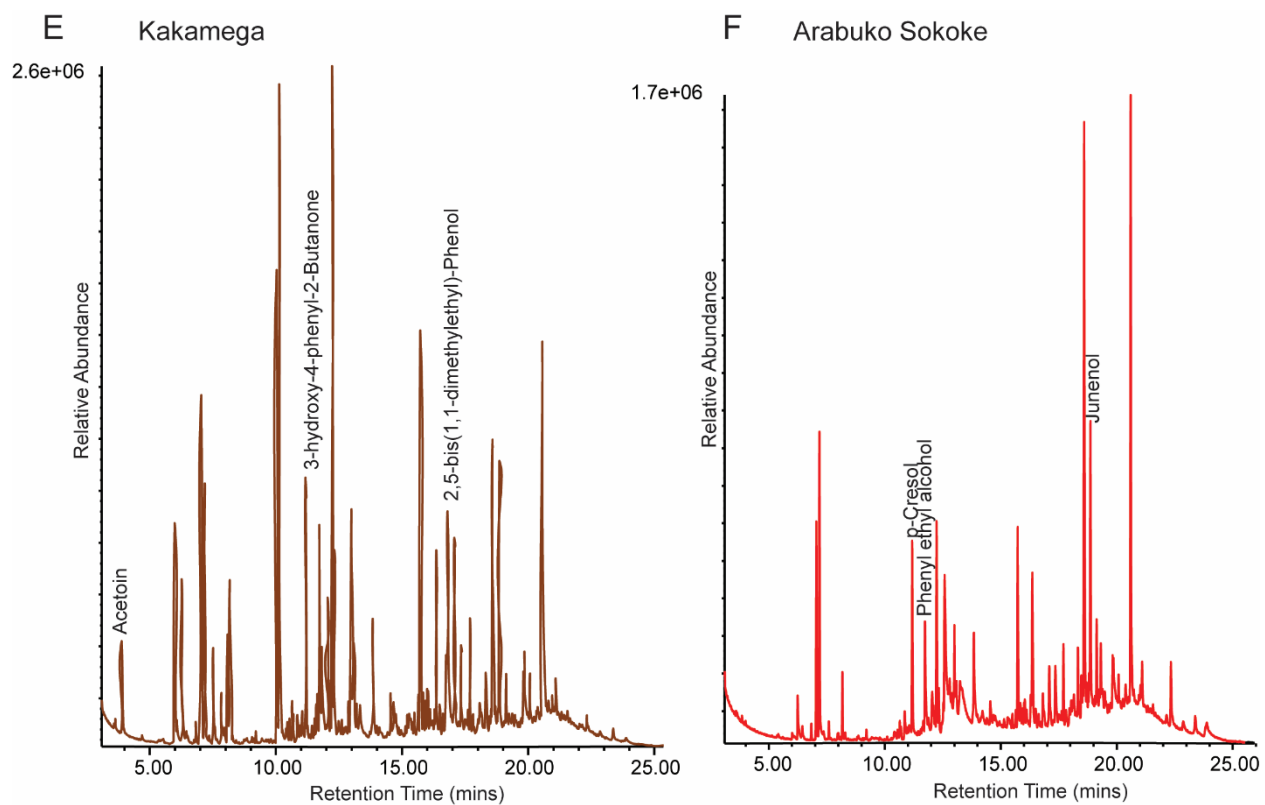**Figure 1**: Chromatograms from GC-MS analysis of the volatile compounds in honey showing some of the major compounds with antibacterial activity in each honey.