MICROBIAL DIVERSITY IN HIVE-STORED POLLEN OF INDIAN HONEY BEE, *Apis cerana indica* (Fabricius)

By

ABHISHEK V.
(2019-11-009)

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2021

MICROBIAL DIVERSITY IN HIVE-STORED POLLEN OF INDIAN HONEY BEE, *Apis cerana indica* (Fabricius)

by

ABHISHEK V.
(2019-11-009)

THESIS

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2021

DECLARATION

I hereby declare that this thesis entitled **"Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
Date: 26-11-2021

Abhishek V.
(2019-11-009)

CERTIFICATE

Certified that this thesis entitled **"Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)"** is a record of research work done independently by **Mr. Abhishek V. (2019-11-009)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

Vellanikkara
Date: 26-11-2021

Dr. Mani Chellappan
(Major Advisor, Advisory committee)
Professor and Head
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Abhishek V. (2019-11-009)** a candidate for the degree of **Masters of Science in Agriculture**, with major field in Agricultural Entomology, agree that the thesis **"Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)"** may be submitted by **Mr. Abhishek V. (2019-11-009)**, in partial fulfillment of the requirement for the degree.

Vellanikkara
Date: 26-11-2021

Dr. Mani Chellappan
(Chairman, Advisory committee)
Professor and Head
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara, Thrissur

Dr. Ranjith M.T.
(Member, Advisory committee)
Assistant Professor
Department of Agricultural Entomology
College of Agriculture, KAU
Vellanikkara, Thrissur

Dr. K. Surendra Gopal
(Member, Advisory committee)
Professor and Head
Department of Agricultural Microbiology
College of Agriculture, KAU
Vellanikkara, Thrissur

Dr. Seeja Thomachan
(Member, Advisory committee)
Assistant Professor
Department of Community Science
College of Agriculture, KAU
Vellanikkara, Thrissur

ACKNOWLEDGEMENT

With the blessings of almighty, as I complete this venture, I express my heartfelt gratitude towards all the people who helped, guided and inspired me to complete this endeavor successfully.

Interdependence is certainly more valuable than independence. This thesis is the result of constant hard work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them. my major advisor, esteemed teacher and an eminent scholar ***Dr. Mani Chellappan,*** *chairman, Professor and Head, Department of Agricultural Entomology. I am especially indebted for his patience in handling my shortcomings, which have helped me immensely towards developing my research skills. His generosity, affection and understanding will always remain engrained in my memory. I am really blessed to get an opportunity to work under his guidance. I am deeply indebted to him forever. I owe him lots of gratitude for having me shown this way of research. I am again thankful to Almighty God, who provided me chairman like you.*

I wish my sincere thanks to ***Dr. Surendra Gopal****, Professor and Head, Department of Agricultural Microbiology and member of my Advisory Committee for providing facilities, giving valuable suggestions, encouragement and wholehearted help throughout this work.*

With a deep sense of gratitude, I extend my warmest thanks to ***Dr. Ranjith, M. T.****, Assistant Professor, Department of Agricultural Entomology, for his kind co-operation, authentic guidance, keen interest and valuable criticism during the course of investigation and thesis writing. My sincere thanks to* ***Seeja Thomachan,*** *Assistant Professor and Head, Department of Community Science, for her support, valuable suggestions and co-operation throughout the research work and during the formulation of thesis.*

Whole hearted thanks to my teachers ***Dr. Haseena Bhaskar****,* ***Dr. Berin Pathrose, Dr. Deepthy K. B., Dr. Smitha M.S., Smitha Revi and Dr. Madhu Subramanian*** *for their expert teaching, moral support and co-operation throughout the period of study.*

My special thanks to the laboratory staff Ancy chechi, Divya chechi, office staff, non-teaching staffs, especially ***Rema chechi****, Surya chechi and all the other members of Department of Agricultural Entomology who have helped me directly/indirectly for their motherly affection and encouragement throughout my study.*

*I am grateful to thank **Ravi sir, Sharan anna, Venkateswara reddy anna,** Bintu chechi, Nisha chechi, Anju chechi, Nisha chechi, Dharma anna, Lokesh anna, Sravanthi akka.*

*I also use this opportunity to thank my batchmates **Rahul raj, Sreesha, Sikha Raju and Sreelakshmi** whose helping hands, love and affection fetched a remarkable place in my heart. I take this opportunity to extend my gratitude to my seniors Anusree chechi, Vineetha chechi, Bindu akka, Anju chechi, Nimisha chechi for their support in different stages of the study.*

*Words are hard to express my feelings towards my best friend **Jonnada Likhita** for her affection, constant support and inspiration which played a key role during the course of study.*

*The lack of vocabulary utterly fails to express gratitude to my **friends Akhil, Pooja, Janmitha, Ankitha, Chaitra, Gokul, Arya, Rahul, Kanavi, Mallesh** for their valuable assistance, motivation and kind of co-operation during entire period of my investigation.*

*I would like to thank my family for all their love and encouragement. I feel a deep sense of that matter in life. My beloved father, **Venkateshaiah** who raised me with a love of science and supported me in all my pursuits, gratitude for my mother, **Nagarathnamma** who formed part of my vision and taught me the good things which has been extremely strong and supportive for me.*

*I sincerely thank the facilities rendered by the **library of College of Agriculture**, Vellanikkara.*

*I gratefully recall the assistance provided by all members of the **Student's Computer Club, Library, Office of COH, and KAU Central Library** employees.*

A statement of thanks to everyone who assisted me in the successful completion of this undertaking and an apology to those I have not mentioned in person.

*The award of **ICAR-NTS fellowship** is thankfully acknowledged.*

Abhishek V.

DEDICATED TO MY PARENTS, GUIDE & TEACHERS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

	11d. 1000µl Micropipettes	
	11e. Laminar Air Flow (LAF)	
	11f. Pouring of 1ml diluent into sterile petri plates	
12	12a. Primary stain-Crystal Violet	47-48
	12b. Moderant-Gram's iodine	
	12c. Decolourising agent-Ethyl alcohol	
	12d. Counter stain- Safranin	
13	Chemical for fungal staining	47-48
14	Observation of microscopic slides under compound microscope	47-48
15	15a. Pollen of Arjun tree-*Terminalia arjuna*	70-71
	15b. Pollen of Flea tree- *Albizia lebbeck*	
	15c. Pollen of Tabasco pepper- *Capsicum frutescens*	
	15d. Pollen of Lemon- *Citrus limon*	
	15e. Pollen of Cucumber- *Cucumis sativus*	
	15f. Pollen of Daisy-*Helianthus aspera*	
16	16a. Pollen of Wattles- *Acacia* sp.	70-71
	16b. Pollen of Asian palmyra palm-*Borassus flabellifer*	
	16c. Pollen of Indian beech- *Pongamia pinnata*	
	16d. Pollen of Slender amaranth-*Amaranthus viridis*	
	16e. Pollen of Senna tora-*Cassia tora*	
17	17a. Pollen of Singapore daisy-*Sphagneticola trilobata*	70-71
	17b. Pollen of Asian pigeonwings- *Clitoria ternatea*	
	17c. Pollen of Peacock flower- *Caesalpinia pulcherrima*	
	17d. Pollen of Holy Basil- *Ocimum sanctum*	
	17e. Pollen of Common zinnia - *Zinnia elegans*	
18	18a. Pollen of little tree plant (*Biophytum sensitivum*	70-71
	18b. Pollen of White orchid tree- *Bauhinia acuminata*	
	18c. Pollen of Yellow elder-*Tecoma stans*	

	18d. Pollen of Moss-rose purslane- *Portulaca grandiflora*	
	18e. Pollen of Shame plant- *Mimosa pudica*	
19	19a. Microscopic image of Gram-negative bacteria	70-71
	19b. Microscopic image of Gram-positive bacteria	
20	20a. Isolate B1	70-71
	20b. Isolate B2	
	20c. Isolate B3	
	20d. Isolate B4	
21	21a. Phylogenetic tree of *Pseudomonas aeruginosa*	70-71
	21b. Phylogenetic tree of *Bacillus megaterium*	
	21c. Phylogenetic tree of *Bacillus aryabhattai*	
22	22a. Isolate T1	70-71
	22b. Isolate W1	
	22c. Isolate A1	
23	23a. Isolate T1	70-71
	23b. Isolate W1	
	23c. Isolate A1	
24	24a. Phylogenetic tree of *Trichoderma reesei*	70-71
	24b. Phylogenetic tree of *Westerdykella multispora*	
	24c. Phylogenetic tree of *Neurospora crassa*	

LIST OF ABBREVIATIONS AND SYMBOLS USED

PKD	-Palakkad
WYD	- Wayanad
ml	- milli liter
h	- hours
g	- gram
kg	- kilo gram
lbs	- Pound
mm	- milli meter
mg	- milligram
m	- meter
µm	- micro meter
mmol	- millimolar
nm	- nano meter
meq	- milliequivalent
Linn.	- Linnaeus
var.	- Variety
df	- degrees of freedom
^{0}C	- Celsius
w	- weight
A. cerana	- *Apis cerana*
FAO	- Food and Agricultural Organisation

APEDA	- Agricultural and Processed Food Products Export Development Authority
USD	- United States Dollar
Mt	- Metric tonnes
CD	- critical difference
Km	- kilometer
AICRP	- All India Coordinated Research Project
ICAR	- Indian Council of Agricultural Research
NSC	- Nectar Sugar Concentration
KVIC	- Khadi and Village Industries Commission
CBRTI	- Central Bee Research and Training Institute
ICBB	- International Commission for Bee Botany
UK	- United Kingdom
SASO	- Saudi Arabian Standards Organization
%	- per cent
$	- dollar
et al.	- *et alia* (Latin- 'and others')
SEM	- Scanning Electron Microscopy
PDA	- Potato Dextrose Agar
NA	- Nutrient Agar
SDA	- Sabouraud Dextrose Agar
MEA	- Malt Extract Agar
CYA	- Czapek Yeast Extract Agar
AIA	- Actinomycetes Isolation Agar
HMF	- Hydroxy Methyl Furfural
AOAC	- Association of Official Agricultural Chemists
RI	- Refractive Index
a_w	- water activity
IR	- Infrared
CP	- Crude Protein
mM	- milli Molar
Cfu	- Colony forming units

sp.	- species
PCA	- Plate Count Agar
B.	- *Bacillus*
NaOH	- Sodium Hydroxide
PCR	- Polymerase Chain Reaction
NCBI	- National Center for Biotechnology Information
BLAST	- Basic Local Alignment Search Tool
v	- volume

INTRODUCTION

CHAPTER I

INTRODUCTION

Honey bees are well-known, widely distributed flying beneficial insects which are regarded as superorganisms with their colony as a biological unit rather than each individual. Honey bees are eusocial insects that live in permanent colonies made up of a queen, workers and drones. There are mainly six types of honey bee species in India that are commercially important. *Apis dorsata* (Rock bee), *Apis laboriosa*, (Himalayan giant honey bee), *Apis cerana indica* (Indian hive bee), *Apis florea* (Dwarf bee), *Apis mellifera* (European bee) and *Tetragonula iridipennis* (Dammer or stingless bee).

Apis cerana indica Fabricius, the Indian bee is a domesticated honey bee species that are economically important in Southern India (I'anson Price and Gruter, 2015). China, Japan, India, Bangladesh, Nepal, Papua New Guinea and Malaysia are the countries using the services of Indian bees (Theisen-Jones and Bienefeld, 2016). Indian honeybee is the most common bees domesticated in India, Pakistan, Nepal, Myanmar, Bangladesh, Sri Lanka, Thailand and Mainland Asia because of its non-aggressive nature and less swarming behaviour.

Insects such as wasps, ants, flies, butterflies, beetles and bees as well as termites are estimated to be responsible for one-third of the human food supply (Jivan, 2013; Said *et al*., 2015). Honey bees collect pollen and nectar from flowers using their modified legs and mouthparts during which they assist in pollination of plants (Dalio, 2015 and Kumar and Sharma, 2016).

Honeybees play a critical role in preserving biodiversity through assisted pollination of wide variety of agricultural and horticultural crops, therefore increasing agricultural production and profitability (Hroncova *et al.*, 2019). Honey bees are essential to mankind because of biological and economic reasons (Eimanifar *et al.*, 2018). The global monetary value of entomophily has been estimated at €153 billion per year (almost 10 per cent of total agricultural productivity). In US, honey bees contributing around $15 billion to agriculture production per year (Gallai *et al*., 2008).

In the bee colony, developing young ones are fed with royal jelly and pollen bread which is a blend of pollen and honey. Pollen is a protein supply for honeybees which allow them to survive. Indian honey bee is known as an excellent pollinator of many crops including spice crops, fruits, nuts, oilseeds, cauliflower, okra and onion. *Apis cerana* are efficient pollinators due to several factors, foremost is having limited foraging range. Within a smaller range, each worker spends more time with the same plants and has higher floral fidelity than *Apis mellifera.* Smaller colony size of Indian bee is being an advantage, as it makes transport of pollen and honey easier. It also has a longer foraging period (in a day) than *Apis mellifera.* Indian bee begins foraging activity early in the morning and continues till evening and which prefers to forage at lower temperatures when compared to *Apis mellifera* (Hemalatha *et al*., 2018).

Pollen provides nutrients to honeybees for their normal growth, development and reproduction. Honeybees forage nectar and pollen during blooming period to make honey and bee food which they store in their hives. Majority of cultivated crops and commercial plantations provide pollen and nectar which is consumed by bees as a food. Honeybees are important pollinating insects which accounts for 91.26 per cent of all insects that visit crops (Lakshmi and Rao, 1998). The peak foraging activity of *Apis cerana* was observed at 09.00 h as pollen collectors. The abundance of all insect pollinators regardless of species is higher on onion crops between 12.00 and 14.00 h (Priti, 1998). *Apis cerana* was found to be the most dominant forager (46.66%) followed by *A. mellifera* (8%) and *A. florea* (7%) (Deuri *et al.,* 2018).

Apart from pollination, beehive products like honey, pollen and propolis have high economic potential (Jones, 2012). Honeybees produce a variety of products such as bee wax, bee venom, royal jelly and honey. These products have high demand in and around India. Honey is a popular food with high commercial and economic value. Honey's healing properties are influenced by its physical and chemical properties (Basualdo *et al*., 2007). Honey is golden coloured having translucent and sour taste which is rich in carbohydrates, vitamins, minerals, organic acids, amino acids and enzymes (Gomes *et al*., 2010). *Apis cerana indica* produces 6-8 kg of honey per year per hive.

According to the FAO (2017-18), India was the eighth largest producer of honey in the world (64.9 thousand tonnes), whereas China ranked first (551 thousand tonnes). India has the capacity to generate 200 million bee colonies, compared to the current 3.4 million which has been increased between years 2014-15 and 2017-18. The volume of honey exported increased from 29.6 to 51.5 thousand tonnes (National Bee Board and the Ministry of Agriculture and Farmers' Welfare). During the year 2020-21, the country exported 59,999.24 MT of natural honey to the world, valued at Rs. 716.13 Crore/ 96.77 USD Millions. India sells the majority of its honey to the United States, Saudi Arabia, Bangladesh and Canada (GOI, 2013).

In India, there are 2,63,583 honey production units in which state Kerala accounts for 49,071 units. Punjab stands first by generating 15,000 Mt of honey and employing over 35,000 beekeepers which accounts for 39 per cent of the country's honey output. Kerala honey output fell from 5733.95 metric tonnes in 2010-11, 4208.14 metric tonnes in 2011-12 and 4586.88 metric tonnes in 2012-13 (GOI, 2013).

Pollen studies or melissopalynological research can assist to identify the geographical and botanical origin of honey (Louveaux *et al.*, 1978; von der Ohe *et al.*, 2004). Palynological studies helps to assess the bee pasturage required for practicing apiculture. Pollen grains in honey samples come from several plant families including Euphorbiaceae, Asteraceae, Myrtaceae, Meliaceae, Acanthaceae, Fabaceae, Malvaceae and Liliaceae. The number of pollen grains per 10 g of honey ranged between 15,000 to 7,48,000 (Vijayakumar *et al*., 2020).

The pollen grain diversity in honey varies from place to place. In India, honey from Uttar Pradesh commonly consists of pollen from Antegonon, Moringa (Nair *et al.*, 1974), *Rumex* sp. and members of the Myrtaceae, Liliaceae, Rosaceae and Euphorbiaceae (Sharma *et al.*, 1965, Gaur *et al.* 1989). Honey samples from Himachal Pradesh consists of a major portion of pollen from *Brassica*, *Adathoda*, *Clematis*, *Mussenda* and *Helianthus* sp. (Singh *et al.*, 1994), Citrus, Toona, Eucalyptus and *Medicago* (Mattu *et al.*, 1997), whereas pollen from *Sapind*us, Eucalyptus, *Anacardium* and *Cleome* species are dominated in honey samples analyzed from Andhra Pradesh (Kalpana and Ramanujan, 1997).

In search of pollen sources, honey bees forage up to a radius of 3 km. Palynological identification of pollen provides valuable information about bee pasture in that area. An absolute pollen count was performed to assess the number of pollen present in 1g honey sample. In general, light microscopy is performed to examine pollen grains and scanning electron microscopy is used to characterize the predominant pollen types.

The various physical and chemical properties of honey are its colour, flavor, moisture, protein and sugar content. Nectar and pollen samples from the source plants are used in the determination of physicochemical properties. However, minor variations in constituents are seen in natural honey, which depends upon site, nectar source and climatic conditions. Physicochemical properties of honey help to distinguish between natural honey and artificial honey. These physicochemical properties of honey are also used to characterize and classify different types of honey.

Diverse group of microbial communities are associated with honey-bee gastrointestinal tract and hive stored pollen. In India, however there is a lack of research on microbial symbionts associated with honey bee. Yeast and bacterial communities were frequently isolated from stingless bees from South America (Rosa *et al*., 2003). Only a few microorganisms can grow or survive in honey. These microorganisms are derived from either primary or secondary contamination sources. The primary source of contamination is related to honeybee digestive tracts which contain natural microorganisms and material collection sources such as nectar, pollen, propolis, air, flowers and the environment inside the beehive.

According to scientific reports, many microbes have a natural association with Indian bee colonies and their products, moreover, information on the description and characterization of microbes associated with hive stored pollen of *Apis cerana* is limited. Hence it is proposed to identify the microbial diversity associated with Indian bees with the following objectives.

1. Collection of hive-stored pollen samples from the central midlands and high ranges of Kerala
2. Palynological identification
3. Physicochemical analyses of hive-stored pollen
4. Microbiological determination

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Honeybees are social insects that are found all over the world with whom man has created a peaceful coexistence (Mishra *et al*. 1998). These insects are vital because they not only produce honey but also serve as crucial pollinators for a variety of plants (Jadhav, 1981; Abrol and Bhat, 1985). Pollen, nectar, water and propolis are collected by bees as part of their foraging activity. The honeybee's foraging behaviour is determined by its innate capacity and responses to numerous environmental cues.

In India, beekeeping has a larger potential to flourish as a leading agro-horticulture and forest-based rural business that is particularly suited to rural, tribal and weaker parts of the population. Beekeeping is a multi-dimensional activity that has become the most feasible enterprise due to the worth of various components such as bee pollination, wax production, medicinal benefits of honey and others (Singh, 1997). The diversity of microorganisms found in pollen is little understood and the most frequent microbes found in bee colonies are bacteria, yeast and molds (Menezes *et al*., 2013). This chapter presents a review of the research on the palynological identification of hive stored bee pollen, physicochemical properties of Indian honey bee pollen and microorganisms associated with pollen.

2.1 Collection of hive-stored pollen samples

2.1.1 Beekeeping in India

According to Smith (1953), the Negas tribe in Assam and Burma are not beekeepers, but the first discoverer of a colony of bees gets the right to gather the produce. As it is in many African tribes, chastity must be observed the night before taking a bees' nest or the bees will sting. The people of Indo-China use crude hives. Hollow logs, bamboo, straw or boards are used to construct them (Toumanoff and Nanta, 1933; Toumanoff, 1933). In Borneo, hollow logs with top and bottom stoppers and a hole in the side are utilized (Ransome, 1937).

According to Muttoo (1956), the genus *Apis* contains three species: *A. dorsata* Fabricius*, A. florea* Fabricius and *A.* mellifera. Beekeeping has been practised in India since the dawn of time. It is unclear when it originally began. Hymns in the Vedas, Hindu sacred writings and the world's oldest literature are the earliest records we have. In the north, the typical yield is 15-17 lbs. (7-8 kg) may be consumed as usual, while certain excellent apiarists produce significantly more. A single hive can produce over 100 lbs. (45 kg) of honey, while the production in the south is substantially lower. The average production is only about 4 to 5 lbs. per hive, except in some favourable locations and well-managed hives.

In 1980, the Indian Council of Agricultural Research (ICAR) launched the All India Coordinated Research Project (AICRP) on Honey Bee Research and Training, which is now known as the AICRP on Honey Bees and Pollinators. The AICRP on Honey Bees and Pollinators has 15 coordinating centres and two voluntary centres across the country, with an administrative centre at the Haryana Agricultural University Campus in Hisar, Haryana.

During the 1980s, the Khadi and Village Industries Commission's (KVIC) numerous initiatives supported an estimated one million bee colonies. Beekeeping is concentrated in the country's southwest hills and nearby plains, which are home to rubber, cardamom, and coffee plantations. India has been dubbed the "Honey Land." Honey was the first sweet food the ancient Indians who found it after immense search in those forests. For this divine gift, he chased beehives. In the form of murals by prehistoric man in rock shelters, India possesses some of the earliest traces of the honey industry. Beekeeping has been performed in its most basic form for centuries. Swarms of bees were drawn to clay receptacles, wood logs, and niches in the exterior walls of houses. Honey was obtained after the flowering seasons by scaring the bees away with smoke and squeezing the honey from the combs. There was no attempt to keep bees from fleeing the hive during the process.

According to Sivaram (2001), bees and plants co-evolved during the middle Cretaceous period (Michener, 1974). Bees rely on flowering plants for sustenance, which comes in the form of pollen and nectar. In the same way, plants rely on bees for pollination. The mutual reliance of anthophilous insects and entomophilous

angiosperms facilitated their co-evolution (Suryanarayana, 1986). The relationship between bees and plants stems from cretaceous times, roughly 100 million years ago (Velthius, 1992). The percent Nectar Sugar Concentration (NSC) of all the nectar-yielding plants was determined and the NSC ranges from 16.24 and 58.50 in *Tecoma stans* and *Syzygium cumini*, respectively. According to Tej *et al*. (2017), *Apis* bee spp. was the only rearable bee in India before the arrival of the Italian bee. It is domesticated in many parts of Pakistan, Nepal, Burma, Bangladesh, Sri Lanka and Thailand, in addition to India. These are non-aggressive and do not move around a lot. These bees build several parallel combs in dark areas such as clay pots, logs, walls, tree openings and so on and each colony may produce 7-9 kg of honey per year.

Thomas (2001) reported that traditionally, farmers have been keeping this species in logs, walls and box hives. *Apis cerana* is a good pollinator and has a high survival capacity due to its co-evolution with native floral sources, pests and predators accustomed to the same climatic conditions. In South India, a large number of beekeepers are earning their living from this indigenous bee. The attack of Thai sac brood disease on *Apis cerana* in 1991 was severe and the fast epidemic disease prevailed over the entire potential beekeeping areas of Kerala, Tamil Nadu, and Karnataka. Almost 90 per cent of the bee colonies perished, affecting honey production.

According to Thomas *et al*. (2002), the technology used in beekeeping varies by location. *Apis dorsata, Apis cerana, and Apis mellifera* provide the majority of the harvest. *Apis cerana* beekeeping is a burgeoning industry in Central and Southern India. Recognizing the possibility of beekeeping as a village enterprise, the Khadi and Village Industries Commission (KVIC) established a well-organized beekeeping programme across India, with over a million bee colonies being raised by lakhs of beekeepers. Lower and middle-class individuals in Karnataka, Kerala and Tamil Nadu are active in *Apis cerana* beekeeping, which is quite popular and produces a considerable amount of honey from rubber estates. The Thai sac brood disease attack on *Apis cerana* was severe in 1991 and the disease spread quickly across the entire potential beekeeping territories of Kerala, Tamil Nadu, and Karnataka.

According to Makkar *et al*. (2010), India has all four major honey bee species: *Apis mellifera, Apis cerana, Apis dorsata and Apis florea.* Honey bees, together with other pollinators, provide an estimated $194.6 billion to global agriculture each year (Gallai, 2008). In 2009, the country produced 72,000 tonnes of honey of which 32,000 tonnes were sold to 47 countries (Chhuneja, 2009). According to Veer and Jain (2017), beekeeping is one of the useful activities that can bring additional income to a large number of rural and tribal producers. The honey, wax, and pollination services provided by bees, which boost the productivity and income of many agricultural and horticultural crops, are the most valued returns of beekeeping. Haryana is one of the pioneer states in honey production, producing 275 Mt of honey with only 28,000 colonies in 2004-05 and increasing to around 3,05,000 bee colonies annually yielding about 4100 Mt of honey.

Traditional beekeeping with *Apis cerana* is widely practiced by rural people, according to Tiwari *et al*. (2013), who conducted a survey in the Chamoli district of Uttarakhand and found that (14%) of total families in the district were involved in traditional beekeeping as a secondary occupation to agriculture. The majority of people (85%) learned beekeeping from their parents, elders, or neighbours, while 10 per cent developed their abilities via experience and 5 per cent received training from various organizations. The peasants' sole source of honey comes from beekeeping.

According to Agarwal (2014), beekeeping in India is primarily based in the forest. As a result, nature provides the raw material for honey manufacturing. Beehives do not require more land or compete for resources with agriculture or animal husbandry. To look after the bee colonies, the beekeeper simply needs to devote a few hours per week. There are 150000 beekeepers and 600000 beehives, with an average annual honey production of 8.5 kg per beehive.

Thakur (2014) stated that the Mahabaleshwar Bee Research Centre (MBRC) opened in 1952 and was upgraded to the Apiculture Research Laboratory in 1954. On November 1, 1962, KVIC founded the Central Bee Research and Training Institute (CBRTI) in Pune with the support of the Maharashtra State Khadi and Village Industries Board (MSKVIC). The importance of the beekeeping business was

recognized by Mahatma Gandhi, who included it in his rural development program. Many commercial beekeepers in states like Himachal Pradesh, Bihar and South India use migratory beekeeping. Honey production in India is around 70,000 tonnes per year, with exports of 25,000-27,000 tonnes per year to over 42 countries. In India, there are around 1.6 million bee colonies, which produce an estimated 65,000 metric tonnes of honey each year, including honey from wild honey bees. Punjab, Haryana, Uttar Pradesh, Bihar and West Bengal are the biggest honey producing states.

In India, beekeeping is mostly practiced as full-time employment and an engaging hobby to create attractive money and table honey, according to Tej *et al*. (2017). Honeybees are a unique gift to humanity because beekeeping may be used for both pollination and the production of valuable items such as honey, beeswax, propolis and bee venom. In India, these goods are widely used in a variety of local and large-scale enterprises. The only unpleasant aspect of beekeeping is the sting of bees. Honeybees sting to defend their colony, but this bitterness will only last for the first few weeks of beekeeping, and after that, only the sweetness of honey will be tasted. Over time, most beekeepers build up a tolerance to bee venom and become less sensitive to discomfort and swelling. So, learning about honey bee science means getting to know and comprehending one of nature's most hardworking and intriguing creatures.

2.1.2 Beekeeping in Kerala

According to Nair (2003), Kerala that produces the greatest amount of honey. However, the quality is worse, which has lowered the price of Kerala honey in the Indian market possibly due to the honey's reliance on rubber (*Hevea braziliensis*), which produces nectar from the extra floral nectaries in the leaf, while the coconut palm provides pollen. Flower sources and woodland habitats are the most ideal in terms of greater market price and honey quality. Floral honey is mostly made from wild plants and Kerala lacks a scientific study in this area.

In the years 2007-2008, the Kerala Khadi and Village Industries Board ran the “Keralam Then Kalavara” initiative, which focused on training beekeepers to be more diligent in assuring the quality of honey produced. The market would respond positively to such honey. In 2007-2008, the Board agreed to hold 20 training programmes in ten

districts (Thiruvananthapuram, Kollam, Pathanamthitta, Kottayam, Eranakulam, Palakkad, Malappuram, Thrissur, Kozhikkode and Kannur). Each batch has a 10-person intake capacity.

For commercial beekeeping in the state, the Indian honey bee, *Apis cerana indica,* was utilized. At the time, a devastating epidemic of a terrible disease in Indian honeybees wiped out 95 per cent of all bee colonies, nearly eradicating commercial beekeeping in the state. The number of bee colonies in the state is believed to be around 6 lakhs. Commercial beekeeping in the state was done with the Indian honey bee, *Apis cerana indica* (GOI, 2013).

2.1.3 Foraging activity

During the wet season, worker bees carry fewer pollen pellets (Sharma, 1989). During the winter season, both black and yellow strains pollen carrying capacity was at its peak. Dhaliwal claimed that the *Apis cerana* hill station collects the most pollen and that the weight of the pollen load is determined by pollen sources and weather conditions during the season.

According to Deuri *et al*. (2018), the peak time for *A. cerana* to visit mango was between 09:00 and 10:00 a.m. Honey bees were at their most active foraging in the pollination of guava between 09:00 and 11:00 a.m. (Ahmad, 2011). The greatest number (09.75) of *A. cerana* per sq. m. per minute was reported at 09:00-10:00 h and the lowest number (01.90) around 13:00-14:00 h. On apple blossoms, *Apis cerana* had the highest visitation frequency and quantity compared to *A. mellifera* (Naveen and Joshi, 2010. The activity began at 06:00 h, with the peak being between 09:00 and 11:00 h (16-20 bees/panicle) and the lowest occurring between 14:00 and 17:00 h (2-5 bees/panicle) and none after 18:00 h.

Hemalatha *et al*. (2018) measured bee foraging activity by counting the number of worker bees leaving and returning to the hive with and without pollen loads every five minutes using a hand tally counter and stopwatch. Nectar gatherers were regarded as bees that returned without pollen burdens.

During the whole study period, Hemalatha *et al*. (2018) evaluated foraging flora once a week. From 6.00 am to 6.00 pm, observations were made by documenting the different flowering plant species on which the bee forages. According to Balchandra *et al*. (2014), bee foraging plants were validated by bee visits and successful foraging of at least three bees within a 10-minute observation period. Bee pasturage was divided into 41 nectar producers, 29 pollen producers and 183 nectar and pollen producers.

Tiwari *et al*. (2012) performed a survey in Kamad, Bagi, Kumarkot, Thandi, Bhadkot, Jalang, Med, Sem, Marwari and Niwalgaon to collect honey samples from wall hives of domesticated *Apis cerana indica* colonies. All of the honey samples were unprocessed. The samples were taken from the honey extraction sites in sterile plastic bottles. To eliminate suspended particles such as dirt, bee wax and other contaminants, the honey was filtered using a single-thickness fine cloth. It was kept at room temperature in sealed containers.

In the beehive box, Hosamani *et al*. (2019) examined the foraging behaviour of *A. cerana* of uniform strength across five colonies. At hourly intervals commencing at 06:00 to 18:00 h, observations on the number of bees returning with pollen load and without pollen load for every five minutes in each hive were recorded at the hive entrance. Pollen foragers are arriving bees with pollen burdens on their legs (corbicula), whereas nectar foragers are those without pollen loads.

2.2 Palynological studies

Melissopalynology or Melittopalynology is a branch of palynology that covers different research on the botanical and geographical origin of honey by exposing honey sediment, and therefore pollen and other fungus *imperfecti* found within, to microscopic examination (Lakshmi, 2012).

Bee pollen is an apicultural product that may be used for its nutritional value in the human diet (Diana *et al*., 2011; Sarmento *et al*., 2006). Pollen gathered by honeybees has a chemical makeup that makes it a possible source of energy and proteins for human use (Abreu, 1992; Block *et al*., 1994). The chemical composition of honeybee pollen is influenced by plant species, environmental conditions during pollen

formation, plant age at pollen development, nutritional state of the plant, pollen extraction technique and pollen storage (Oliveira *et al.,* 2009). Proline is the most abundant free amino acid in honey and pollen. Pollen pellets collected from flowers are the most significant source of proteins and free amino acids for honeybees (Herbert and Shimanuki, 1978; Gonzalez *et al*., 2006).

Melissopalynology, which is concerned with the study of pollen grains found in honey samples, is highly significant in the beekeeping business. When combined with essential field research including phenology and floral biology, pollen loads collected by honey bees from a given location provide accurate information on the flower kinds that serve as significant or minor nectar and/or pollen sources for the bees. Melissopalynological research has shown the many varieties of floral honey that may be found in different seasons, as well as the flow and dearth periods in a given area. The pollen spectrum of honey is created to reflect the diverse character of floral components, their connections, and the geographical spread of pollen Saklani and Mattu (2018).

2.2.1 Pollen density

Suryanarayana *et al*. (1981) used the approach of Kalpana *et al*. (1990) to report absolute pollen counts of honey samples. *Apis cerana* honey had an absolute pollen count of 5,37,000 per gram. All three honey samples are superior to Group V according to the ICBB grading standards of 1970. According to several studies, the pollen density in honey samples varies greatly. The pollen content of honey samples obtained in Tamil Nadu ranged from 10000 to 77000 grains per ml (Singh and Suryanarayana, 2002), whereas the pollen density of honey samples collected in Annamalainagar ranged from 2464 to 4826 grains per ml (Hariprasad *et al*., 2006). The pollen density of 10 honey samples from Karnataka ranged from 4000 to 80,000 grains per millilitre (Bhargava *et al*., 2009). The absolute pollen count of 10 honey samples from Ahmednagar confirmed this, ranging from 48,000 to 12,50,000 grains per millilitre (Kolhe *et al*., 2011). Tiwari *et al*. (2012) found a similar large variance in pollen count in honey samples from different Himalayan regions, with pollen density ranging from 500 to 5,500 grains/ml.

2.2.2 Palynological analysis of pollen loads

Chaudhary (1978) gathered pollen samples from Punjab and analysed 5,200 pollen loads obtained from the Indian hive bee, *Apis cerana* and the results showed that 56 per cent of pollen loads contained pollen from several plant species. Barth (1990) gathered almost 400 samples of honey from various parts of Brazil. A pollen spectrum of honey samples revealed that 190 of them were monofloral honey. *Eucalyptus sp.* and *Citrus sp*. were the most prevalent sources for this honey. Ramalho *et al*. (1991) gathered 256 samples of honey from various locations in southern Brazil. Based on the sources of pollen grains contained in the samples, the honey was categorized as Eucalyptus honey (54 samples), Citrus honey (49 samples) and wild honey (153 samples).

According to Seethalakshmi (1980), melissopalynological studies of 12 different apiaries extracting honey from eight different states revealed that Indian honey falls into the International Commission for Bee Botany's Group-I to Group-III categories, i.e., honey with absolute pollen counts ranging from 10,000 to 5,00,000 per 10 g of honey.

Pollen analysis was performed on 6 summer pollen samples obtained from rock bee combs in a tropical dry deciduous forest region of Andhra Pradesh, India, by Jhansi *et al*. (1991). All of the pollen samples were multi-floral and included 57 different pollen types. Ramanujam *et al.* (1993) collected four pollen samples from *Apis cerena* colonies for pollen analysis in the winter season. This investigation revealed that three of the four samples were *Sapindus emarginatus* unifloral honey (71-84 % total pollen) while the fourth contained 35 per cent *S. emarginatus* pollen. Five summer samples were identified as unifloral, with three being from *Cocos nucifera* (48-58 % pollen) and two from *Borassus flabellifera* (63 and 77%).

Singh *et al*. (1994) reported that analytical studies of pollen have both unifloral and multifloral types. Melissopalynological studies of 21 pollen samples collected from ten localities in Himachal Pradesh revealed the presence of dominant sporomorphs such as *Brassica* sp., *Adathoda* sp., *Clematis* sp., *Mussenda* sp., *Helianthus* sp.

Subir Bera *et al*. (1997) investigated the qualitative and quantitative characteristics of seven *A. cerana* pollen samples obtained in Sikkim and West Bengal's sub-Himalayan regions. *Trifolium, Calendula*, and *Brassica* were the most common pollen types found in unifloral samples. The nectar sources in this location looked to be *Clematis, Rubus, Centauiti, Sechium, Moringa,* and *Leucas*.

Joshi *et al*. (1998) from Pune studied melissopalynology using 38 pollen samples, 28 of which were unifloral and 10 of which were multi-floral. During the peak of the honey flow season, significant nectar sources such as *Bombax, Lannea, Limonia, Moringa, Peltofonm, Pongamia, Syzygium,* and *Tamarindus* were all present (February-July). During the minor honey flow season, *Eucalyptus* and *Alternanthera* pollen were the most prevalent (September-December).

Pollen is a nectar source for honeybees. A classic example of pollen load research (Suryanarayana *et al.,* 1991) is Hodges (1955), who offered a thorough and practical guide to locating pollen sources in the UK. Pollen grains can also be used to determine the botanical and geographical provenance of bee products (Borges *et al*. 2006). Pollen grains are harvested in loads, transported to hives and stored in cells along with the honeycombs (Dorea *et al*., 2010). A well-established melissopalynological approach for determining the sources of pollen for honey bee colonies is the microscopic examination of pollen loads gathered by foragers.

While researching the palynological characteristics of eucalyptus honey, Terrab *et al*. (2003) discovered that eucalyptus honey must contain at least 70 per cent eucalyptus pollen to be classified as unifloral pollen. Pollen analysis revealed the presence of six pollen types, but the majority of the samples (75%) contained more than 90 per cent eucalyptus pollen.

Vijaya *et al.* (2008) found that pollen from *Melilotua alba* (47%), *Ageratum conyzoides* (57.5%), and *Aspidopteris indica* (82.5%) were present in more than 45 per cent of the winter samples, which was referred to as the major pollen type. *Melilotus, Aspidopteris*, and *Ageratum* honey are other names for these. Summer honey is

dominated by pollen from *Phoenix sylcestris* (53.75-83.75%), *Dillenia pentagyna* (90%), *Schleichera oleosa* (53.75%) and *Gardenia lucida* (47.5%).

Krishna and Patil (2011) collected fourteen samples, out of which eleven samples appeared to be uniforal with predominant pollen and three were multifloral. In the samples of Irde (DK-Ir-01), Mithanadka (DK-Mi-05) and Mitoor (DK-Mt-14), *Ixora coccinea* was the predominant pollen, with pollen ranging from 46 to 60 per cent. Samples were collected from Betampady (DK-Be-03) and Kadaba (DK-Ka-09). *Cocos nucifera* was the predominant pollen, ranging from 50 to 60 per cent. Some of the pollen, such as *M. pudica, P. guajava, A. catechu, C. nucifera* and *I. coccinea* were predominant and can be considered an important source of forage for honey bees in Dakshina Kannada.

Tiwari *et al*. (2012) reported that the results of pollen analysis consist of various types of pollen which were collected from *Alnus nepalensis* (16.37 to 64%), *Sarcococca coriacea* (6.5 to 18%), *Impatiens spp.* (5.7 to 18%), *Chenopodium album* (4.6 to 16%), *Prinsepia utilis* (16 to 24%), *Fagopyrum dibotrys* (3.5 to 16%), *Coriandrum sativum* (3.9 to 16.3%), *Rhamnus sp*. (3 to 18.2 per cent), *Symplocos* sp. (16.3 to 19 per cent), Asteraceous (53%), Poaceous (20%) and Lamiaceous (3.9%). *Alnus nepalensis, Chenopodium album, Sarcococca coriacea, Fagopyrum spp., Impatiens spp., Citrus spp*., Asteraceous, Rosaceous and Poaceous, plants are the major source of forage for honey bees.

Balasubramanyam (2013) gathered pollen samples from various locations and processed them using the method outlined by Louveaux *et al.* (1978) for determining the floral source of the honey using pollen grains. Melissopalynological analysis of samples from the study area revealed the presence of plantations (*Cocus nucifera, Coffea arabica, Tectona grandis*), forest flora (*Syzygium caryophyllatum, Borassus flabellifera, Sapindus emarginatus*), and commercial crops (*Musa paradisiaca, Ricinus communis, Anacardium occindentale).*

Suryanarayana *et al.,* (1991) collected a total of 40 species were used as pollen sources for the project. *Zea mays, Brassica spp, Phoenix sylvestris* (L) Roxb, and *Borassus flabellifer* were the most important pollen sources for *A. cerana*, in order of significance. *Zea mays* contributed 34 per cent of total pollen loads and were a reliable source of pollen almost every month. *Cyanotis* sp., *Cosmos bipinnatus*, Fabaceae, Cucurbitaceae and *Cocos nucifera* were medium-importance pollen suppliers, contributing a significant amount of pollen.

Mamatha *et al*. (2018) scanned eleven pollen samples for palynological studies and identified 32 pollen taxa, including *Hygrophila auriculata* (Schumach.) Hence, *Justicia procumbens* L., *Amaranthus viridis* L., *Borassus flabellifer* L., *Cocos nucifera* L., *Phoenix sylvestris* (L.) Roxb., and *Coriandrum sativum* L. Pollen was found in 75-80 per cent of these kinds.

Saklani and Mattu (2018) performed a pollen analysis of 17 pollen samples collected from Hamirpur hills of Himachal Pradesh, India during 2011-12 were investigated to ascertain the honey pollen sources for honeybees. During this investigation, 84 pollen types belonging to 41 different families were recorded. The highest pollen types frequency was observed for the family Fabaceae followed by Asteraceae. Among 84 pollen types, 66 were identified up to genus and species level whereas, the rests were up to family level. *Woodfordia fruticosa, Prunus* sp., *Adhatoda vasica*, *Eucalyptus camaldulensis*, *Brassica* sp. and Rutaceous member were the most common pollen types, while others were secondary, important minor, and minor pollen types.

Jamwal and Mattu (2020) gathered pollen content from 11 pollen samples obtained from *Apis cerana* F. hives in different parts of the Kullu mountains in Himachal Pradesh. A total of 46 pollen taxa from 27 families were discovered. There are 42 nectariferous plants and four non-nectariferous plants among them. Seven of the 11 honey samples were unifloral, whereas four were multifloral.

Jamwal and Mattu (2020) collected pollen contents of 12 autumn honeys from different areas of Himachal Pradesh's Kullu hills and identified 5 pollen types as predominant, 17 as secondary, and 38 as important minor and minor pollen sources.

2.2.3 Microscopic analysis

Sharma and Nair (1965) conducted identification of pollen in helps, which leads to the identification of the sources and analysis of the bee pollen loads, which reveals the pollen sources of an area. Honey pollen analysis provides an index of the honey-yielding plants in a particular locality. The microscopic examination of pollen grains in honey is termed melissopalynology or pollen analysis.

Louveaux (1978) and Barbattin *et al*. (1991) have given a minimum pollen count was found in sample KA BDIII-13 and a maximum pollen count was found in sample KA-BDI-11, and honey samples were categorized into Group 1 (pollen count 2000), Group 11 (2000-10,000), Group 111 (10,000-50,000), Group IV (50,000-10,00,000) and Group V (> 10,00,000), which indicate extremely poor, poor and richness of pollen in honey. Pollen types were classified as major (>45%), secondary (16–45%), important minor (3–15%) and minor (3%) based on pollen frequency.

Lieux (1980) established a microscopical pollen analysis method for processing honey samples from the United States. The approach includes an acetolysis schedule and enables the gathering of both quantitative and qualitative data in a single step. The benefits and drawbacks of the acetolysis technique were discussed.

Kalpana *et al*. (1990) performed pollen identification by dispersing each pollen load in water and then acetolysing. Two pollen slides were prepared for each load and examined microscopically. Pollen loads with one pollen type were called unifloral, with two pollen types as bifloral and loads with more than two pollen types as multi floral or mixed (Sharma, 1970). Identification and confirmation of the pollen grains recovered and pollen loads were based upon comparison with reference slides of acetolysed pollen grains of the Osmania University campus flora and concluded that *A. cerana* pollen was characterized by the presence of *Mangifera indica* (Anacardiaceae, 68.7%) as the

predominant pollen type. *Phoenix sylvestris* (Palmae) is the important minor pollen type of this honey.

Garg (1996) carried out pollen analysis in the manner indicated by Garg and Nair (1994). Individual pollen grains were dissolved in water and acetolysed using a conventional acetolysis technique (Erdtman, 1952 and Nair, 1960). Pollen that had been acetolysed was split into two pieces. For light microscopy, one portion of the pollen is fixed on a slide using glycerine jelly, while another section is mounted with brass studs and coated with gold for SEM.

According to Nagamitsu and Inoue (1999), pollen grains from every sample were acetolysed and mounted in silicon oil on a slide. Slides were observed under a light microscope. From each of the slides, 200 pollen grains were randomly selected and identified at least one genus (Nakamura, 1980a, 1980b; Nagamitsu and Nagamasu, 1994). According to the method of O'rourke and Buchmann (1991), the proportion of pollen volume represented by plant taxa in every sample was calculated and it was concluded that *Apis cerana* preferred *Celastrus, Euonymus* spp., *Aesculus* and *Acer* spp. in May and June.

Vijaya *et al.* (2008) amassed eleven pollen samples were collected from the various mandals of the Khammam district. To analyse the pollen content, three pollen slides were prepared for each sample and examined. With the help of a reference slide collection of the local flora and relevant literature (Kirtikar and Basu, 1993), pollen types were identified as far as possible at the genus or species level. The frequencies and frequency classes of the pollen types were determined by Louveaux *et al*. (1978).

Chandra and Sharma (2011) collected pollen and nectar samples from various areas of Uttar Pradesh and prepared slides, finding that the main pollen and nectar sources are *Brassica compestris* Linn var. *sarson* Prain, *Cajanus cajan* (Linn.) *Mill* sp, *Cassia occidantalis* Linn., *Bauhinia vareigata* Linn. and *Psidium guajava* Linn. in winter and *Azadirachta indica* A. Juss., *Sorghum vulgare* Linn., *Cassia tora* Linn. and *Dalbergia sissoo* Roxb. in summer season. In the bee colonies of the area, members of the Caesalpiniaceae family provide the majority of pollen and nectar.

Chauhan and Trivedi (2011) created permanent pollen slides in glycerine jelly and sealed them with paraffin wax for microscopic inspection. The pollen grains in the samples were identified using reference pollen slides from the BSIP-Herbarium as well as pollen photos from the published literature (Chauhan and Bera, 1990; Nayar, 1990). Samples having 45 per cent or more pollen of a single kind is considered unifloral by the International Commission for Bee Botany (ICBB).

Kalpana e*t al*. (1990) prepared five pollen slides from each pollen sample and identified them based on the pollen morphological characters and also by comparing them with the reference slides prepared from the local flora. The absolute pollen count of each sample was determined by the richness of pollen per gram of honey according to the method suggested by Suryanarayana *et al*. (1981) and the absolute pollen count of all the 15 samples from the study area ranged between 58,400-1,48,000 per gram of honey.

Tiwari *et al*. (2012) used the acetolysis technique to create pollen slides (Erdtman, 1960). The pollen sediment was collected on a glycerin jelly pellet and placed to the center of 75x25 mm slides with a thickness of 0.8 mm. The melted jelly with pollen sediment was covered with cover glass after being slightly warmed (22 mm). Later, paraffin wax was used to seal the cover glass (Nair, 1960). Pollen grains were recognized using standard slides made from local flora and classified into four pollen frequency classes: dominant (45%), secondary (16-45%), important minor (3-16%), and minor pollen types (3%).

2.3 Physicochemical analysis

The most commonly studied physicochemical parameters of the Indian bee include moisture content, pH, electrical conductivity, sugar content, Hydroxy Methyl Furfural (HMF), free acidity and ash content. The least studied parameters include colour, enzyme activity, nitrogen content, soluble solids and Fiehe's test (Nordin *et al*., 2018; Julika *et al*., 2019; Shamsudin *et al*., 2019).

2.3.1 Moisture Content

Herbert and Shimanuki (1978) estimated the total per cent moisture in pollen and bee bread by placing 5 g of each sample in a hot air oven at 100°C for 24 h. After the samples were removed from the oven, they were allowed to reach room temperature and then weighed. The difference between dry weight and the initial weight was used to calculate the per cent moisture. Moisture levels, for example, ranged from 21.7 per cent in Wyoming to a high of 27.0 per cent in California.

Al-Khalifa and Al-Arify (1999) reported moisture in pollen using a refractometer (AO), Model (10480 S/N) (AOAC, 1995). Moisture content varied due to the type of sample. As a result, the average moisture ranged from 14.0 to 16.90 per cent, while current Saudi regulations require a minimum moisture of 21 per cent.

Andrade *et al*. (1999) stated that the pollen samples possessed moisture from 14.6 to 19.9 per cent with an average of 17.83 per cent which means a proper degree of maturity and agrees with the reported higher moisture in Ericaceae (Crane, 1975, 1990).

Conti (2000) reported that moisture values vary from 16.36$\pm$0.02 per cent (range of 16.00±18.04 per cent) when determined using a special refractometer. Terrab *et al*. (2002) experimented on water content (moisture) with an Erma refractometer reading at 20˚C, using the Wed more table (AOAC, 1990). Water content, a parameter related to the maturity degree (White, 1975) shows values between 14 and 21.3 per cent; two samples with a water content over 20 per cent were found, the maximum allowed by the Spanish regulations.

Anupama *et al*. (2003) measured the moisture content of pollen samples by the refractometer method. A hand refractometer reading of 20˚C was recorded. The moisture content ranged between 17 and 22.6 per cent. F and I samples had high values for viscosity (13.8 and 12.1 Pas) corresponding to 17.0 and 17.6 per cent moisture, respectively. Commercial samples examined in this study had higher moisture content than reported values.

Nanda *et al*. (2003) reported that moisture in pollen was determined with a refractometer reading at 20˚C and obtaining a corresponding per cent moisture from the table (AOAC, 1990). Trifolium pollen showed the highest value of moisture content as compared to other types. The mean values of the moisture content of Eucalyptus (17.09%) and Brassica (14.63%) pollen were lower than the values of 19.4 per cent (*Eucalyptus*) and 21.8 per cent (*Brassica*) reported by Singh and Bath (1997). Estupinan and Sanjuan (1998) reported the moisture content of pollen in the range of 13-25 per cent.

Downey *et al*. (2005) performed the determination of moisture content with an Abbe refractometer reading at 20˚C using the Wedmore table (AOAC, 1990). High moisture content renders pollen liable to fermentation, spoilage and flavour loss, resulting in a significant decrease in quality (Costa *et al*., 1999). Kinds of pollen from first year had an average moisture content in the range of 15.6 to 18.8 per cent w/w, indicating optimum harvesting and a good degree of maturity. Moisture levels in the second year ranged from 16.3 to 20.6 per cent.

Acquarone *et al*. (2007) determined water content by refractometry, measuring the Refractive Index (RI) according to AOAC Methods (AOAC 969.38B, 1996), using a standard model Abbe type refractometer at 20˚C. Most of the water content values were below 18 per cent and all of the values were below 21 per cent which is the maximum fixed by the Codex Standards (1981/2001).

Ouchemoukh *et al*. (2007) determined water content (moisture) by refractometry using an Abbe-type refractometer (RF 490, Euromexholland). Values ranged between 14.64 to 19.04 per cent, corresponding to a refractive index between 1.4889 and 1.4999. Oued-Des pollen (H01) has the highest moisture content as compared to other samples.

Kahraman *et al.* (2010) stated that the moisture content of pollen is a highly important factor contributing to its stability against fermentation and granulation during storage (Singh and Bath, 1997). In this study, 4.29 per cent (3/70) of samples were in

an unacceptable range. The moisture content of samples at permitted levels was found to be in the range of 13.6-9.4 per cent.

Cherian *et al*. (2011) analysed the moisture content of pollen extracted from pollen and reported that the sample value ranged from 19.1 to 23.1. The moisture percentage of capped pollen ranges from 19.1 to 19.8 and uncapped or immature pollen ranges between 21.3-23.1. The moisture content of the sample was found to be 21 per cent higher than the maximum allowable content for pollen.

Gairola *et al*. (2013) reported that the moisture percentage or water content of pollen was observed to be between 19 and 25 per cent. These values were in the range of earlier reported values of 23 per cent by Natarajan and Yesuvadian (1978) from Kerala, India. According to Singh and Bath (1997), similar moisture content values were determined to be 18.7-21.8 per cent for some Indian pollen. Generally, Indian pollen has a significantly higher moisture content (20-25%) in comparison to Western kinds of pollen, *i.e*., 12-15 per cent (Manjunath, 1999). The water content depends upon the environmental factors during production, such as weather and humidity inside the hive, but also on nectar conditions.

2.3.2 Water activity

Conti (2000) reported that water activity could be determined using a Rotronic detector (Rotronic Instruments Corporation, A2101 set, USA), which is based on the combination of a microprocessor-based indicator with a ventilated probe. Water activity was very low in all 30 samples analyzed with a mean value of 0:57$\pm$0:016.

Acquarone *et al*. (2007) reported the water activity of liquid pollen samples was measured with an Aqualab (Decagon Devices, Inc.) at 20^{o}C and a_w varied from 0.513 to 0.597.

Estevinho *et al*. (2012) determined water activity by using a model, Rotronic Hygroskop DP (Labmaster, Turku, Finland). All pollen samples studied had higher values for water activity (ranging from 0.32 to 0.55) than the value of 0.27 established by Serra Bonvehi and Jorda (1997).

Barajas *et al*. (2012) determined that the water activity of the samples was measured using a water activity meter (model ms1, Novasina AG, Lachen, Switzerland). The calibration was performed using a salt standard (Gleiter *et al*. 2006). Measurements were made in triplicate. a_w values according to the treatment, fresh (0.73-0.78), dried at 35^0C.

Barbara *et al*. (2015) estimated water activity by placing each pollen sample directly into a water activity meter (Rotronic HygroPalm. Bassersdorf CH-8303) (AOAC,1990) and values are with 0.86 ± 0.02 per cent (values obtained at 26.7 ± 0.8°C).

2.3.3 pH

According to Al-Khalifa and Al-Arify (1999), pH was measured by a pH meter (Corning M240) from a solution containing 5 g pollen in 10 ml of CO_2 free distilled water. Kinds of pollen are acidic, having a pH in the range of 3.5±5. Thus, all samples fell within the Saudi legal regulations for pH value, except that Sidir Aseer pollen exceeded 6.06 pH value.

Andrade *et al*. (1999) stated that the pH found in all samples (3.60±4.46) corresponded to that of oral kinds of pollen (Crane, 1990).

Conti (2000) mentioned that the pH was assessed using a potentiometric pH meter (Mettler Delta 345; Mettler Toledo SpA, Novate Milanese, Milano, Italy). The majority of analysed kinds of pollen showed a pH value of between 4 and 5 with a mean value of 4.3 ± 0.5.

Terrab *et al*. (2002) reported that the pH was measured by a pH meter (Orion 420 A) in a solution containing 10 g pollen in 75 mL of CO_2 free distilled water (AOAC, 1990). pH values, of great importance during pollen extraction and storage due to their influence on texture, stability and endurance, range between 2.25 and 4.71.

Anupama *et al*. (2003) added 10 g of homogenized pollen to 50 ml of distilled water and the pH was read directly from the pH meter. The instrument was calibrated with standard buffer solutions of pH 7 and pH 4, before measuring the pH of the

samples. Samples varied in pH from 3.62 to 4.00, except sample J, which had a pH of 5.46, and the values are comparable to those reported by Esti *et al*. (1997), who reported pH in the range of 3.05-4.50. Mateo and Bosch-Reig (1998) and Lopez *et al*. (1996) reported pH in the range of 3.61-4.97.

Downey *et al*. (2005) indicated that the kinds of pollen tested were most likely of floral origin since pollen dew kinds of pollen generally have a higher ash content than floral, resulting in with less active acidity and therefore a higher pH (White, 1978). The pH values recorded were similar to those obtained by Iglesias *et al*. (2004) for floral kinds of pollen collected in the centre.

Acquarone *et al*. (2007) obtained pH values by performing potentiometrically a 20^0C using a pH-meter (Mettler Toledo Delta 320) in (a) a 10 per cent (w/v) solution of pollen in freshly boiled distilled water according to local regulations (Norma IRAM 15938, 1996). (b) pollen samples diluted with freshly boiled distilled water in concentrations ranging from 10% to 100 per cent (w/v). The values range from 3.12 to 3.88, which means it is acidic.

Ouchemoukh et al. (2007) used a pH metre (CRISON 2000) to determine the pH of a 10 per cent (w/v) solution of pollen in distilled water The pH of dew and blossom pollen blends is between 3.5 and 4.5. The pH of honeydew is between 4.5 and 5.5 (Gonnet, 1986).

Cherian *et al*. (2011) measured pH by performing at 20±0.1°C on the stirred solution obtained after dissolving 10 g of sample in 75 ml of distilled water by potentiometric titration with a 0.1M NaOH solution until pH reached 8.3 (Bogdanov, 2002). The pH value fell within the normal range, i.e., 3.5 to 3.6.

2.3.4 Ash Content

Sulfated ash in samples was determined according to AOAC Method No. 29.014. Todd and Bretherick (1942) reported that the ash content of bee-collected pollen ranged from 0.91 to 6.36 per cent with a mean of 2.70 per cent. The ash content ranged from 2.4 to 3.5 per cent (with a mean of 3.17%) (Herbert and Shimanuki, 1978).

White *et al*. (1962) and White (1967) found levels of ash of over 1 per cent in pollen samples.

Ash content was determined by the methods of Bogdanov *et al*. (1997). The ash content was determined by heating 5 g of pollen at 625˚C in a muffle furnace. The ash content of the studied pollen samples differs widely. It ranged between 0.06 and 0.54 per cent. The maximum value is found for the sample H01, followed by the sample H02 (0.23%) (Ouchemoukh *et al*., 2007).

Andrade *et al*. (1999) stated that the range of values for total ash content in samples is of 0.04 ± 0.52 per cent. Al-Khalifa and Al-Arify (1999) performed ash percentage by calcination overnight at 550ºC in a furnace to constant mass (AOAC, 1995). Farm and Magra Aseer pollen had the highest ash content when compared with others. Pollen normally has a low ash content and this depends on the material collected by the bees during foraging.

Nanda *et al*. (2003) measured the ash content by calcination, overnight at 550˚C in a furnace to constant mass (AOAC, 1990) range of values for ash content (0.12-0.28 per cent) fell within the limit allowed for floral pollen (0.6%) and indicated the cleanness of pollen samples and possibly the lack of adulteration with molasses (Krauze and Zalewski, 1991). Potassium was the most abundant of the elements present in all pollen types while copper was present in the lowest amount.

According to Downey *et al.* (2005), ash content is a quality criterion of particular relevance for pollen of stated botanical origin (White, 1978); blossom pollen has a lower (60.6%) ash content than honeydew (61.2%). Samples 7, 13 and 17 (year 1) exhibited ash content of 0.07, 0.16 and 0.11 per cent w/w with corresponding *T. repens* pollen levels of 71 per cent, 78 per cent and 69 per cent. While the frequency distribution of ash values exhibits an approximately equal occurrence of ash values in the range of 0.05–0.26 per cent.

Acquarone *et al*. (2007) performed ash content by using samples of 5-10 g which was weighed into ignited crucibles and placed under an IR lamp with variable voltage input. Voltage was slowly increased until samples were black and dry and then

they were incinerated in a furnace a 600ºC to constant weigh, cooled and weighed. The result was expressed as g ash/100 g of pollen (AOAC 920.181, 1996). Values were below 0.6 per cent in all cases, as expected for pollen (CAA, Codex and EU Standards). The samples CaM (Group I) and Vv (Group II), from regions with different soil characteristics, presented the highest values for ash content that could be attributed to different clime and/or floral origins.

Kahraman *et al*. (2010) mentioned that the ash content depends upon the botanical and geographical origins of pollen samples. The ash content in pollen is generally small and depends on the nectar composition of predominant plants in its formation (Al-Khalifa and Al-Arify, 1999). In our study, the ash content of samples (mean 0.28%) was in the acceptable range.

Gairola *et al*. (2013) measured ash content by utilizing ignition of pollen samples in a muffle furnace at 550˚C to a constant weight. Generally, it depends on the nectar composition of dominant plants in their formation (Al-Khalifa and Al-Arify, 1999). The ash content in the pollen samples varied between 0.09 and 0.18 per cent. The ash content is associated with the botanical and geographical origins of pollen samples.

Vijayakumar *et al*. (2020) estimated the ash content of the pollen sample by burning 5–10 g of the sample with a few drops of pure olive oil in a silica or platinum dish. The ash content of pollen samples collected from different species of honeybee showed a range from 0.15 to 0.44 per cent, with an average of 0.32±0.07 per cent. Samples are on par with Pongamia-*A. dorsata* pollen (BaDF/Pp) (0.40%).

2.3.5 Total acidity

Al-Khalifa and Al-Arify (1999) performed total acidity according to SASO (1990) by the titrimetric method; 10 g of the sample was accurately dissolved in 75 ml of CO_2-free distilled water and titrated with 0.1 N NaOH. High total acidity may mean that the pollen has fermented at some point and that the resulting alcohol was converted into organic acid (Rodger, 1979). Talh Medina pollen had the highest total acidity, whereas Magra Aseer had the lowest value. Andrade *et al*. (1999) showed that the total

acidity was within limits (below 40 meq/kg of pollen), indicating the absence of undesirable fermentation.

Terrab *et al*. (2002) mentioned that total acidity was determined by the titrimetric method: the addition of 0.05 M NaOH is stopped at pH 8.50 (free acidity); immediately a volume of 10 ml of 0.05 M NaOH is added and without delay, back-titrated with 0.05 M HCl to pH 8.30 (lactonic acidity). Total acidity ranges between 12.6 and 115 meq/kg.

Anupama *et al*. (2003) determined the acidity percent as formic acid. The percent by mass of total reducing sugars and sucrose were determined as per the method given in Indian Standards as formic acid varied from 0.03 to 0.15 per cent; five samples (I, E, G, K and A) had more than 0.1 per cent acidity. There was a negative correlation between pH and percent acidity (r = 0.62). Sample J, having the highest pH (5.46), had the lowest acidity (0.03%).

Nanda *et al*. (2003) determined total acidity by the titrimetric method: by adding 0.05 N NaOH and stopping at pH 8.5 (free acidity), immediately pipette in 10 ml of 0.05 N NaOH, and without delay, back-titrate with 0.05 N HCL to pH 8.3 (lactone acidity). Total acidity results from adding free plus lactone acidities (AOAC, 1990). Results were expressed as meq/kg. The total acidity of *Citrus* and *Helianthus* pollen was higher than the other pollen types. Citrus pollen had the highest free acid content, with the lowest value being Brassica pollen. El-Sherbiny and Rizk (1979) reported that total acidity was higher in cotton pollen than in clover pollen, which indicates the influence of floral types on total acidity.

Downey *et al*. (2005) determined total acidity by the sum of free and lactonic acidities (Terrab *et al*., 2002) and the total acidity ranged from 26.8 to 55.9 meq/kg and 21.2 to 52.4 meq/kg in year 1 and year 2, respectively.

Total acidity was determined by the titrimetric method with 0.05 N NaOH to pH 8.5 (free acidity). Total acidity resulted from adding free and lactone acidities. The results were expressed as milliequivalents/kg (meq/kg). total acidity values of below 40

milliequivalents/kg. samples CaE, CaM, SL and Vv showed very high values of total acidity. A linear correlation was found between aw, total acidity and free acidity (total acidity = 1.993 + 1.22) (Acquarone *et al*., 2007).

Kahraman *et al*. (2010) reported that the total acidity of pollen is due to the presence of organic acids, particularly gluconic acid, in equilibrium with their lactones or esters and inorganic ions such as phosphate and chloride (Al-Khalifa and Al-Arify, 1999). The mean value of total acidity was found at 24.1, with a range of 6.94 to 29.6 meq/kg.

Vijayakumar *et al*. (2020) reported total acidity content by following the titration method and values varied from 0.06 to 0.32 per cent with an average value of 0.20±0.07 per cent. *Suregada angustifolia-A. cerana* pollen (BaCE/Sa) and *Pongamia pinnata-A. cerana* pollen (BaCF/Pp-2) had the maximum acidity level, whereas the minimum was in Eucalyptus-*A. cerana* pollen (BaMM/E).

2.3.6 Protein content

Herbert and Shimanuki (1978) estimated total nitrogen content by the AOAC Micro-Kjeldahl method (Association of Official Agricultural Chemists, 1960). Todd and Bretherick (1942) analyzed and found that the content of crude protein ranged from 7.02 to 29.87 per cent.

According to official legislation (AOAC, 1995; Directive 93/28/CEE), the protein content of honeybee pollen was determined using the Kjeldahl technique (Saa-Otero *et al*. 2000). Pollen protein concentrations are high in all beehives tested, ranging from 27 per cent (MC2) to 22 per cent (LG1). This is more than 5 per cent higher than in other parts of the Iberian Peninsula, where it ranged between 18.2 and 12.7 per cent (Serra-Bonvehi *et al*. 1986).

Roulston *et al*. (2000) determined the protein content of honey bee pollen by analyzing total nitrogen by combustion using an LECO 600 carbon and nitrogen analyzer (LECO, St. Joseph, Michigan) or micro-Kjeldahl analysis with a TechniCon Auto Analyzer II. A multiplier of 6.25 was employed to convert nitrogen levels to

protein, which is the most widely used multiplier for plant pollen (Buchmann 1986). Pollen has a protein level ranging from 2.5 to 61 per cent.

Ouchemoukh *et al*. (2007) determined protein content by the method of Azeredo *et al.* (2003). A volume of 0.1 ml of protein extract (pollen sample 50%w/v) was added to 5 ml of Coomassie Brillant Blue. After 2 min of incubation, the number of proteins was estimated at 595 nm with the bovine serum albumin standard curve. The protein content of pollen is normally less than 5 mg/g (Anklam, 1998). The protein contents of analyzed pollen samples were between 3.7 and 9.4 mg/g.

According to Maria *et al*. (2008), pollen protein concentration varies considerably depending on the botanical origin. Only approximately a tenth of total protein is made up of free amino acids. The protein content is calculated using the Kjeldahl technique and a conversion factor of 6.25 or 5.6. (Rabie *et al*., 1983).

The nitrogen content of 0.5 g of sample was measured by Martins *et al*. (2011) after acid hydrolysis and Kjeldahl distillation. 12.28-27.07 g of total protein per 100 g. The protein level of the samples obtained in Parana and Santa Catarina was lower, whereas the lipid content was higher in Rio Grande do Sul. Lower protein levels were seen in Minas Gerais and Espirito Santo.

The macro-Kjeldahl technique was used by Estevinho *et al*. (2012) to determine the crude protein content (N = 6.25). Using a Soxhlet device, the crude fat was measured by extracting the pollen with petroleum ether. Protein content did not differ statistically between pollen from Covilha and Braganca, ranging from 24.23 to 34.18 per cent.

Barbara *et al*. (2015) used the Kjeldahl technique to estimate the protein content of bee pollen (230-Hjeltec Analyzer, Foss Tecator, Hognass, Sweden). Using the conversion factor of 6.25 (N = 6.25), the crude protein (CP) content was determined (AOAC, 1995). Protein content was 21.2 percent on average

Mayda *et al*. (2020) calculated total protein content using AOAC Official Method 990.03. (AOAC, 2007). The total protein content of BP samples ranged between 17.6

per cent (BP3) and 22.2 per cent (BP4 and BP5, respectively). In BB samples, the results ranged from 17.5 per cent (BB3) to 21.2 per cent (BB5).

Ochungo *et al.* (2021) determined the protein content of bee bread samples by crushing them and placing 0.025 g in a microcentrifuge tube. Protein from the samples was extracted by applying it to each of the samples. Then, 4 ml of 30 mM TE buffer was added and vortexed to ensure uniform mixing. The sample mixture was centrifuged for 20 min at 3000x g and afterward, the supernatant was collected in 0.1 mL aliquots. We added 5 mL of Bradford reagent, leaving the setup for 2 minutes at room temperature (Bradford, 1976). The samples were measured using a spectrophotometer at an absorbance of 595 nm. Higher protein percentages were observed in pollen during the long rainy seasons of May and November, as opposed to the dry seasons in January and June.

2.4 Microbial determination

In any habitat or "micro-niche" for insect species, microorganisms play a vital role. Fungi may effectively colonize terrestrial ecosystems and penetrate the substrates to thrive (Paz-Gonzalez *et al*. 2005). Lactic acid fermentation by bacteria, yeasts and molds resulted in the conversion of pollen to bee bread (Foote, 1957; Haydak, 1958; Gilliam *et al*., 1989). In the beehive, actinomycetes generate bioactive chemicals that serve as antibiotics (Promnuan *et al*., 2013). In social bees, symbiotic bacterial species promote colony homeostasis and fitness (Leonhardt and Kaltenpoth, 2014).

Actinomycetes are one of the most extensively dispersed families of microorganisms in nature, accounting for a significant portion of the microbial population in soil and aquatic environments, including rivers, lakes and other freshwater ecosystems (Goodfellow and Williams, 1983).

2.4.1 Bacteria

Serra and Jorda (1997) diluted ten grams of pollen in 90 ml of 0.1 per cent sterile peptone water (Difco-1807, Difco Laboratories, Detroit, MI) and poured 0.1 ml of the solution into each of two Petri plates and then added 10-15 ml of plate count agar

(Difco-0479). Plates were solidified and incubated at 30°C for 72 h before counting *Streptococci* bacterial colonies.

Kacaniova *et al*. (2009) used the plate dilution technique for quantitative Cfu count measurement of respective groups of microorganisms in 1 g of honey and pollen. In triplicates, 1 ml of honey and pollen samples were inoculated on the surface of the nutrient medium in petri plates. After incubation for 48 h, the mean colony population of bacteria observed was composed of anaerobic (3.16), aerobic (3.39), *Escherichia coli* (0.62) and coliform bacteria (1.22).

Estevinho *et al*. (2012) enumerated aerobic mesophilic bacteria counted on a standard Plate Count Agar (PCA; Himedia, Mumbai, India) and incubated at 30^0C for 48 h. Colony-forming units per gram of bee pollen (Cfu/g) were used to represent microbial numbers. Aerobic mesophiles of the bacterium were detected in 40 per cent of the samples, ranging from $2.8x10^3$ to $7.6x10^2$ Cfu/g.

Nogueira *et al*. (2012) performed enumeration on Plate count agar (Himedia) and then incubated it for 48 h at 37°C. Colony forming units per gram of bee pollen (Cfu/g) were used to calculate microbial counts. Only 12.5 per cent of the samples included aerobic mesophiles and the number of colony-forming units varied between 10 and $0.87x10^4 \pm 7.8x10^4$ Cfu/g.

Anderson *et al*. (2014) enumerated a serial dilution series and spread plated in triplicate at concentrations of 10^3, 10^4 and 10^5 on both media types. Pollen grains per gram were used in combination with microbial counts per gram to estimate the absolute number of microbes per pollen grain. The majority of the samples (75 of 120 or 62.5%) produced Colony forming units (Cfu's).

Kis *et al*. (2018) counted aerobic mesophilic bacteria on Plate Count Agar (PCA agar; Biokar, Beauvais, France) incubated at 30±2°C for 72 h. Spore forming bacteria (mainly *Bacillus* spp.) of the Enterobacteriaceae family make up the microflora linked with honey bees and their products. *Clostridium botulinum* colonies obtained from honey are in low quantities throughout the incubation period.

Ceausi *et al.* (2009) counted the total number of mesophilic and aerobic microbes obtained from decimal dilutions in peptonated water and distributed a 1 cm3 volume of each dilution into two Petri plates using sterile droppers. Melted agar was put onto each plate which was then cooled at 40-45^{0}C homogenized and incubated for 24 h at 37^{0}C. *Bacillus cereus*, *Escherichia colli* and coagulase-positive *Staphylococci* were used to demonstrate that a variety of samples included various microbes.

Sinacori *et al*. (2013) determined the Total Mesophilic Count (TMC) on Plate Count Agar (PCA) and incubated it aerobically at 30^{0}C for 72 h. PCA revealed the greatest concentrations of bacteria. Bacteria belonging to Enterobacteriaceae family and *B. amyloliquefaciens, B. megaterium and B. pumilus* were obtained from two samples.

2.4.2 Fungus

Nasser (2004) enumerated and identified molds in pollensamples by spreading eight pieces (approximately 0.5 g each) of each sample over the surface of two 10 per cent sucrose-agar Czapek's plates and incubating them at 28°C for 7 days. The number of developing fungus was determined. *Aspergillus* was the genus with the most species and the highest frequency. *Emericella nidulans*, *Fusarium oxysporum*, *Humicola grisea*, *Penicillium corylophilum*, *P. funiculosum* and *Trichoderma hamatum* were identified in very high numbers which accounts for 8.4 per cent of the total molds.

Kacaniova *et al.* (2009) immersed 5 g of powdered pollen in 45 ml of sterile tap water containing 0.02 per cent for 30 minutes before inoculating 1 ml aliquots on three plates of Czapek-Dox agar with streptomycin to determine fungal Colony forming units (Cfu). The spread plate is used and it is incubated at 25^{0}C for 72 h. *Alternaria alteranata* (42.48%), *Cladosporium cladosporioides* (33.10%) and *Penicillium* spp. were the most common colonies found in the total quantity of 10,75,680 (100%) microscopic fungus isolated from pollen (16.63%).

Felsociova *et al*. (2012) used serial dilution to add 0.1 ml each of 10^{-1} and 10^{-2} aliquots to stiff Malt Extract Agar (MEA) plates. All of the tests were done twice. Finally, the plates were kept in the dark for 5 to 7 days at 25$\pm$1°C. Members of the

Aspergillus genus were isolated on CYA (Czapek Yeast Extract agar, Samson *et al*., 2010) and MEA (Malt Extract Agar, Samson *et al*., 2010) diagnostic media while isolates of the *Penicillium* genus were isolated on MEA and CYA respectively and the contamination level of samples by fungi was law with a charge variable between 0.5 x 10^1 and 1 x (average 1.1 x 10^1 Cfu/g).

Sinacori *et al*. (2013) gathered fungal colonies and streaked them over PDA by observing them at 24 h intervals and transferring them to a new petri dish with the same substrate after they reached a diameter of around 2 mm. At the genus level, isolates from groups II, XI and XV were identified as *Arthinium*, *Emericella* and *Penicillium,* respectively.

Kis *et al*. (2018) used Dichloran 18 per cent mass fraction glycerol agar to count molds and yeasts (DG18; Biokar, Beauvais, France). The results were reported in Colony forming units per gram of honey (Cfu/g). Yeast counts varied from 18 to 1300 Cfu/g in 17 samples, whereas mold counts ranged from 18 to 182 Cfu/g. *Cladosporium* was the most common mold found, followed by *Alternaria, Penicillium, Mucor, Aureobasidium* and *Stachybotrys*

2.4.3 Actinomycetes

Promnuan *et al*. (2009) used the dilution plate method on starch casein agar (Kuster and Williams 1964) supplemented with 25 mg/ml of nystatin and nalidixic acid to isolate actinomycetes from 1 g of each sample. Nakajima *et al*. (1999) disclosed genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and PCR product sequencing. *Streptomyces* was the most common genus of actinomycetes and *S. drozdrwiczii, S. albidoflavus* and *S. badius* were discovered in bees of *A. cerana*.

Patil *et al*. (2010) isolated actinomycetes by using Actinomycete Isolation Agar (AIA) enriched with cycloheximide (50 mg/ml) and rifamycin (5 mg/ml). Filamentous bacterial colonies that appeared powdery, fuzzy or leathery were chosen and purified after being incubated at 30^0C for 3-7 days. At least one Cfu of actinomycete was carried by 70 per cent of the bees during any of the four seasons. The intestines included an actinomycete that was similar to *Nocardiopsis alba.* At this site, between 28-58 per cent of the bees generated at least one actinomycete isolate with measurable bioactivity.

Promnuan *et al*. (2011) isolated actinomycetes on starch-casein agar (Kuster and Williams, 1964) supplemented with 25 g/ml each of nystatin and nalidixic acid using the usual dilution plate method. The isolate was transferred and purified after being cultured at 30^0C for 21 days. The majority of the isolates were identified as belonging to the *Streptomyces* genus with a few less frequent isolates classified as belonging to the *Nonomuraea* and *Nocardiopsis* genera. The categorization of IM17-1T within the genus *Actinomadura* was based on these morphological and chemotaxonomic features.

According to Khan *et al*. (2020), numerous microorganisms such as bacteria, actinomycetes thrive in the anatomical components of honey bees (larvae, adults), food supply (pollen, beebread and honey), honeycombs and floral nectar. The morphological, physiological, chemical and molecular methods were used to describe 32 actinomycetes isolates. *Streptomyces* was the most common genus found in all isolates followed by *Actinomadura*, *Nonomuraea* and *Nocardiopsis* (Promnuan, 2011).

2.4.4 Yeast

Serra and Jorda (1997) measured yeast count by adding a 0.1-ml aliquot of the aerobic colony count solution to each of three Petri plates, followed by the addition of 10-12 ml of Sabouraud agar modified (Difco-0747). Plates were incubated for five days at 22°C once they had hardened. Colonies were mapped out and named.

Estevinho *et al*. (2012) enumerated total molds and yeasts on DG18 (Himedia) and incubated them at 25^0C for 5 days. Molds and yeasts were found in 60 per cent of the samples, whereas Hervatin (2009) discovered molds and yeasts in 100 per cent of the bee pollen samples examined in Brazil.

Felsociova *et al*. (2012) looked at several types of yeast contaminated samples and found that their levels were typically higher ranging from 0.5×10^1 to 5.7×10^2 Cfu/g (average: 7.6×10^1 Cfu/g) and *Zygosaccharomyces mellis* yeast was found.

Nogueira *et al*. (2012) enumerated mold and yeast were counted on DG18 (Himedia) and cultured at 25°C for 5 days. Colony forming units per gram of bee pollen (Cfu/g) were used to calculate microbial counts. *Candida magnoliae* sp. and

Zygosaccharomyces rouxii sp. were found in 37.5 per cent and 25 per cent of the samples, respectively. *Candida norvegensis* sp., *Cryptococcus humicola* sp., and *Saccharomyces cerevisiae* sp. were found in 12.5 per cent of the samples.

MATERIALS AND METHODS

CHAPTER III

MATERIAL AND METHODS

Studies on the physicochemical properties, palynology and microbiological identification of hive stored pollen of the Indian honeybee (*Apis cerana indica)* were conducted in the Insectary laboratory, Department of Agricultural Entomology, College of Agriculture, Kerala Agricultural University, Vellanikkara. The materials and techniques adopted during the course of this study are presented in this chapter.

3.1. Survey for the collection of samples

3.1.1 Survey

A random survey was conducted on the hive-stored pollen from the hives of Indian honeybees. Samples were collected from Palakkad and Wayanad districts, which represented the central midlands and highland areas of Kerala. Samples were collected from managed colonies of commercial beekeepers (Plate 1).

3.1.2 Samples collection and storage

Pollen samples were collected from three Indian honeybee (*Apis cerana indica*) colonies in Palakkad and Wayanad. Pollen samples were collected from bee comb cells using sterile forceps and sterile spatulas cautiously and transferred to sterile glassware.

3.1.3 Labelling

Labelling was done by giving the samples a code for easy identification. The samples collected from Palakkad were given the code PKD, which includes PKD-1: Mannarkkad, PKD-2: Nellipuzha, PKD-3: Nottanamala and similarly, from Wayanad as WYD, which includes WYD-1: Kenichira, WYD-2: Kayakunnu and WYD-3: Cheengode (Table 1).

Tabel 1. Survey locations for collection of samples

Sl. No.	Zone of collection	Survey area	Sample code	Location of collection
1	Central midlands	Palakkad	PKD-1: Mannarkkad	10°59'37"N 6°27'36"E
			PKD-2: Nellipuzha	10°59’26"N 76°28’07"E
			PKD-3: Nottanamala	10°59’26"N 76°28’07"E
2	High ranges	Wayanad	WYD-1: Kenichira	10°59'40"N 76°27'32"E-
			WYD-2: Kayakunnu	11°44’48"N 76°07’19"E
			WYD-3: Cheengode	11°43’49"N 76°07’54"E

3.1.4 Storage

The collected samples were kept in the refrigerator at 4°C to avoid any contamination and were used for further research studies.

3.2 Palynological identification

3.2.1 Preparation of microscopic slides

Pollen samples were collected and preserved in 70 per cent alcohol. A set of two reference pollen slides were prepared from these pollen samples, with one slide stained with safranin and the other without stain. An Olympus® CX43 Trinocular Microscope was used to photograph pollen.

For scanning electron microscopy, the pollen samples (1 ml) were taken in centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. The supernatant was decanted off. The samples were subjected to glacial acetic acid wash and subjected to centrifugation at 3000 rpm for 10 minutes. The supernatant was decanted off and washed with distilled water three times, which was followed by centrifugation at 3000

rpm for 10 minutes. Later the pollen samples were washed and dehydrated in ascending hydroethanolic series of 50, 70, 90 and 100 per cent for 10 minutes at each step (Plate 2).

3.2.2 Sputter coating

Before placing the slides in SEM, sputter coating has to be done. The nitrogen cylinder was opened and set at 1 bar. With the vent knob, the vent chamber was opened to place the sample stub. Start and wait till the vacuum reaches $8x10^{-2}$ mbar and set the timer for 60 sec. Press the plasma push button and rotate the leak knob anticlockwise till the current reaches 10 mA. Release the plasma push button and press the start button. After completion of the process, close the leak knob and vent the chamber (Plate 3).

3.2.3 Characterization and identification of bee pollen

Pollen grains of plant species reveal great variation not only in shape and size but also in exine ornamentation. The pollen was stored in Eppendorf® tubes containing 70 per cent alcohol after sampling. The general characteristics of pollen were examined using an Olympus® CX43 Trinocular Microscope and compared with the pollen reference slides. The pollen grains were described based on the following features (Lakshmi, 2012).

a. Nature - Monad, tetrad or polyad.

b. Size - Based on diameter classified as small (10-24µm), medium (25- 49µm) or large (50-99µm).

c. Shape - Prolate, oblate, spheroid based on polar and equatorial axes

d. Aperture - No-aperture, porate, calpate, calporate

e. Sculpture/ornamentation- In general pollen grains have two walls, the inner intine and outer exine wall. Ornamentation is the projection of the outer exine wall and it varies from species to species. The basic exine ornamentations are psilate, reticulate, equinate, striate and punctate.

3.2.4 PalDAT

The pollen images obtained during SEM were identified by using the PalDAT database (the world's largest database which consists of a comprehensive resource on pollen and palynology; it consists of a huge amount of data for more than 22,000 plant species). Predominant pollen samples were thus recorded.

3.3 Physicochemical analyses of hive-stored pollen

Physicochemical analysis, microbe isolation and characterization works were carried out at the Insectary laboratory, Department of Agricultural Entomology, College of Agriculture, Kerala Agricultural University, Vellanikkara.

Physicochemical parameters like moisture content, water activity, pH, ash content, total acidity and protein content were carried out. All the values were recorded for three replications and mean values were calculated.

3.3.1 Moisture content

Moisture content was measured by using a Sartorius® moisture content analyzer at 37^0C. After opening the drawer knob, 5g of the prepared sample was placed in the drawer and closed, which was further analysed for measuring moisture content (Terrab *et al*., 2002) (Plate 4).

3.3.2 Water activity

Water activity was measured by using the Aqualab® Pre-Water activity analyzer at 25.4°C. After opening the drawer knob, 5g of prepared sample was placed in a drawer and closed, which was further analysed for water activity (Acquarone *et al*., 2007) (Plate 5).

3.3.3 pH values

The pH of the pollen sample was measured using a pH meter. The pH of the meter was set by using a neutralizing buffer. Two grams of bee bread were dissolved

in 18 ml of distilled water for analysis of pH in the pollen sample (Al-Khalifa and Al-Arify, 1999) (Plate 6).

3.3.4 Ash content

Ash content was determined by using a muffle furnace. Initially, 1 g of pollen was placed in a porcelain dish and placed in the furnace for 3-4 h before final values were obtained (Nanda et al., 2003) (Plate 7).

3.3.5 Total acidity

The titration method was used for the estimation of total acidity. Total acidity was estimated by weighing 10 g of the sample was dissolved in 75 ml of distilled water and titrated against 0.1 N NaOH. Colour change was regarded as the endpoint (Al-Khalifa and Al-Arify, 1999) (Plate 8).

3.3.6 Protein content

Micro Kjeldahl was used for the estimation of protein content. 3 gm of a bee pollen sample was transferred to a hydrolysis tube containing 10 ml of 6 N HCL. The samples were treated with performic acid to avoid the decomposition of Sulphur-containing amino acids (AOAC, 1990). The hydrolysis tube was sealed under vacuum, heated in an oven at 110^0C for 24 h and then cooled to room temperature, then dissolved in 1 ml distilled water and evaporated again to remove the acid traces. The contents were filtered through Whatman No. 1 filter paper to remove visible sediments. The tube and precipitates on filter paper were then washed with deionized water. The combined filtrate and wash were diluted to the volume of 25 ml in a volumetric flask. Five ml of the filtrate was transferred to a 50 ml beaker. Dry residues were dissolved in 1 ml of lithium citrate buffer (pH 2.2). Twenty ml of the solution were loaded onto the cation exchange column and then four lithium citrate buffers with pH 2.2, 2.8, 3.3 and 3.7 were successively applied to the column at a flow rate of 0.2 ml/min. The ninhydrin flow rate was 0.2 ml/min and pressure of reagent was 150 bar. The pressure of buffer was from 0 to 50 bar and 130^0C reaction temperature. Results were obtained by using the 5.60 factor for conversion into crude protein (El-Kazafy *et al*., 2019) (Plate 9).

3.3.7 Statistical analysis

The pollen sample analysis was carried out with three replications. Using R software, the obtained data were analysed using Welchs' s two sample t test.

3.4 Isolation of microbes

The Indian honeybee pollen was collected from Palakkad and Wayanad districts and the bacteria, fungi, actinomycetes and yeast were isolated.

3.4.1 Materials and media

3.4.1.1 Glassware

The glasswares, *viz.*, conical flasks, beakers, measuring cylinders, test tubes and petri plates used were "Borosil" grade for the isolation of microbes.

3.4.1.2 Media

Readymade media was used for the isolation of different organisms. Nutrient agar (HiMedia Laboratories Pvt. Ltd.) (MOO1), Potato Dextrose Agar (HiMedia Laboratories Pvt. Ltd.) (MHO96), Kenknight and Munaier's medium (HiMedia Laboratories Pvt. Ltd.) (M695) and Sabouraud Dextrose Agar, granulated medium (HiMedia Laboratories Pvt. Ltd.) (GM063) were used to isolate bacteria, fungi, actinomycetes and yeast, respectively (Plate 10).

The media were prepared according to the recipe and autoclaved for 20-30 minutes at 121°C and 15 lbs of pressure. The medium was stored at room temperature for further use**.**

3.4.2 Serial dilution and plate counting technique

One gram of Indian bee pollen samples was serially diluted up to 10^{-4} and a series of dilutions was performed. The samples collected from each location were plated carefully in respective media for the isolation of bacteria, fungi, actinomycetes and yeast using Nutrient Agar (NA), Potato Dextrose Agar (PDA), Kenknight and Munaier's medium and Sabouraud Dextrose Agar (SDA), respectively. Three

replications were maintained for each sample. After incubation, colony-forming units were calculated (Anderson *et al*., 2014) (Plate 11).

3.4.3 Preparation of pure culture

Bacterial colonies were sub-cultured by performing quadrant streaking method on Nutrient Agar (NA) media and purified by performing repeated streaking on NA slants.

Fungal colonies were sub-cultured by placing the hyphal disc at the centre of Potato Dextrose Agar (PDA) media and purified by placing the hyphal discs in PDA slants.

3.4.4 Characterization of microbial colonies

3.4.4.1 Morphological characterization

Native bacterial isolates of Indian bee pollen samples were characterized using a ready-made gram staining kit (Spectrum Reagents and Chemicals Pvt Ltd). The Gram staining was carried out as per the protocol mentioned in the staining kit. After air drying, the smear of suspension was prepared on the clean slide with a loopful of sample. After heat fixing, the crystal violet was poured and kept for about 30 seconds to 1 minute and rinsed with water. Flood the gram's iodine for 1 minute and wash with water. Then, wash with 95 per cent alcohol or acetone for about 10-20 seconds and rinse with water. Add safranin for about 1 minute and wash with water. Allow to air dry before blotting dry and inspecting under a microscope. Results of these tests were scored either as positive (blue stain) or negative (red stain) by observing under a phase-contrast microscope and grouped accordingly (Plate 12) (Plate 14).

A small bit of fungal mycelia was kept in a glass slide and stained with lactophenol and sealed with the help of DPX mountant. The stained fungal spores were observed under a phase-contrast microscope. Morphological characters of fungi were recorded (Plate 13) (Plate 14).

3.4.4.2 Sequencing of ITS region of fungi and bacteria

The 16S rRNA and ITS regions of bacteria and fungi were amplified with universal primers specific to bacteria and fungi. Purified cultures of bacteria and fungi were given for sequencing at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala.

Genomic DNA of bacteria was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. Part of the culture was taken in a microcentrifuge tube. At 56°C, 180 µl of T1 buffer and 25 µl of proteinase K were added and incubated in a water bath until they were completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. After incubating at 70°C for 10 minutes, 200 l of B3 buffer was added and incubated for another 10 minutes. Then 210 µl of ethanol (100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. The wash step was repeated using 600 µl of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded onto a 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front had migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel Documentation System (Bio-Rad). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) by using universal primers (Table 2).

Table 2. Primer sequences for bacteria

16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR conditions maintained during amplication of bacterial DNA was

95^{o}C	-	5.00 min	
95^{o}C	-	30 sec	} 35 cycles
60^{o}C	-	40 sec	
72^{o}C	-	60 sec	
72^{o}C	-	7.00 min	
4^{o}C	-	∞	

Five µl of PCR product was mixed with 0.5µl of ExoSAP-IT and incubated at 37^{o}C for 15 minutes followed by enzyme inactivation at 85^{o}C for 5 minutes.

About 100 mg of the fungal tissue or mycelium was homogenized using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 was added and vortexed for one minute. Ten microlitres of RNase A solution was added and inverted to mix. For 10 minutes, the homogenate was incubated at 65^{o}C.The lysate was transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid was collected and the filter was discarded. Four hundred and fifty microlitres of buffer PC were added and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid was discarded. The column was filled with 400 microlitres of buffer PW1, centrifuged at 11000 x g for 1 minute, and the liquid flows through the column, though the liquid was discarded. Then 700 µl PW2 was added, centrifuged at 11000 x g and the flow through liquid was discarded. Finally, 200 µl of PW2 was added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65^{o}C for 5 minutes. The column was then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4^{o}C.

Gel electrophoresis was carried out in the same way for bacterial DNA. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) by using universal primers (Table 3).

Table 3. Primer sequences for fungus

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

The PCR conditions maintained during amplification of fungal DNA was

98°C	-	30 sec	
98°C	-	5 sec	} 40 cycles
58°C	-	10 sec	
72°C	-	15 sec	
72°C	-	60 sec	
4°C	-	∞	

Agarose Gel electrophoresis of PCR products

The PCR products were checked on 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. For loading, 1 µl of 6X loading dye was mixed with 4 µl of PCR products and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel Documentation System (Bio-Rad).

Exo SAP-IT Treatment

Exo SAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal

of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

3.4.4.3 NCBI BLAST

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1. The rRNA homology searches were performed using the BLAST program.

3.4.4.4 Construction of Phylogenetic tree

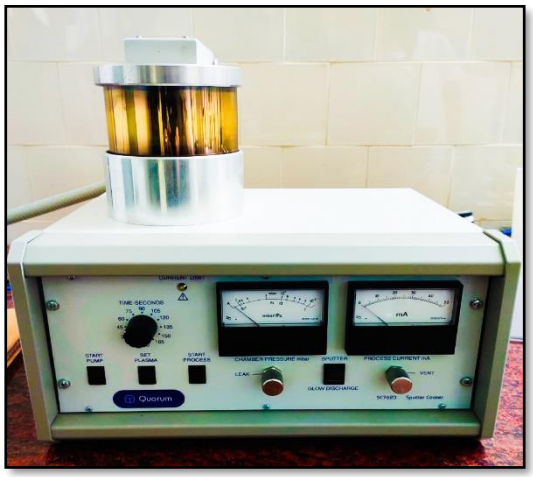
After BLAST, the sequences of the organisms were subjected to the construction of a phylogenetic tree to find out the evolutionary relationship between those organisms. Using MAFFT software, a phylogenetic tree was constructed at the centre of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Vellanikkara.

1a. Collection of hive stored pollen of *A. cerana indica* from Palakkad

1b. Collection of hive stored pollen of *A. cerana indica* from Wayanad

Plate 1. Survey on hive stored pollen of *A. cerana indica*

Plate 2. Scanning Electron Microscope



3a. Sputter coater

3b. Placing of pollen sample for sputter coating

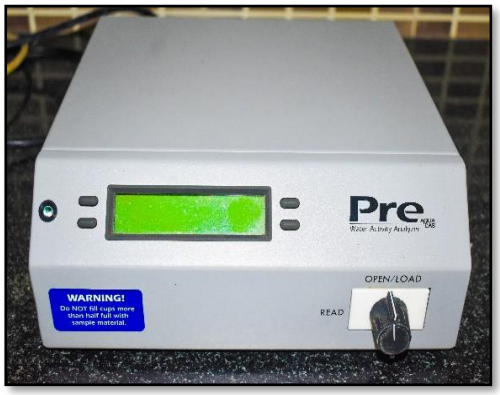
3c. Placing of Sputter coated pollen samples in SEM

Plate 3. Preparation of samples for SEM

4a. Sartorius® moisture content analyser

4b. Placing of sample in moisture analyser

Plate 4. Analysing moisture content of hive stored pollen sample



5a. Aqualab® Pre-Water activity analyser

5b. Placing of sample in Water activity analyser

Plate 5. Analysing water activity of hive stored pollen sample

Plate 6. Analysing pH of hive stored pollen sample

7a. Muffle furnace

7b. Placing of samples inside Muffle furnace

Plate 7. Analysing ash content of hive stored pollen sample

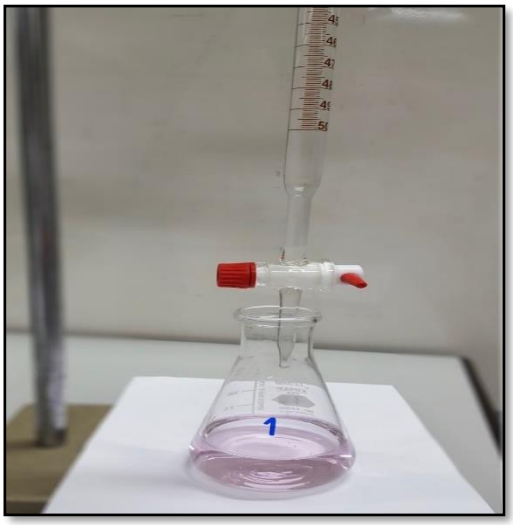
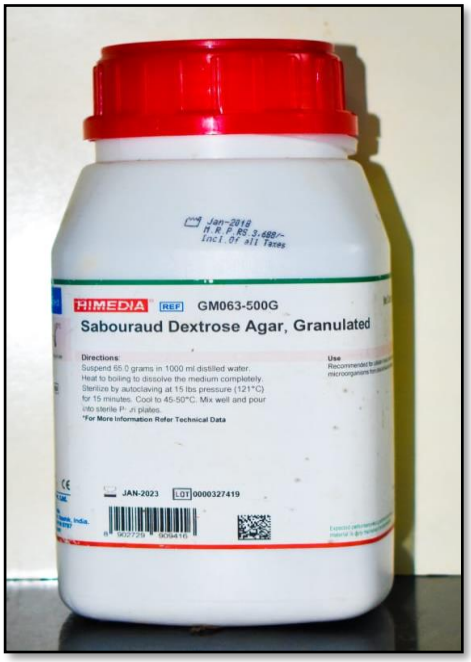


Plate 8. Analysing total acidity of hive stored pollen sample

Plate 9. Analysing protein content of hive stored pollen sample

10a. Nutrient Agar (NA) medium

10b. Potato Dextrose Agar (PDA) medium



10c. Sabouraud Dextrose Agar (SDA)

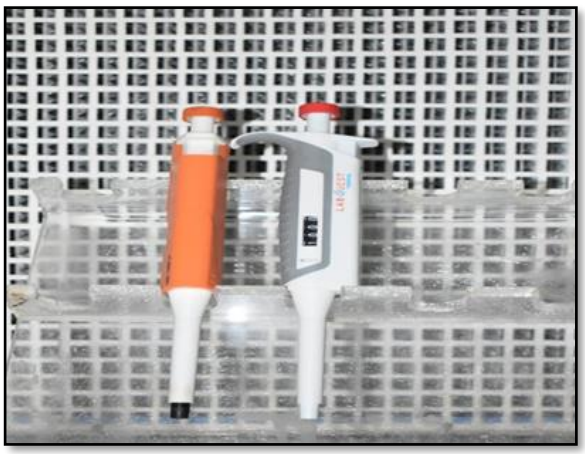
10d. Kenknight & Munaier's Medium

Plate 10. Media for isolation of microbes

11a. Weighing balance

11b. Hive stored bee pollen

11c. Sterile water blanks



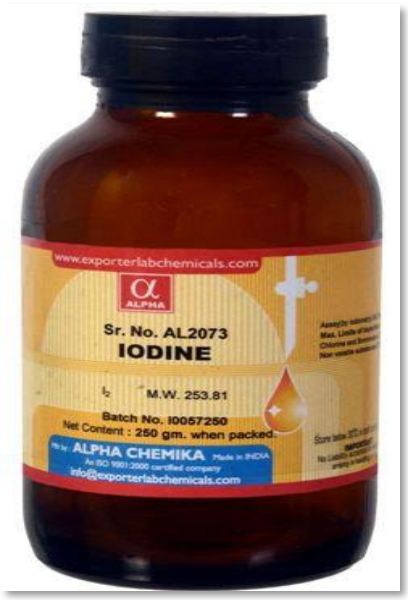
11d. 1000µl Micropipettes

11e. Laminar Air Flow (LAF)

11f. Pouring of 1ml diluent into sterile petri plates

Plate 11. Serial dilution and Pour plating technique

12a. Primary stain-Crystal Violet



12b. Moderant-Gram's iodine

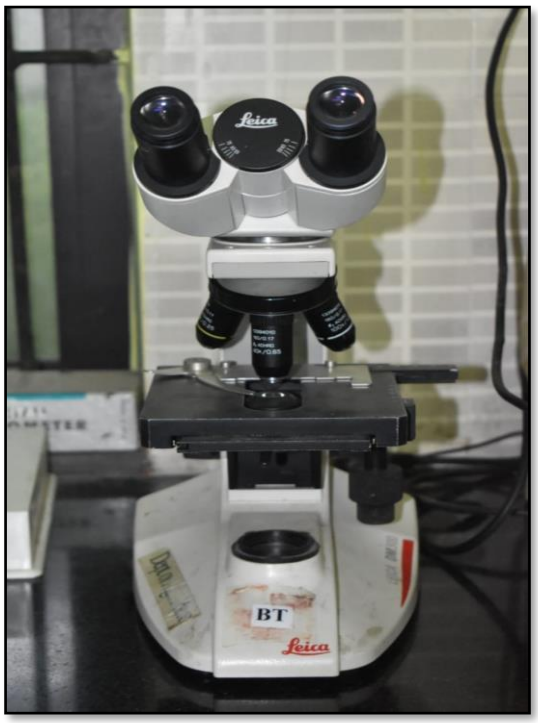
12c. Decolourising agent-Ethyl alcohol

12d. Counter stain- Safranin

Plate 12: Chemicals for Gram staining

Lactophenol Cotton Blue stain

Plate 13. Chemical for fungal staining



Compound microscope

Plate 14. Observation of microscopic slides under compound microscope

RESULTS

CHAPTER IV

RESULTS

Study on 'Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)' was carried out at Department of Agricultural Entomology, College of Agriculture, Kerala Agricultural University, Vellanikkara. The salient findings of the research work are presented in this chapter:

4.1 Palynological identification of hive stored pollen

Light microscopic images of hive stored pollens captured during the study subjected to the PalDAT analysis revealed the identity of plant sources from which the honey bee *Apsis cerana indica* collected the pollen during their foraging activity. The analysis of hive stored pollen sample collected from Palakkad revealed the presence of pollen from the flowers of Singapore daisy (*Sphagneticola trilobata),* Peacock flower (*Caesalpinia pulcherrima),* Common zinnia (*Zinnia elegans),* Holy Basil (*Ocimum sanctum),* Asian pigeonwings *(Clitoria ternatea*), Arjun tree (*Terminalia arjuna*), Flea tree (*Albizia lebbeck)*, Tabasco pepper (*Capsicum frutescens),* Lemon (*Citrus limon),* Cucumber (*Cucumis sativus)* and Daisy (*Helianthus aspera)* (Table 4 and Plate 15).

Table 4. Pollen sources of hive stored pollen of *Apis cerana indica* from Palakkad

Location	Common name	Scientific name	Family
Palakkad	Singapore daisy	*Sphagneticola trilobata*	Asteraceae
	Asian pigeonwings	*Clitoria ternatea*	Fabaceae
	Peacock flower	*Caesalpinia pulcherrima*	Fabaceae
	Holy Basil	*Ocimum sanctum*	Lamiaceae
	Common zinnia	*Zinnia elegans*	Asteraceae
	Arjun tree	*Terminalia arjuna*	Combretaceae
	Flea tree	*Albizia lebbeck*	Fabaceae
	Tabasco pepper	*Capsicum frutescens*	Solanaceae
	Lemon	*Citrus limon*	Rutaceae
	Cucumber	*Cucumis sativus*	Cucurbitaceae
	Daisy	*Helianthus aspera*	Asteraceae

Similarly, the hive stored pollen samples collected from Wayanad consisted of pollen belonged to the plant species *viz.*, Little tree plant (*Biophytum sensitivum),* White orchid tree (*Bauhinia acuminata*), Shame plant (*Mimosa pudica),* Yellow elder (*Tecoma stans),* Moss-rose purslane (*Portulaca grandiflora),* Wattles (*Acacia* sp.), Asian palmyra palm (*Borassus flabellifer),* Indian beech (*Pongamia pinnata),* Slender amaranth (Amaranthus *viridis)* and Senna tora (*Cassia tora*) (Table 5 and Plate 16).

Table 5. Pollen sources obtained from hive stored pollen of *Apis cerana indica* from Wayanad sample

Location	Common name	Scientific name	Family
Wayanad	Little tree plant	*Biophytum sensitivum*	Oxalidaceae
	White orchid tree	*Bauhinia acuminata*	Fabaceae
	Yellow elder	*Tecoma stans*	Bignoniaceae
	Moss-rose purslane	*Portulaca grandiflora*	Portulacaceae
	Mimosa pudica	Fabaceae	Shame plant
	Wattles	*Acacia* sp.	Fabaceae
	Asian palmyra palm	*Borassus flabellifer*	Arecaceae
	Indian beech	*Pongamia pinnata*	Fabaceae
	Slender amaranth	*Amaranthus viridis*	Amaranthaceae
	Senna tora	*Cassia tora*	Fabaceae

4.1.1 Characterization of hive stored pollen of *Apis cerana indica*

The scanning electron microscopic images captured during the study were fed to the PalDAT for characterizing the morphological features of pollen identified from the various plant sources. A total of nine monad pollen and one tetrad pollen were identified during the PalDAT analysis (Table 6).

4.1.1.1 Monad pollen

Pollen grains are termed as monad when during their maturity, few pollens do not remain united and are dissociated into single pollen grain.

The hive stored pollen samples collected from PKD-1: Mannarkkad (10°59’37” N-76°27’36” E) consisted of pollen from the Singapore daisy, *Sphagneticola trilobata* (Family: Fabaceae). It was a monad pollen with spherical or circular shape. The size of the pollen was 24.17 µm with a tricolporate aperture. The pollens were echinate with sharp spines and conical base exine (rigid outer coating of pollen) (Plate 17a). Similarly, another monad type of pollen with triangular shape belonged to the Asian pigeonwings, *Clitoria ternatea* (Family: Fabaceae) was also identified from the hive stored pollen collected from Mannarkad. The size of the pollen was 52.1 0 µm with a tricolpate aperture and they were microrugulate, fossulate (slightly hollowed or grooved) and with granulate exine pattern (roughening of surface with little tubercles like grains) (Plate 17b).

The hive stored pollen samples collected from PKD-2: Nellipuzha (10°59’26"N -76°28’07"E) consisted of pollen from the Peacock flower, *Caesalpinia pulcherrima* (Family: Fabaceae). It was a monad pollen with spheroid, prolate shape. The size of the pollen was 49.20 µm with a tricolporate aperture. The pollens were Synaperturate, aperture membrane with ornamentations (Plate 17c). Similarly, the sample also consisted another monad pollen beloned to the Holy Basil, *Ocimum sanctum* (Family: Lamiaceae). The size of the pollen was 33.72 µm with a hexacolpate aperture. The pollens were with exine pattern of aperture membrane which are ornamented, bireticulate, reticulate and with micro reticulate exine pattern (Plate 17d).

The hive stored pollen samples collected from PKD-3: Nottanamala (10°59’26"N-76°28’07"E) consisted of pollen from the Common Zinnia, *Zinnia elegans* (Family: Asteraceae). It was a monad pollen with Spherical/ Circular shape. The size of the pollen was 28.06 µm with a tricolporate aperture. The pollens were with exine pattern of echinate, sharp spines with conical base exine (Plate 17e).

The hive stored pollen samples collected from WYD-1: Kenichira (10°59'40"N-76°27'32"E) consisted of pollen from the Little tree plant, *Biophytum sensitivumi*

(Family: Oxalidaceae). It was a monad pollen with spheroidal shape. The size of the pollen was 41.10 µm with a tricolpate aperture. The pollens were with exine pattern of reticulate exine (Plate 18a).

The hive stored pollen samples collected from WYD-2: Kayakunnu (11°44’48"N-76°07’19"E) consisted of pollen from the White orchid tree, *Bauhinia acuminata* (Family: Fabaceae). It was a monad pollen with cylindrical/circular shape. The size of the pollen was 42.80 µm with a tricolpate aperture. The pollens were with exine pattern of reticulate exine (Plate 18b). Similarly, another monad type of pollen belonged to the Yellow Elder tree, *Tecoma stans* (Family: Bignoniaceae) was also identified from the sample collected at Kayakunnu. The size of the pollen was 42.6 µm with a tricolpate aperture. The pollens were with Echinate and perforate exine (Plate 18c).

The hive stored pollen samples collected from WYD-3: Cheengode (11°43’49"N- 76°07’54"E) consisted of pollen from the Moss-rose purslane, *Portulaca grandiflora* (Family: Portulacaceae). It was a monad pollen with Spheroidal shape. The size of the pollen was 56.62 µm with a Pantocolpate aperture. The pollens were with the exine pattern of pantoaperturate echinate exine (Plate 18d).

4.1.1.2 Tetrad pollen

Pollen grains are termed as tetrad when four pollen grains unite to form a single structure. These tetrads are the unseparated product of meiosis.

The hive stored pollen samples collected from WYD-3: Cheengode (11°43’49"N 76°07’54"E) consisted of pollen from the Shame plant, *Mimosa pudica* (Fabaceae). It was a tetrad pollen with spheroid, prolate shape. The size of the pollen was 7.08 µ with an Inaperturate aperture. The pollens were with exine pattern of areolate exine (Plate 18e).

All the pollen sources identified from hive stored pollen sample collected from Palakkad possessed monad dispersal unit, whereas, pollen sample from Wayanad had both monad and tetrad type of dispersal unit.

Table 6. Characterization and identification of hive stored pollen of *Apis cerana indica*

Sl. No.	Taxa	Pollen grain shape	Dispersal unit	Size (µm)	Aperture	Exine pattern
1	*Sphagneticola trilobata*	Spherical/Circular	Monad	24.17	Tricolporate	Echinate, sharp spines with conical base
2	*Caesalpinia pulcherrima*	Spheroid, prolate	Monad	49.20	Tricolporate	Synaperturate, aperture membrane ornamented
3	*Zinnia elegans*	Spherical/ Circular	Monad	28.06	Tricolporate	Echinate, sharp spines with conical base
4	*Ocimum sanctum*	Circular	Monad	33.72	Hexacolpate	Aperture membrane ornamented, bireticulate, reticulate, microreticulate
5	*Clitoria ternatea*	Triangular	Monad	52.10	Tricolpate	Microrugulate, fossulate, granulate
6	*Biophytum sensitivum*	Spheroidal	Monad	41.10	Tricolpate	Reticulate
7	*Bauhinia acuminata*	Cylindrical/ Circular	Monad	42.80	Tricolpate	Reticulate
8	*Mimosa pudica*	Spheroid, prolate	Tetrad	7.08	Inaperturate	Areolate

9	*Tecoma stans*	Spheroidal	Monad	42.6	Tricolpate	Echinate and perforate
10	*Portulaca grandiflora*	Spheroidal	Monad	56.62	Pantocolpate	pantoaperturate Echinate

The images of hive stored pollen sample collected from the two locations captured using Scanning Electron Microscopy and characterized based on their nature, size, shape, aperture and exine ornamentation. Nature of the different pollen types varied from monad to tetrad, out of which monad was the dominant one. The shape of pollen shapes varied from spherical, circular, triangular, spheroidal, cylindrical, prolate to elliptical. Out of which, spherical shaped pollens were dominant. The size of pollen ranged from 7.08 to 56.62 µm, which varied greatly within plant sources as well as with locations. The pollen of *Mimosa* recorded the smallest size (.08 µm), whereas the largest pollen identified belonged to *Portulaca grandiflora (*56.62 µm). Pollen aperture vary from tricolporate, hexacolpate, tricolpate, inaperturate, pantocolpate. Out of which, tricolpate aperture type pollens were dominant. Exine ornamentations have a diversified forms from echinate, synaperturate, bireticulate, reticulate, microreticulate, pantoaperturate. Out of which, echinate, sharp spines with conical base pollens were dominant.

4.1.2 Relative abundance of predominant families of pollen sources

The twenty one pollen sources which contributed to hive stored pollen, which were collected and identifed during the study belongs to twelve different families. Fabaceae, Asteraceae, Lamiaceae, Combretaceae, Solanaceae, Rutaceae, Oxalidaceae, Bignoniaceae, Portulacaceae, Arecaceae, Amaranthaceae and Cucurbitaceae were identified as the predominant families of pollen sources. Among twenty-one pollen sources, eight species were belonged to Fabaceae, three belonged to Asteraceae, one each was belonging to Lamiaceae, Combretaceae, Solanaceae, Rutaceae, Oxalidaceae, Bignoniaceae, Portulacaceae, Arecaceae, Amaranthaceae, Cucurbitaceae. Among twelve families, a major share of 38.09 per cent was occupied by Fabaceae which was followed by Asteraceae with a share of 14.28 per cent.

Table 7. Relative abundance of predominant families of pollen sources

Sl.No.	Family	Relative abundance (%)
1	Fabaceae	38.09
2	Asteraceae	14.28
3	Lamiaceae	4.76
4	Combretaceae	4.76
5	Solanaceae	4.76
6	Rutaceae	4.76
7	Oxalidaceae	4.76
8	Bignoniaceae	4.76
9	Portulacaceae	4.76
10	Arecaceae	4.76
11	Cucurbitaceae	4.76
12	Amaranthaceae	4.76

The identified pollen sources belonged to the family, Fabaceae were Peacock flower (*Caesalpinia pulcherrima),* Asian pigeonwings *(Clitoria ternatea*), Flea tree (*Albizia lebbeck)*, White orchid tree (*Bauhinia acuminata*), Shame plant (*Mimosa pudica),* Wattles (*Acacia* sp.), Indian beech (*Pongamia pinnata),* Senna tora (*Cassia tora*), which accounted for 38.09 per cent of the total pollen sources. It was followed by the pollen sources belonged to the family Asteraceae *viz.*, Singapore daisy (*Sphagneticola trilobata),* Common zinnia (*Zinnia elegans),* and Daisy (*Helianthus aspera)*, which accounted for 14.28 per cent of the total pollen sources. One pollen source each belongs to the family Combretaceae, Solanaceae, Rutaceae, Oxalidaceae, Bignoniaceae, Portulacaceae, Arecaceae, Cucurbitaceae and Amaranthaceae which accounted for 4.76 per cent of total identified pollen sources.

4.2 Physiochemical properties of hive stored pollen of *Apis cerana indica*

4.2.1 Moisture content

The estimated moisture content of the hive stored pollen samples collected from Palakkad varied between 15.27 (g/100g) and 15.30 (g/100g) with a mean moisture content of 15.29 (g/100g) whereas moisture content of samples collected from Wayanad varied between 15.33 (g/100g) to 15.44 (g/100g) with a mean moisture content of 15.38 (g/100g) (Table 8). Among the sample collected from Palakkad, PKD 2 and PKD3 recorded the highest moisture content (15.3 g/100g) which was followed by PKD-1(15.27 g/100g). WYD-1 recorded the highest moisture content of 15.44 (g/100g) among the samples collected from Wayanad, which was followed by WYD-3(15.37 g/100g) and WYD2 (15.33 g/100g). The statistical analysis revealed that significant difference exists among the moisture content of hive stored pollen samples collected from Palakkad and Wayanad.

Table 8. Moisture content (g/100g) of hive stored pollen of *Apis cerana indica*

Location	Sample code	Value	Mean	t value	p value
Palakkad	PKD-1	15.27	15.29	-5.2706	0.0003825
	PKD-2	15.3			
	PKD-3	15.3			
Wayanad	WYD-1	15.44	15.38		
	WYD-2	15.33			
	WYD-3	15.37			

4.2.2 Water activity

The water activity of the hive stored pollen samples collected from Palakkad ranged between 0.737 (a_w) and 0.739 (a_w)with a mean water activity of (0.73 a_w) whereas water activity of samples collected from Wayanad varied between (0.742 a_w) and (0.743 a_w) with a mean water activity of 0.74 (a_w) (Table 9). Among the sample collected from Palakkad, PKD-2 and PKD-3 recorded the highest water activity (0.739 a_w) which was followed by PKD-1 (0.737 a_w). WYD-1 and WYD-3 recorded the

highest water activity of 0.743 a_w among the samples collected from Wayanad, which was followed by WYD-2 (0.742 a_w). The statistical analysis revealed that significant difference exists among the water activity of hive stored pollen samples collected from Palakkad and Wayanad.

Table 9. Water activity (a_w) of hive stored pollen of *Apis cerana indica*

Location	Sample code	Value	Mean	t value	p value
Palakkad	PKD-1	0.737	0.73	-5.9648	2.619e-05
	PKD-2	0.739			
	PKD-3	0.739			
Wayanad	WYD-1	0.743	0.74		
	WYD-2	0.742			
	WYD-3	0.743			

4.2.3 pH

The estimated pH of hive stored pollen samples collected from Palakkad varied between 3.81 to 3.84 with a mean pH of 3.83 whereas pH of samples collected from Wayanad varied between 3.74 to 3.8 with a mean pH of 3.77 (Table 10). Among the sample collected from Palakkad, PKD-3 recorded the highest pH (3.84) which was followed by PKD-2 (3.83) and PKD-1 (3.81). WYD-2 recorded the highest pH (3.80) among the samples collected from Wayanad, which was followed by WYD-3 (3.78) and WYD-1 (3.74). The statistical analysis revealed that significant difference exists among the pH of hive stored pollen samples collected from Palakkad and Wayanad.

Table 10. pH of hive stored pollen of *Apis cerana indica*

Location	Sample code	Ph	Mean	t value	p value
Palakkad	PKD-1	3.81	3.83	4.6254	0.0006632
	PKD-2	3.83			
	PKD-3	3.84			
Wayanad	WYD-1	3.74	3.77		
	WYD-2	3.80			
	WYD-3	3.78			

4.2.4 Ash content

The ash content of the hive stored pollen samples collected from Palakkad fluctuated between 2.00 (g/100g) to 2.03 (g/100g) with a mean ash content of 2.01 (g/100g) whereas from Wayanad fluctuated between 1.87 (g/100g) and 1.88 (g/100g) with a mean ash content of 1.87 (g/100g) (Table 11). Among the sample collected from Palakkad, PKD-1 recorded the highest ash content (2.03 g/100g) which was followed by PKD-3 (2.02g/100g) and PKD-2 (2 g/100g). WYD-2 recorded the highest ash content of (1.88 g/100g) among the samples collected from Wayanad, which was followed by WYD-3 and WYD-1 (1.87g/100g). The statistical analysis revealed that significant difference exists among the ash content of hive stored pollen samples collected from Palakkad and Wayanad.

Table 11. Ash content (g/100g) of hive stored pollen of *Apis cerana indica*

Location	Sample code	Values	Mean	t value	p value
Palakkad	PKD-1	2.03	2.01	19.834	3.495e-12
	PKD-2	2.00			
	PKD-3	2.02			
Wayanad	WYD-1	1.87	1.87		
	WYD-2	1.88			
	WYD-3	1.87			

4.2.5 Total acidity

The total acidity of the hive stored pollen samples collected from Palakkad differed between 10.12 (mmol/g) and 10.17 (mmol/g) with a mean total acidity of 10.13 (mmol/g) whereas from Wayanad differed between 9.62 and 9.65 with a mean total acidity of 9.64 (mmol/g) (Table 12). Among the sample collected from Palakkad, PKD-2 recorded the highest total acidity (10.17 mmol/g) which was followed by PKD-1 and PKD-3 (10.12 mmol/g). WYD-1 recorded the highest total acidity of (9.65 mmol/g) among the samples collected from Wayanad, which was followed by WYD-3 (9.63 mmol/g) and WYD-2 (9.62 mmol/g). The statistical analysis revealed that significant difference exists among the Total acidity of hive stored pollen samples collected from Palakkad and Wayanad.

Table 12. Total acidity (mmol/g) of hive stored pollen of *Apis cerana indica*

Location	Sample code	Value	Mean	t value	p value
Palakkad	PKD-1	10.12	10.13	39.99	1.888e-15
	PKD-2	10.17			
	PKD-3	10.12			
Wayanad	WYD-1	9.65	9.64		
	WYD-2	9.62			
	WYD-3	9.63			

4.2.6 Protein content

The protein content of the hive stored pollen samples collected from Palakkad ranged between 3.45 (%) and 3.48 (%) with a mean protein content of 3.46(%) whereas from Wayanad ranged between 3.69 (%) and 3.7 (%) with a mean protein content of 3.69 (%) (Table 13). Among the sample collected from Palakkad, PKD-2 recorded the highest protein content (3.48 %) which was followed by PKD-1 and PKD-3 (3.45 %). WYD-2 recorded the highest protein content of (3.7 %) among the samples collected from Wayanad, which was followed by WYD-1 and WYD-3 (3.69 %). The statistical analysis revealed that significant difference exists among the protein content of hive stored pollen samples collected from Palakkad and Wayanad.

Table 13. Protein content (%) of hive stored pollen of *Apis cerana indica*

Location	Sample code	Value	Mean	t value	p value
Palakkad	PKD-1	3.45	3.46	-36.072	1.356e-14
	PKD-2	3.48			
	PKD-3	3.45			
Wayanad	WYD-1	3.69	3.69		
	WYD-2	3.7			
	WYD-3	3.69			

4.3 Microbiological determinations in hive stored pollen of *Apis cerana indica*

In the present study, microbes associated with hive stored pollen of *Apis cerana indica* from Palakkad and Wayanad were identified based on morphological features and molecular characterization. The microbial diversity, microbial load associated with beehives, microbial load to pollen grain ratio and total bacterial and fungal population were also assessed from samples collected from the locations under study.

4.3.1 Microorganism identification

Total four colonies of bacteria were obtained from hive stored pollen of *Apis cerana indica* from the sampling locations. Isolate B1 (from sample PKD-1) and isolate B2 (from sample PKD-3) were obtained from pollen samples collected from Palakkad, whereas isolate B3 (from sample WYD-3) and isolate B4 (from sample WYD-2) were obtained from pollen samples collected from Wayanad.

Total three colonies of fungi were obtained from hive stored pollen of *Apis cerana indica*. Isolate T1 (from sample PKD-2) was obtained from pollen sample collected from Palakkad whereas isolate W1 (from sample WYD-2) and isolate A1 (from sample WYD-1) were obtained from pollen samples collected from Wayanad.

In the present study even after 72-96h of incubation, actinomycetes colonies were not recorded on the plated Kenknights and Munaiers medium. So, no further works were continued in relation to actinomycetes colonies.

In the present study even after 72-96h of incubation, yeast colonies, colonies were not recorded on the plated Sabouraud Dextrose Agar (SDA) medium. So, no further works were continued in relation to yeast colonies.

4.3.1.1 Bacterial isolate differentiation using staining technique from hive stored pollen of Palakkad and Wayanad districts

Out of 4 bacterial isolates, isolate B1 which appeared in red when observed under a compound microscope, which inferred that the bacteria were gram negative in nature, while the isolate B2, isolate B3 and isolate B4 which appeared in crystal violet when observed under compound microscope and inferred that all three bacteria were gram positive (Plate 19).

4.3.1.1.1 Morphological and cultural characters of different bacterial isolates obtained from hive stored pollen of Palakkad and Wayanad districts

Isolate B1 which was obtained from pollen sample of Palakkad (PKD-1) had rod shaped cells with slight yellowish colony colour. They produced large round colonies in the media with irregular margin and flat elevation. (Table 14) (Plate 20a).

Isolate B2, obtained from pollen sample of Palakkad (PKD-3) had rod shaped cells with creamy white colony colour. They produced numerous small round colonies with entire smooth margin and flat elevation (Table 14) (Plate 20b).

Isolate B3 obtained from pollen sample of Wayanad (WYD-3) had rod shaped cells with creamy white colony colour. They produced numerous small round colonies with entire smooth margin and flat elevation (Table 14) (Plate 20c).

Isolate B4 obtained from pollen sample of Wayanad (WYD-2) had rod shaped cells with creamy white colony colour. They also produced numerous small round colonies with entire smooth margin and flat elevation (Table 14) (Plate 20d).

Table 14. Morphological and cultural characters of different isolates obtained from hive stored pollen of *Apis cerana indica*

Location	Bacte-rial isolate	Gram reaction	Sha-pe	Colour	Margin	Elevati-on of colony	Form of colony
Palakkad	**B1**	Negative	Rod	Slight yellowish	Irregular	Flat	Round large colony
	B2	Positive	Rod	Creamy white	Entire	Flat	Numerous small round colony
Wayanad	**B3**	Positive	Rod	Creamy white	Entire	Flat	Numerous small round colony
	B4	Positive	Rod	Creamy white	Entire	Flat	Numerous small round colony

4.3.1.1.2 Identification of isolated bacteria by molecular characterization

Molecular characterization of bacterial isolates were carried out based on 16S rRNA gene sequencing. PCR amplified products resolved on one per cent agarose gel produced crisp bands. Nucleotide sequences obtained from Rajiv Gandhi Centre for Biotechnology; Thiruvananthapuram were used for homology search using BLASTn. The sequence analysis of the isolate B1 showed 100 per cent query coverage and 99. 84 per cent identity to *Pseudomonas aeruginosa* (NCBI accession no. MH010896.1). The isolate, B2 showed 100 per cent query coverage and 100 per cent identity to *Bacillus megaterium* (NCBI accession no. MN826585.1). The isolate B3 showed 99 per cent query coverage and 100 per cent identity to *Bacillus aryabhattai* (NCBI accession no. MT184818.1). The isolate B4 showed 100 per cent query coverage and 99.69 percent identity to *Bacillus megaterium* (NCBI accession no. KJ500011.1) (Table 16). This was also in conformity with their respective cultural and morphological characterization.

Table 15. Multiple sequence alignment of bacterial isolates in hive stored pollen of *Apis cerana indica* obtained from Palakkad and Wayanad districts

Isolate	Sequences
B1	gaattactgggcgtaaagcgcgcgtaggtggttcagcaagttggatgtgaaatccccggg
B2	gaattattgggcgtaaagcgcgcgcaggcggtttcttaagtctgatgtgaaagcccacgg
B3	gaattattgggcgtaaagcgcgcgcaggcggtttcttaagtctgatgtgaaagcccacgg
B4	gaattattgggcgtaaagcgcgcgcaggcggtttcttaagtctgatgtgaaagcccacgg
	******.*****************.***.****. .****. ********* *** **
B1	ctcaacctgggaactgcatccaaaactactgagctagagtacggtagagggtggtggaat
B2	ctcaaccgtggagggtcattggaaactggggaacttgagtgcagaagagaaaagcggaat
B3	ctcaaccgtggagggtcattggaaactggggaacttgagtgcagaagagaaaagcggaat
B4	ctcaaccgtggagggtcattggaaactggggaacttgagtgcagaagagaaaagcggaat
	******* ***. ***..*****. **.** ****.*.* ****.. .*.*****
B1	ttcctgtgtagcggtgaaatgcgtagatataggaaggaacaccagt-ggcgaaggc-gac
B2	tccacgtgtagcggtgaaatgcgtagagatgtggaggaacaccagt-ggcgaaggc-ggc
B3	tccacgtgtagcggtgaaatgcgtagagatgtggaggaacaccagt-ggcgaaggc-ggc
B4	tccacgtgtagcggtgaaatgcgta-agatgtggaggaacaccagtgggcgaaggcgggc
	..****************** * **. *.********** ********* *.*
B1	cacctggactgatactgacactgaggtgcgaaagcgtgggggagcaaacaggattagata
B2	tttttggtctgtaactgacgctgaggcgcgaaagcgt-ggggagcaaacaggattagata
B3	tttttggtctgtaactgacgctgaggcgcgaaagcgt-ggggagcaaacaggattagata
B4	tttttggtctgtaactgacgctgaggcgcgaaagcgt-ggggagc-aacaggattagata
	. ..*** *** ******.******.********** ******* **********

Table 16. Molecular identification of bacterial isolates obtained from Palakkad and Wayanad districts of Kerala

Sl no.	Sample code	Isolate	Bacteria identified	Accessionn number	Query Coverage (%)	Identity (%)
1	PKD-1	B1	*Pseudomonas aeruginosa*	MH010896.1	100%	99.84%
2	PKD-3	B2	*Bacillus megaterium*	MN826585.1	100%	100%
3	WYD-3	B3	*Bacillus aryabhattai*	MT184818.1	99%	100%
4	WYD-2	B4	*Bacillus megaterium*	KJ500011.1	100%	99.69%

4.3.1.1.3 Construction of phylogenetic tree for individual bacterial isolates showing bacterial diversity of hive stored pollen of *Apis cerana indica*

Evolutionary analysis of B1, B2, B3 and B4 was conducted by constructing the phylogenetic trees. These were constructed for individual bacterial isolates by comparing with their closely related species in NCBI with the help of MAFFT software. Length of the phylogenetic tree has significance. Higher the length of phylogenetic tree, more the genetic divergence. It was constructed for individual isolates with their close species to find out the diversity among different species of the same genus (Plate 21).

4.3.1.2 Fungal isolate differentiation using slide preparation from hive stored pollen of Palakkad and Wayanad districts

The fungal colonies were obtained after 72h of incubation were picked for performing microscopic slide observation. Slide preparation was done for each isolate separately by following the protocol of slide preparation. On a clean grease free slide, placed a drop of water and then a small hyphal bit of respective isolate and stained with lactophenol cotton blue. After placing cover slip carefully without any air bubbles formation and then sealed with DPX mountant. Slides were prepared for all the three fungal isolates and observed under compound microscope.

4.3.1.2.1 Morphological and cultural characters of different fungal isolates obtained from hive stored pollen of Palakkad and Wayanad districts

Isolate T1 obtained from pollen sample of Palakkad (PKD-2) had green large round colonies with entire smooth margin having flat elevation (Table 17) (Plate 23a).

Isolate 2 which was obtained from pollen sample of Wayanad (WYD-2) had white small round colonies with entire smooth margin having flat elevation (Table 17) (Plate 23b).

Isolate 3 which was obtained from pollen sample of Wayanad (WYD-1) had orange small round colonies with smooth margin having flat elevation (Table 17) (Plate 23c).

Table 17. Morphological and cultural characters of different fungal isolates obtained from hive stored pollen of Palakkad and Wayanad districts

Location	Fungal isolate	Colour	Margin	Elevation of colony	Form of colony
Palakkad	T1	Green	Entire	Flat	Large round colony
Wayanad	W1	White	Entire	Flat	Small round colony
	A1	Orange	Entire	Flat	Small round colony

4.3.1.2.2 Identification of isolated fungi by molecular characterization

Molecular characterization of fungal isolates were carried out by ITS sequencing. PCR amplified products were resolved on one per cent agarose gel produced crisp bands. Nucleotide sequences obtained from Rajiv Gandhi Centre for Biotechnology; Thiruvananthapuram were used for homology search using BLASTn. The sequence analysis of the isolate T1 showed 87 per cent query coverage and 96.23 percent identity to *Trichoderma reesei* (NCBI accession no. MW048853.1) The isolate W1 showed 100 per cent query coverage and 99.79 per cent identity to *Westerdykella multispora* (NCBI accession no. MH857224.1*)*. The isolate A1 showed 100 per cent

query coverage and 100 per cent identity to *Neurospora crassa* (NCBI accession no. MT557113.1) (Table 19). This was also in confirmity with their respective cultural and morphological characterization.

Table 18. Multiple sequence alignment of fungal isolates in hive stored pollen of *Apis cerana indica* obtained from Palakkad and Wayanad districts

Fungal isolate	Sequences
A1	cggatccccaacg-caagcagagcttgatggttgaaatgacgctcgaacaggcatgctcg
W1	cagtcacccagcaccaagcagagcttgaaggtgtaaatgacgctcgaacaggcatgccct
T1	aaaacgcccaacaccaagcaaagcttgagggtacaaatgacgctcgaacaggcatgccct
	.. . ****.*. ******.******* *** **********.*
A1	ccagaatactggcgagcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaa
W1	acggaataccatagggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaa
T1	ttggaataccaaagggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaa
	..******.. *.**********
A1	ttcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgt
W1	ttcacactacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgt
T1	ttcacactacgtatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgt
	******.**********

Table 19. Molecular identification of fungal isolates obtained from Palakkad and Wayanad districts of Kerala

Sl. No.	Sample code	Isolate code	Fungi identified	Accession number	Query Coverage (%)	Identity (%)
1	PKD-2	T1	*Trichoderma reesei*	MW048853.1	87%	96.23%
2	WYD-2	W1	*Westerdykella multispora*	MH857224.1	100%	99.79%
3	WYD-1	A1	*Neurospora crassa*	MT557113.1	100%	100%

4.3.1.2.3 Construction of phylogenetic tree for individual fungal isolates showing fungal diversity of hive stored pollen of *Apis cerana indica*

Evolutionary analysis of isolates T1, W1 and A1 was conducted by constructing the phylogenetic trees. These were constructed for individual fungal isolates by comparing with their closely related species in NCBI with the help of MAFFT software. Length of the phylogenetic tree has significance. Higher the length of phylogenetic tree, more the genetic divergence. It was constructed for individual isolates with their close species to find the diversity among different species of the same genus (Plate 24).

4.3.2 Microbial diversity and microbial load

4.3.2.1 Microbial diversity of hive stored pollen of *Apis cerana indica* from Palakkad and Wayanad districts of Kerala

In the present study, the pollen sample collected from Palakkad district possessed the bacterial species *viz., Pseudomonas aeruginosa,* and *Bacillus megaterium* and a fungal species, *Trichoderma reesei*. Actinomycetes and yeast colonies were not recorded from samples collected from Palakkad. Whereas pollen sample collected from Wayanad had the bacterial species *viz*., *Bacillus aryabhattai*, and *Bacillus megaterium* and two fungal species, *viz*., *Neurospora crassa* and *Westerdykella multispora.*

Table 20. Microbial diversity of hive stored pollen of *Apis cerana indica* from Palakkad and Wayanad districts of Kerala

Sl. No.	Sample location	Microbial diversity	
		Fungus	Bacteria
1	Palakkad	1	2
2	Wayanad	2	2

4.3.2.2 Microbial load of hive stored pollen of *Apis cerana indica* from Palakkad and Wayanad districts of Kerala

Microbial load refers to the total number of microbial populations present in the given sample.

Table 21. Microbial load of hive stored pollen samples collected from Palakkad and Wayanad

Location	Microbial load (cfu/g)	t value	p value
Palakkad	$3.3x10^4$	-4.7295	4.413e-05
Wayanad	$6.6x10^4$		

The statistical analysis revealed that significant difference exists among the microbial load present in pollen samples collected from Palakkad and Wayand. The pollen samples collected from Wayanad recorded the highest microbial load ($6.6x10^4$ cfu/g) when compared to Palakkad sample ($3.3x10^4$ cfu/g).

4.3.3 Microbial load to pollen grain ratio

The ratio of microbial load to pollen grain was obtained by dividing the total microbial population obtained with the amount of pollen sample taken for serial dilution.

Table 22: Microbial load to pollen grain ratio in hive stored pollen of *Apis cerana indica*

Location	Microbial load to pollen grain ratio (cfu/g)	t value	p value
Palakkad	$3.3x10^4$	-4.7295	4.413e-05
Wayanad	$6.6x10^4$		

Significant difference exists among the estimated microbial load to pollen ratio of pollen samples collected from Palakkad and Wayanad. The highest microbial load to pollen grain ratio was noted in sample collected from Wayanad ($6.6x10^4$ cfu/g) when compared to Palakkad sample ($3.3x10^4$ cfu/g).

4.3.4 Total bacterial, fungal, yeast and actinomycetes population

In the hive stored pollen sample collected from Palakkad, the estimated fungal population varied between 0.6 $x10^4$ (cfu/g) to 1.3 $x10^4$ (cfu/g) with a mean fungal population of 1 $x10^4$ (cfu/g) whereas bacterial population varied between 1.3 $x10^4$ cfu/g and 3.3 $x10^4$ (cfu/g) with a mean bacterial population of 2.3 $x10^4$ (cfu/g).

In the hive stored pollen sample collected from Wayanad, the estimated fungal population varied between 3 $x10^4$ (cfu/g) and 3.6 $x10^4$ (cfu/g) with a mean fungal population of 3.3 $x10^4$ (cfu/g) whereas bacterial population varied between 2.66 $x10^4$ (cfu/g) and 4 $x10^4$ (cfu/g) with a mean bacterial population of 3.3 $x10^4$ (cfu/g).

From both samples of Palakkad and Wayanad districts, actinomycetes and yeast colonies were not obtained.

Table 23. Bacterial and fungal population of hive stored pollen of *Apis cerana indica* obtained from Palakkad and Wayanad districts of Kerala

Sample code	Microbial colonies		Sample code	Microbial colonies	
	Fungi (10^4)	Bacteria (10^4)		Fungi (10^4)	Bacteria (10^4)
PKD-1	1.3	3.3	WYD-1	3.66	4
PKD-2	1	2.3	WYD-2	3.33	3.33
PKD-3	0.6	1.3	WYD-3	3	2.66
Mean	**1**	**2.3**	**Mean**	**3.3**	**3.3**

The fungal population of the hive stored pollen samples collected from Palakkad ranged between 0.6$x10^4$ (cfu/g) to 1.3$x10^4$ (cfu/g) with a mean fungal population of 1$x10^4$ (cfu/g) whereas bacterial population ranged between 1.3$x10^4$ (cfu/g) to 3.3$x10^4$ (/cfu/g) with a mean bacterial population of 2.3$x10^4$ (cfu/g). The fungal population of the hive stored pollen samples collected from Wayanad ranged between 3$x10^4$ (cfu/g) to 3.66$x10^4$ (cfu/g) with a mean fungal population of 3.33$x10^4$ (cfu/g) whereas bacterial population ranged between 2.66 $x10^4$ (cfu/g) to 4$x10^4$ (cfu/g)

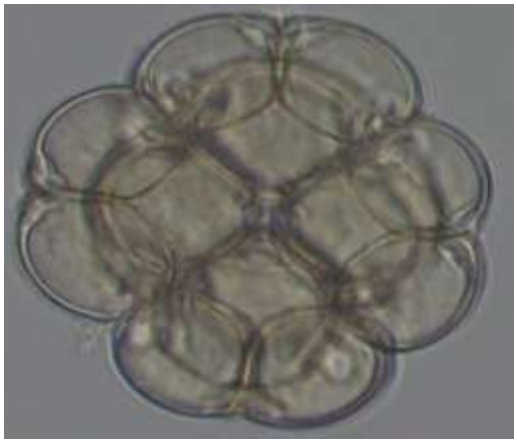
with a mean bacterial population of $3.3x10^4$ (cfu/g). Among the sample collected from Palakkad, PKD-1 recorded the highest fungal population ($1.3\ x10^4$cfu/g) which was followed by PKD-2 ($1x10^4$ cfu/g) and PKD-3 ($0.6x10^4$ cfu/g). Similarly, PKD-1 recorded the highest bacterial population ($3.3x10^4$ cfu/g) which was followed by PKD-2 ($2.3x10^4$ cfu/g) and PKD-3 ($1.3\ x10^4$Cfu/g). WYD-1 recorded the highest fungal population ($3.66x10^4$ cfu/g) which was followed by WYD-2 ($3.33x10^4$ cfu/g) and WYD-3 ($3x10^4$ cfu/g). Similarly, WYD-1 recorded the highest bacterial population ($4x10^4$ cfu/g) which was followed by WYD-2 ($3.33x10^4$ cfu/g) and WYD-3 ($2.66\ x10^4$ cfu/g).

Table 24: Welch two sample t-test statistical analysis of fungal and bacterial population

Sample location	Colony forming units (cfu/g)	
	Fungi	**Bacteria**
Palakkad	$1x10^4$	$2.3x10^4$
Wayanad	$3.3x10^4$	$3.3x10^4$
t value	-5.7155	-2
p value	4.788e-05	0.06473

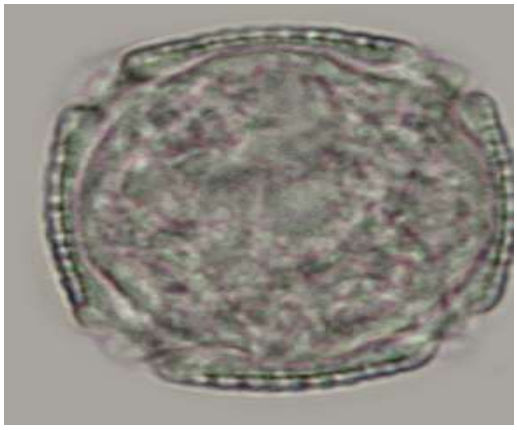
The mean fungal population of the hive stored pollen samples collected from Palakkad was $1x10^4$ (cfu/g) whereas mean bacterial population was $2.3\ x10^4$ (cfu/g). The mean fungal population of the hive stored pollen samples collected from Wayanad was $3.3x10^4$ (cfu/g) whereas mean bacterial population was $3.3\ x10^4$ (cfu/g).

15a. Pollen of Arjun tree-*Terminalia arjuna*



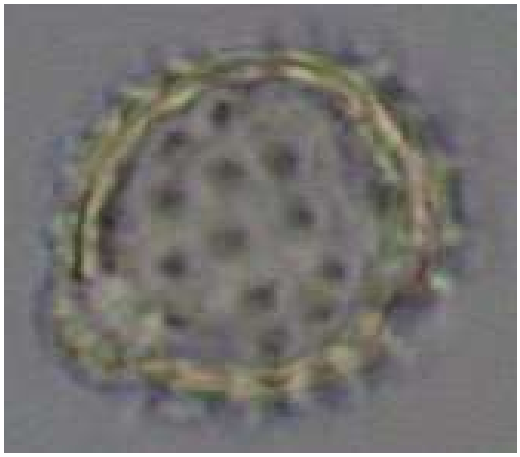
15b. Pollen of Flea tree- *Albizia lebbeck*

15c. Pollen of Tabasco pepper- *Capsicum frutescens*



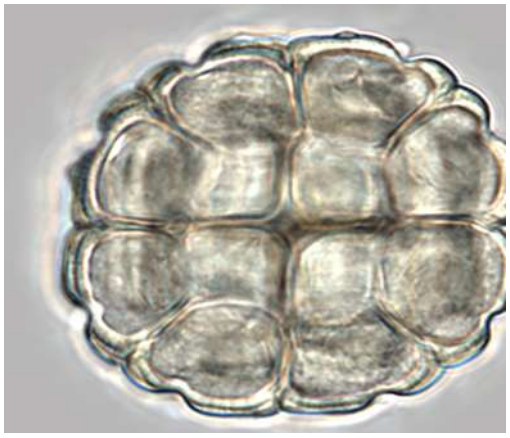
15d. Pollen of Lemon- *Citrus limon*

15e. Pollen of Cucumber- *Cucumis sativus*

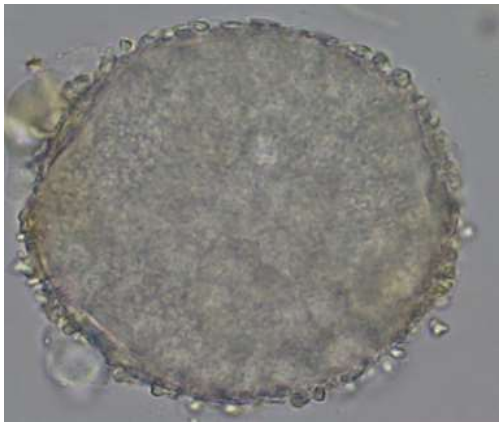


15f. Pollen of Daisy-*Helianthus aspera*

Plate 15. Light microscopy images of pollen collected from hives of *A. cerana indica* from Palakkad

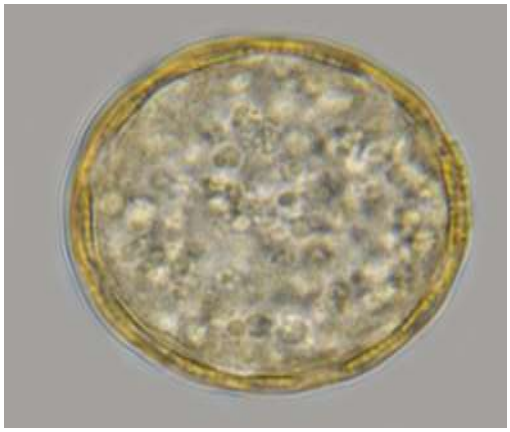


16a. Pollen of Wattles- *Acacia* sp.



16b. Pollen of Asian palmyra palm- *Borassus flabellifer*

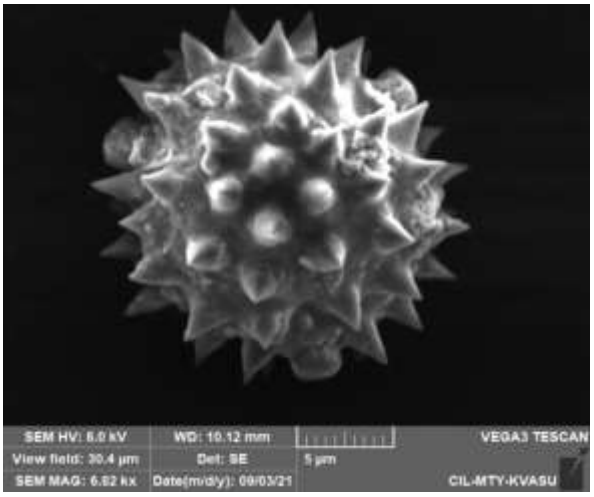
16c. Pollen of Indian beech- *Pongamia pinnata*



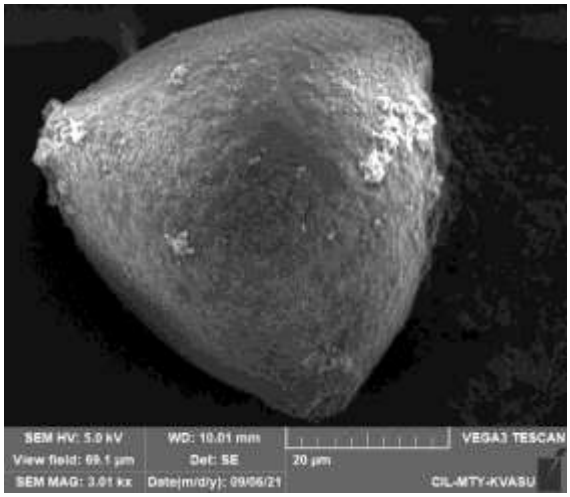
16d. Pollen of Slender amaranth- *Amaranthus viridis*

16e. Pollen of Senna tora-*Cassia tora*

Plate 16. Light microscopy images of pollen collected from hives of *A. cerana indica* from Wayanad

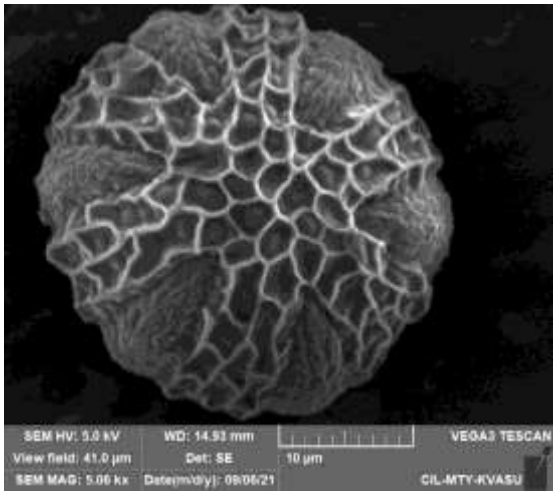


17a. Pollen of Singapore daisy- *Sphagneticola trilobata*

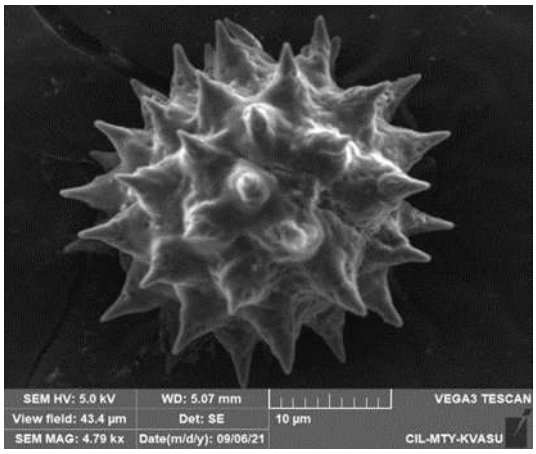


17b. Pollen of Asian pigeonwings- *Clitoria ternatea*

17c. Pollen of Peacock flower- *Caesalpinia pulcherrima*

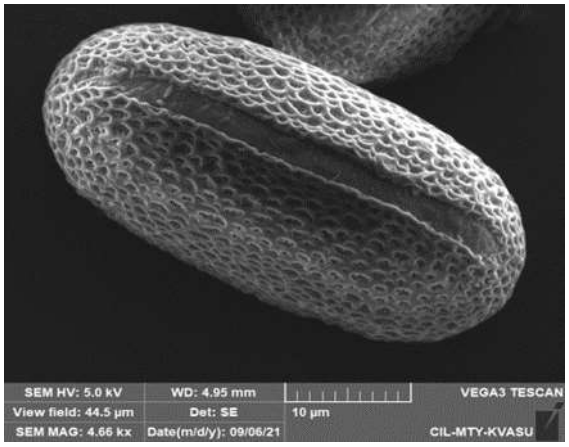


17d. Pollen of Holy Basil- *Ocimum sanctum*

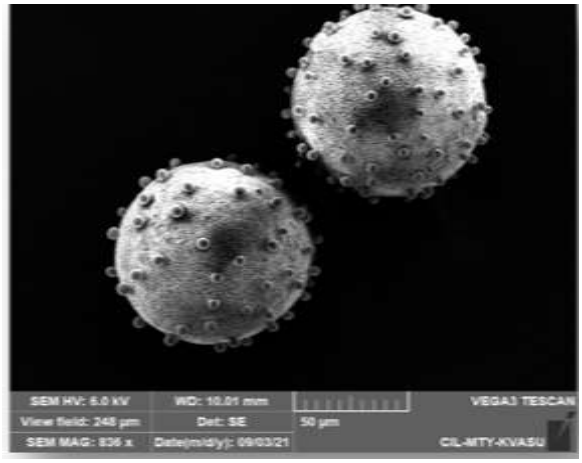


17e. Pollen of Common zinnia - *Zinnia elegans*

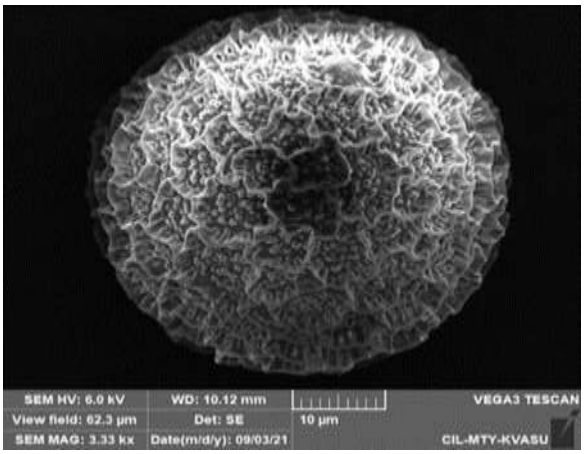
Plate 17. Scanning Electron Microscopy (SEM) images of pollen collected from hives of *A. cerana indica* from Palakkad



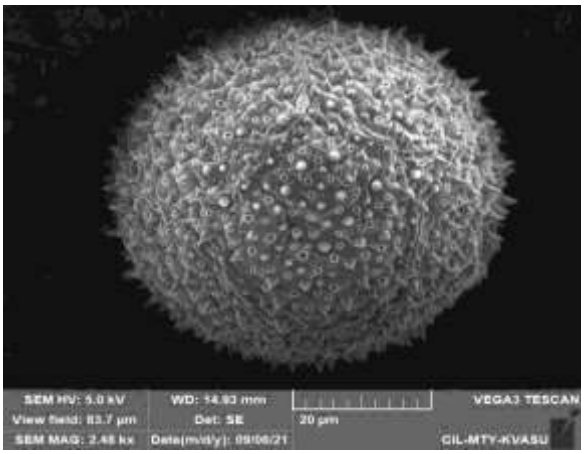
18a. Pollen of little tree plant (*Biophytum sensitivum*



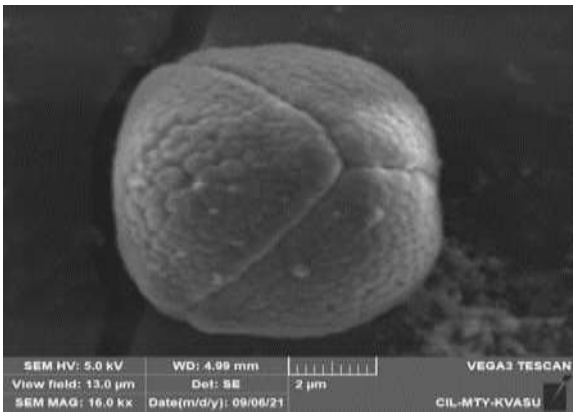
18b. Pollen of White orchid tree- *Bauhinia acuminata*



18c. Pollen of Yellow elder-*Tecoma stans*



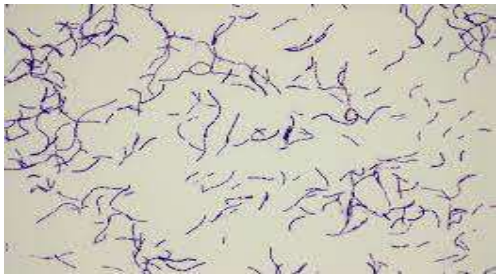
18d. Pollen of Moss-rose purslane- *Portulaca grandiflora*



18e. Pollen of Shame plant- *Mimosa pudica*

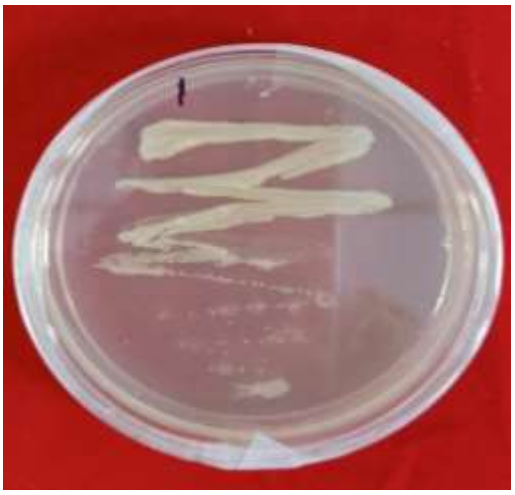
Plate 18. Scanning Electron Microscopy (SEM) images of pollen collected from hives of *A. cerana indica* from Wayanad

19a. Microscopic image of Gram-negative bacteria

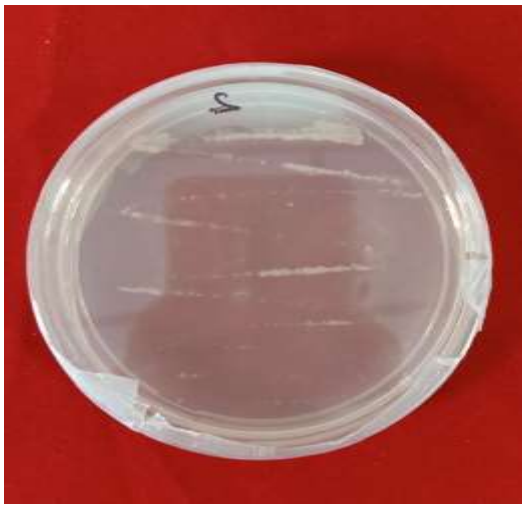


19b. Microscopic image of Gram-positive bacteria

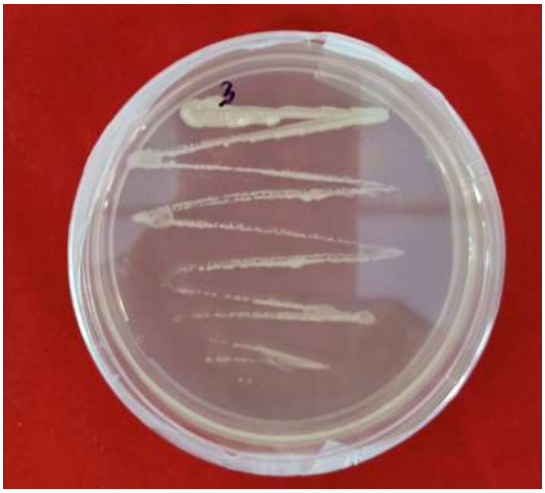
Plate 19. Microscopic images of bacterial isolates obtained from hives of *A. cerana indica*



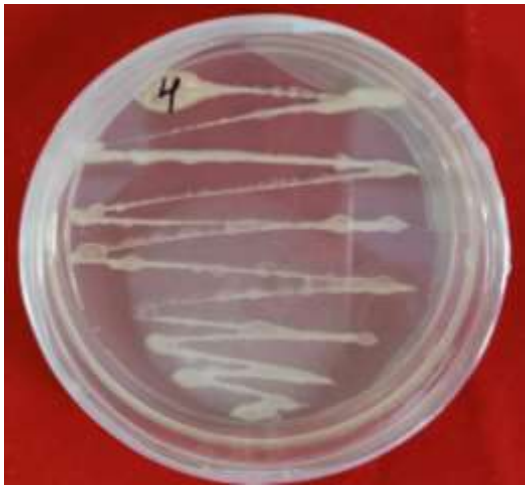
20a. Isolate B1



20b. Isolate B2

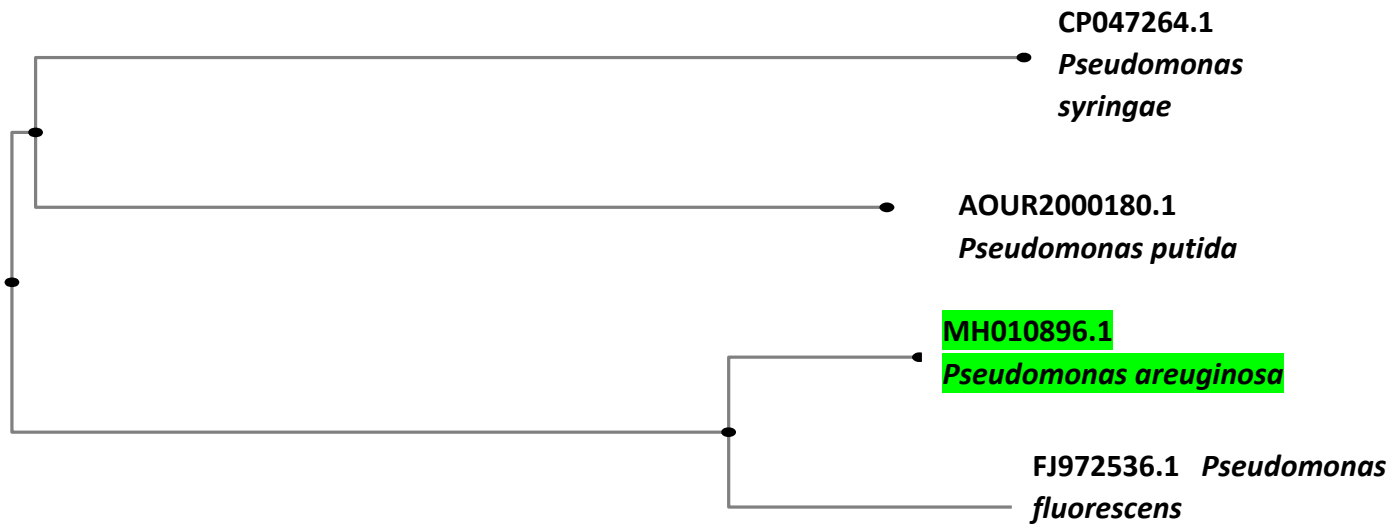


20c. Isolate B3

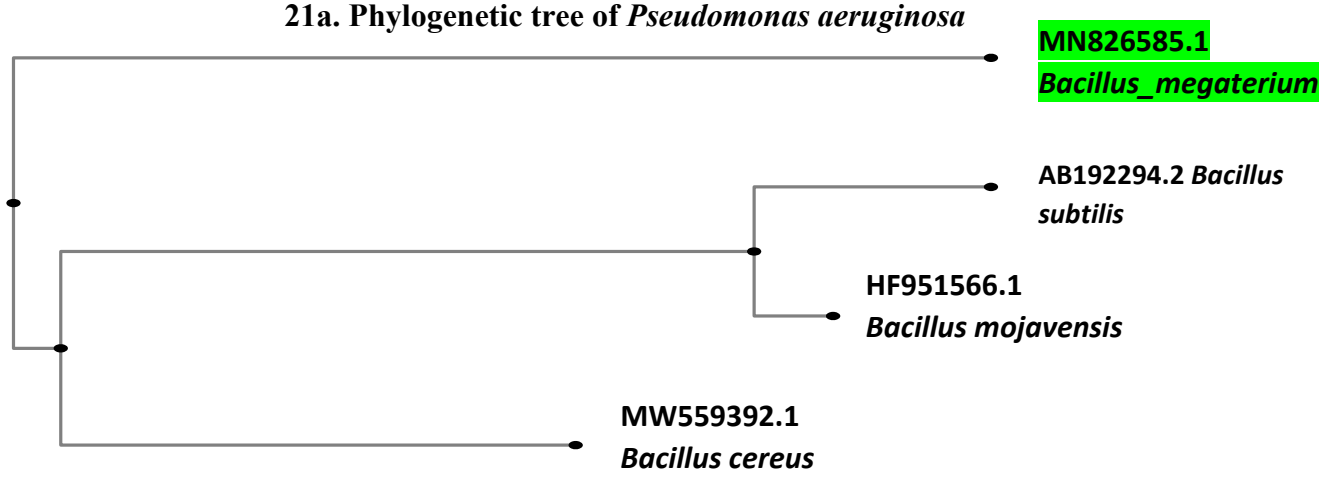


20d. Isolate B4

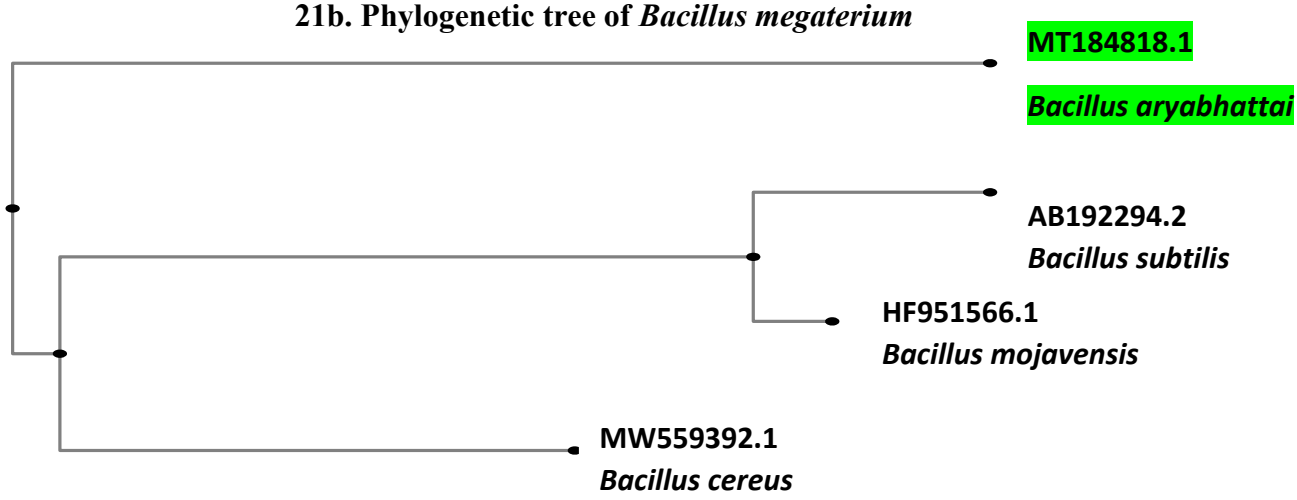
Plate 20. Bacterial isolates obtained from the hives of *A. cerana indica*



21a. Phylogenetic tree of *Pseudomonas aeruginosa*

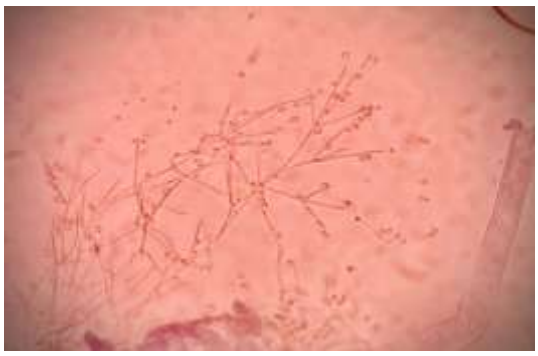


21b. Phylogenetic tree of *Bacillus megaterium*



21c. Phylogenetic tree of *Bacillus aryabhattai*

Plate 21. Phylogenetic tree of bacterial isolates obtained from hives of *Apis cerana indica*

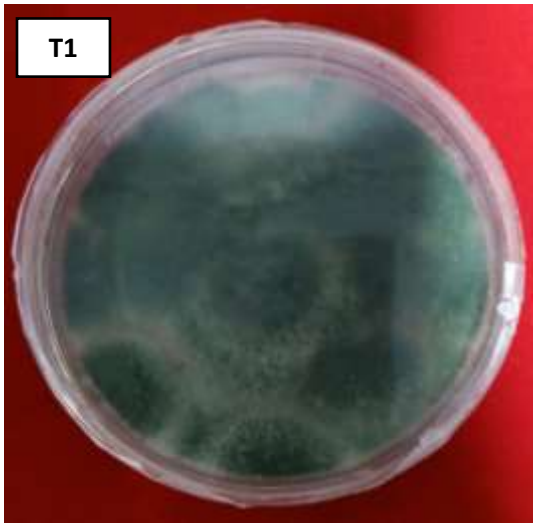


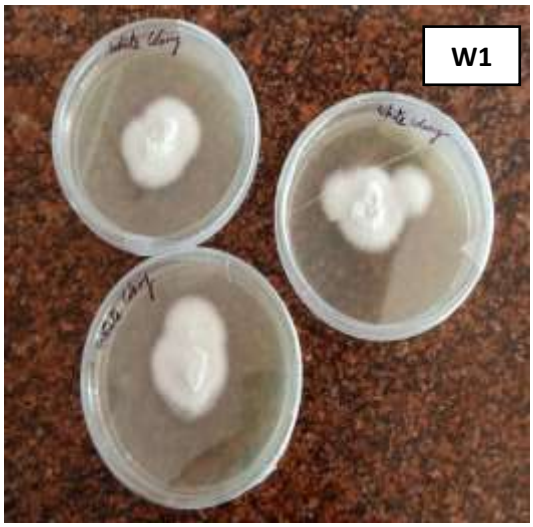
22a. Isolate T1

22b. Isolate W1

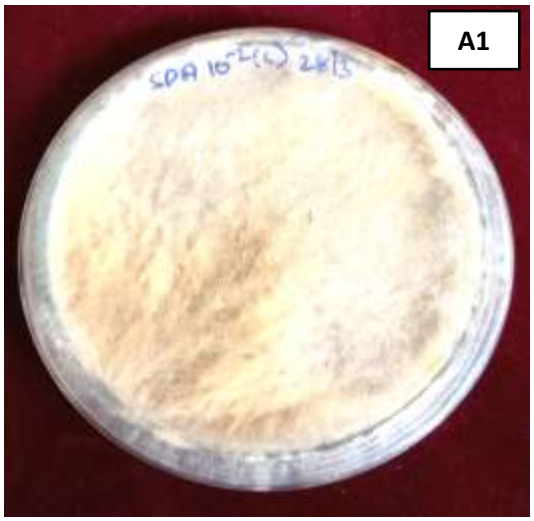
22c. Isolate A1

Plate 22. Microscopic images of fungal isolates obtained from hives of *A. cerana indica*



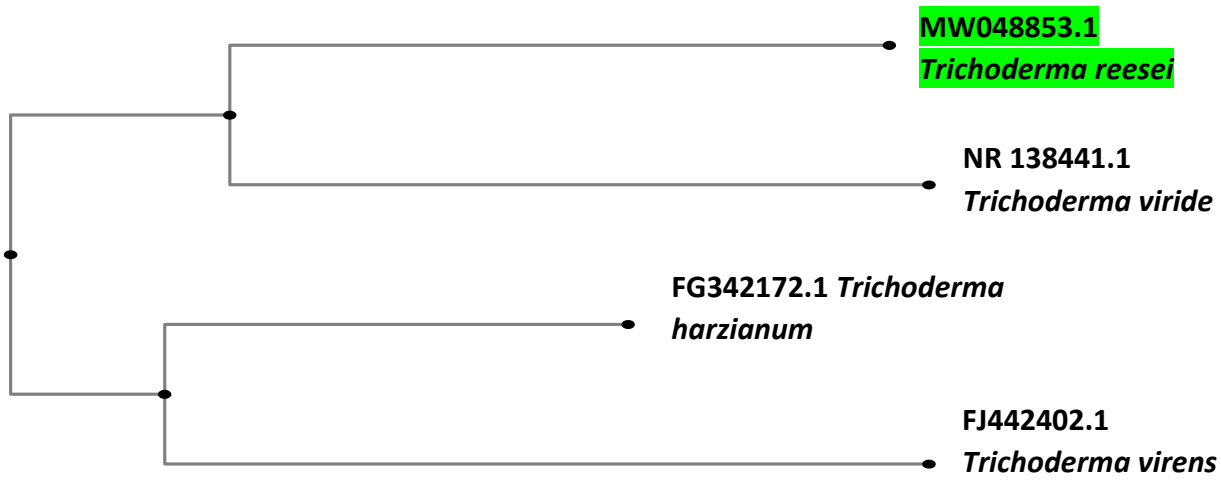


23a. Isolate T1 **23b. Isolate W1**

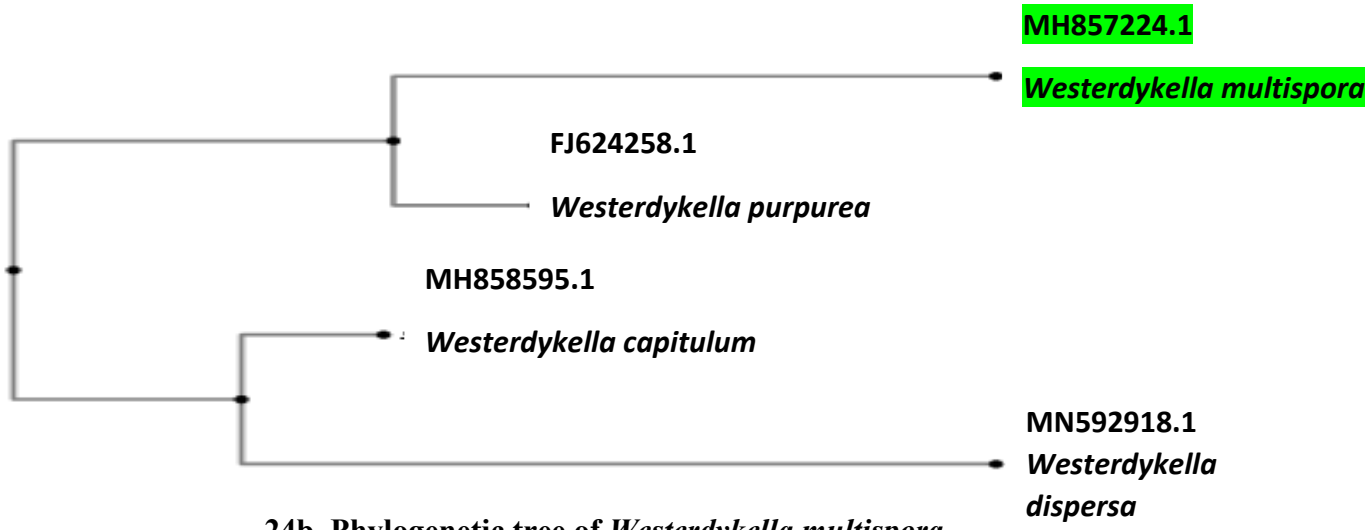


23c. Isolate A1

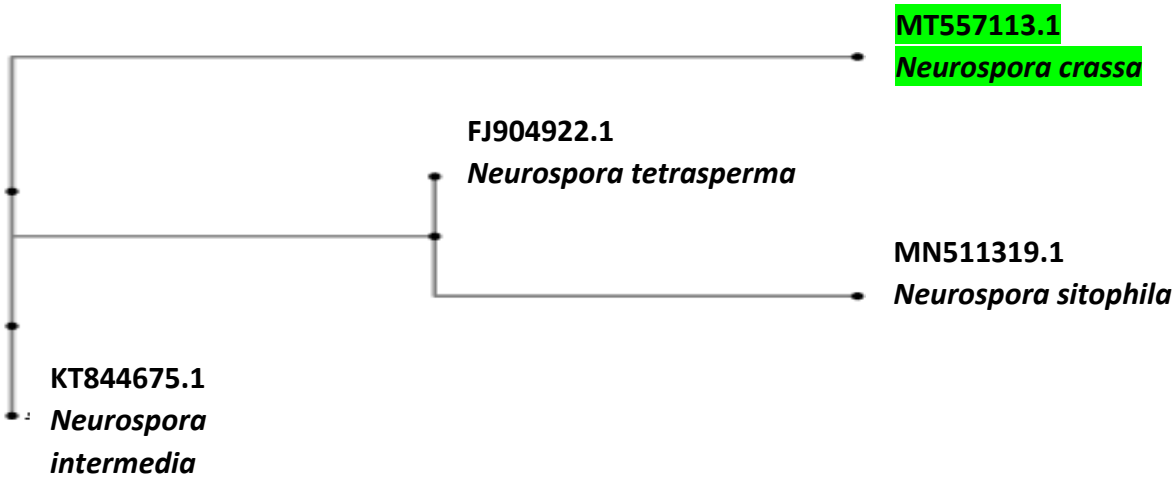
Plate 23. Fungal isolates obtained from hives of *Apis cerana indica*



24a. Phylogenetic tree of *Trichoderma reesei*



24b. Phylogenetic tree of *Westerdykella multispora*



24c. Phylogenetic tree of *Neurospora crassa*

Plate 24. Phylogenetic tree of fungal isolates obtained from hives of *Apis cerana indica*

DISCUSSION

CHAPTER V

DISCUSSION

The present investigation entitled 'Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)' was carried out during the period from June 2020- to September 2021 and the results presented in the previous chapter are discussed hereunder with published scientific literatures.

5.1 Palynological identification of hive stored pollen

The identification and counting of pollen grains in honey sediment by microscopic analysis was widely employed to ascertain its botanical origin (Bambara, 1991; Bolchi Serini and Salvi, 1990; Feller-Demalsy and Parent, 1989). In the present study, the palynological identification of hive stored pollen samples collected from Palakkad and Wayanad revealed the presence of twenty-one pollen sources belonged to 12 families. Identified pollen sources were Singapore daisy (*Sphagneticola trilobata),* Peacock flower (*Caesalpinia pulcherrima),* Common zinnia (*Zinnia elegans),* Holy Basil (*Ocimum sanctum),* Asian pigeonwings *(Clitoria ternatea*), Arjun tree (*Terminalia arjuna*), Flea tree (*Albizia lebbeck),* Tabasco pepper (*Capsicum frutescens),* Lemon (*Citrus limon),* Cucumber (*Cucumis sativus),* Daisy (*Helianthus aspera),* Little tree plant (*Biophytum sensitivum),* White orchid tree (*Bauhinia acuminata*), Shame plant (*Mimosa pudica),* Yellow elder (*Tecoma stans),* Moss-rose purslane (*Portulaca grandiflora),* Wattles (*Acacia* sp.), Asian palmyra palm (*Borassus flabellifer),* Indian beech (*Pongamia pinnata),* Slender amaranth (Amaranthus *viridis)* and Senna tora (*Cassia tora*). Suryanarayana *et al*. (1991) identified the pollen sources from hive stored pollen samples collected from the surrounding places of Muzaffarpur, Bihar and revealed the presences of pollen from plants viz., *Parthenium hysterophorus, Brassica* spp, *Phoenix sylvestris, Borassus flabellifer, Cajanus cajan, Mangifera indica, Zea mays* as major sources of pollen. Balasubramanyam *et al*. (2013) reported that *Citrus* species, *Borassus flabelliformis, Tectoma stans, Tectona grandis, Cocus nucifera, Anacardium occidentale, Coffea arabica, Tribulus terrestris* as major sources of pollen for honey bees in Chick ballapur region of Karnataka. Saklani and Mattu (2018) conducted survey in Hamirpur hills of Himachal Pradesh and palynological

identification revealed that *Acacia* sp., *Citrus* sp. and *Terminalia* sp. were the major sources of pollen present in the beehive cell. Balasubramanyam *et al*. (2013) reported the pollen yielding plants species viz., *Syzygium cumini, Butea monosperma, Ageratum conyzoides, Mangifera indica, Grevillea robusta, Psidium gaujava, Taraxacum officinale, Ocimum* sp., *Seasamum* sp. and *Sechium edule,* which act as major pollen source to honeybees had been reported by. Vijayakumar *et al*. (2020) reported that *Ceasalpinia* sp., *Jatropha* sp., *Eucalyptus* sp., *Coriandrum sativum, Tamarindus* sp., *Glyricidia* sp., *Parthenium hysterophorus* and *Schinus terebinthifolius* species acts as pollen sources for *A. cerana indica* in Doddaballapura and Bangalore districts of Karnataka. Kishan tej *et al.* (2014) reported that plants viz., - *Coriandrum sativum, Cucumis melo, Allium cepa, Cucurbita* spp., *Raphanus sativus, Guizotia abyssinica, Carthamus tinctorius, Medicago sativa* and *Trifolium* spp. also act as pollen sources to *Apis cerana indica* from Coimbatore region of Tamil Nadu. Honeybees are known to collect the pollen and nectar from the available predominant sources in the vicinity of their hives. The present findings are in agreement with the earlier studies.

5.1.1 Characterization of hive stored pollen of *Apis cerana indica*

In the present study, we made a pioneer attempt to characterize the hive stored pollen of *Apis cerana indica*. The images of hive stored pollen sample collected from the two locations captured using Scanning Electron Microscopy and characterized based on their nature, size, shape, aperture and exine ornamentation. Nature of the different pollen types varied from Monad to tetrad, whereas the shape of pollen ranged from spherical, circular, triangular, spheroidal, cylindrical, prolate to elliptical. The size of pollen from various plant sources ranged between 7.08 to 56.62 µm. The pollen of *Mimosa* recorded the smallest size (7.08 µm), whereas the largest pollen identified belonged to *Portulaca grandiflora (*56.62 µm). Pollen aperture varied from tricolporate, hexacolpate, tricolpate, inaperturate, pantocolpate. According to study conducted by Shubharani *et al.,* (2013), morphology of *Acacia* sp. was polyad type with individual 16 cells of sub-globose shape in periphery and square in centre, polyads not in the form of pollinia, morphology of *Bombax malabaricum* was colporate, prolate, per-oblate aperture with bilateral symmetry and morphology of Hibiscus sp. was pantoporate and pores are echinate with radial symmetry.

5.1.2 Relative abundance of predominant families of pollen sources

In the present study, the identified pollen sources belonged to the family, Fabaceae were Peacock flower (*Caesalpinia pulcherrima),* Asian pigeonwings *(Clitoria ternatea*), Flea tree (*Albizia lebbeck),* White orchid tree (*Bauhinia acuminata*), Shame plant (*Mimosa pudica),* Wattles (*Acacia* sp.), Indian beech (*Pongamia pinnata),* Senna tora (*Cassia tora*), which accounted for 38.09 per cent of the total pollen sources. It was followed by the pollen sources belonged to the family Asteraceae *viz.*, Singapore daisy (*Sphagneticola trilobata),* Common zinnia (*Zinnia elegans),* and Daisy (*Helianthus aspera),* which accounted for 14.28 per cent of the total pollen sources. One pollen source each belongs to the family Combretaceae, Solanaceae, Rutaceae, Oxalidaceae, Bignoniaceae, Portulacaceae, Arecaceae, Cucurbitaceae and Amaranthaceae which accounted for 4.76 per cent of total identified pollen sources (Fig 1). According to Tiwari *et al*. (2012), Asteraceous, Rosaceous and Poaceous plants were major source of pollen for Indian honey bees in Kamad, Uttarkashi of Garhwal Himalayas whereas, Chandra and Sharma (2011), observed that that the members of the families Acanthaceae, Asteraceae, Fabaceae, Solanaceae and Myrtaceae were as the minor sources of pollen and nectar for *A. cerana indica* from Shahjahanpur district of Uttarpradesh. Garg (1996) reported that the major source of pollen collected from Bhimal, India belonged to the family Asteraceae (34.7 per cent) - which was followed by Gramineae, Labiatae and Leguminosae with a share of 10 per cent each. Kalpana *et al*. (1990) identified twenty-four pollen types from twenty families of plants *viz.*, Compositaceae, Cucurbitaceae, Loranthaceae, Palmae, Acanthaceae, Amaranthaceae, Bombacaceae, Rubiaceae, Meliaceae, Labiatae, Zygophyllaceae, Simarubaceae, Mimosaceae, Boraginaceae, Caesalpiniaceae, Rutaceae, Myrtaceae, Labiatae, Nyctaginaceae, Rubiaceae from the pollen loads collected from Adikmet area, Hyderabad.

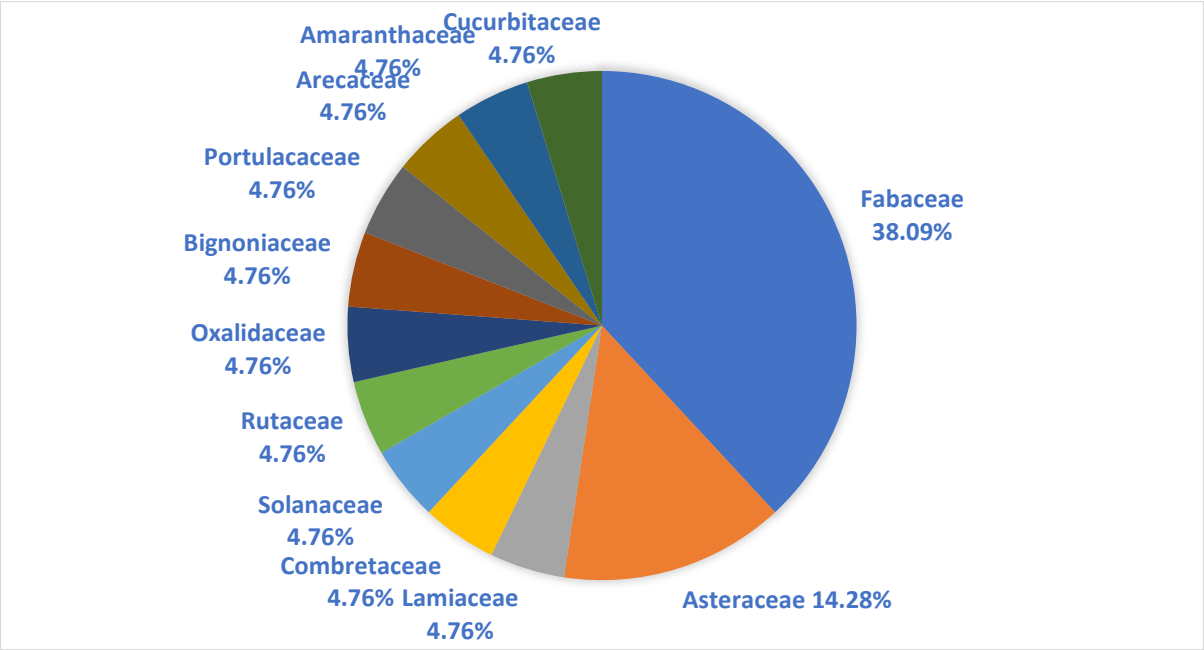


Fig 1. Relative abundance of pollen in *Apis cerana indica* hives

5.2 Physicochemical properties of hive stored pollen of *Apis cerana indica*

Physiochemical properties of hive stored pollen of *Apis cerana indica* was collected from two different districts *viz*., Palakkad and Wayanad. The variation with respect to their moisture content, water activity, pH, total acidity, ash content, protein content of hive stored pollen samples have been discussed in the following subsections

5.2.1 Moisture content

In the present study, the estimated moisture content of the hive stored pollen samples collected from Palakkad varied between 15.27 (g/100g) and 15.30 (g/100g) with a mean moisture content of 15.29 (g/100g), whereas moisture content of samples collected from Wayanad varied between 15.33 (g/100g) and 15.44 (g/100g) with a mean moisture content of 15.38 (g/100g) (Fig 2). According to Codex Standards (1981/2001), the moisture content of the pollen samples should be less than 22 per cent. Mendes *et al*. (1998) collected various pollen samples from different regions of Portuguese and reported that moisture content varied from 13.6 to 19.2 per cent. Al-Khalifa and Al-Arify (1999) reported that moisture content of pollen samples collected from Saudi Arabia ranged from 14.0 to 16.9 per cent. Andrade *et al*. (1999) reported that the moisture content of pollen samples collected from Portugal ranged between

14.6 and 19.9 per cent with an average moisture content of 17.83 per cent. According to Yilmaz and Kufrevioglu (2000) pollen samples collected from Turkey had a moisture content, of 16 per cent. Nanda *et al*. (2003) assessed the moisture content of the pollen samples and it found varied between 14.0 and 18.7 per cent. Ozcan *et al*. (2006) reported that pollen samples collected Turkey had a moisture content of 15.4 per cent and also observed that samples with more moisture content will have less density. Jasim *et al*. (2007) analysed the moisture content in the hive stored pollen of *Apis cerana indica* collected from Karnataka and observed a mean moisture content of 20.12 per cent in the pollens sample. Moisture content plays a crucial role in preservation of pollen. It also depends upon the prevailing weather conditions, which ultimately reflects in composition of associated microbial flora (Townsend, 1970).

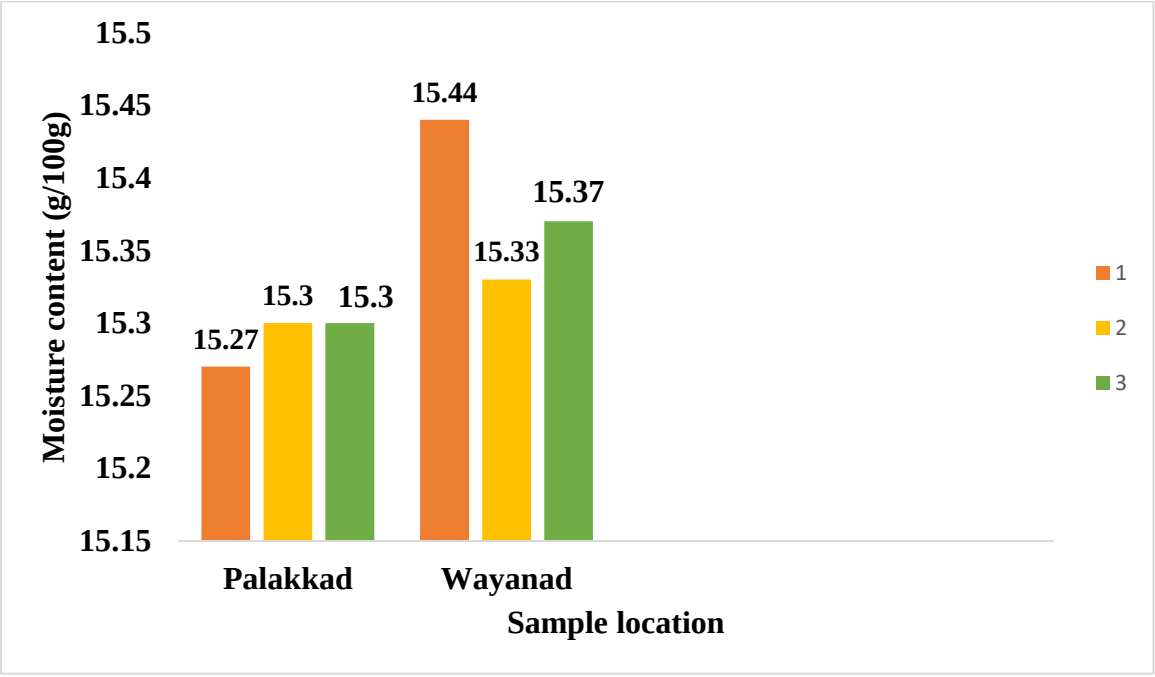


Fig 2. Variation in moisture content (g/100g) of hive stored pollen of *Apis cerana indica*

5.2.2 Water activity

In the present study, the estimated water activity of the hive stored pollen samples collected from Palakkad ranged between 0.737 (a_w) and 0.739 (a_w)with a mean water activity of (0.73 a_w) whereas water activity of samples collected from Wayanad varied between (0.742 a_w) and (0.743 a_w) with a mean water activity of 0.74 (a_w) (Fig 3). Abramovič *et al*., 2008; Velasquez Giraldo, Vélez Acosta and Zuluaga Gallego, 2013 estimated water activity of pollen from Slovesian and it ranged from 0.5 to 0.65 and found that it prevented the appearance of mold, yeast and bacteria, but is not low enough to avoid the contamination with osmophilic yeasts since they are able to grow above minimal water activity of 0.6. Barajas *et al*. (2012), estimated the water activity of pollen collected from Zipaquira and La Calera, which ranged between 0.73 and 0.78. Feas *et al*. (2012) reported that water activity of pollen collected from Spain ranged from 0.21 to 0.54 and inferred that that low a_w inhibits the growth and presence of microorganisms in the pollen. Sagona *et al*. (2017) reported that the water activity of beehive pollen collected from apiaries of *A. cerana indica* from Slovok Republic, and it ranged from 0.574 to 0.665. Water activity (a_w) is a parameter useful to assess honey quality and to control the bacterial replication. Water activity is a crucial factor which determines enzyme activity, growth and survival of microorganisms in pollen. It is used as an indicator of the risk of contamination with microorganisms. Usually, higher the water activity, the faster the growth of various microbes like bacteria, yeast and mould which will further increase the level of contamination.

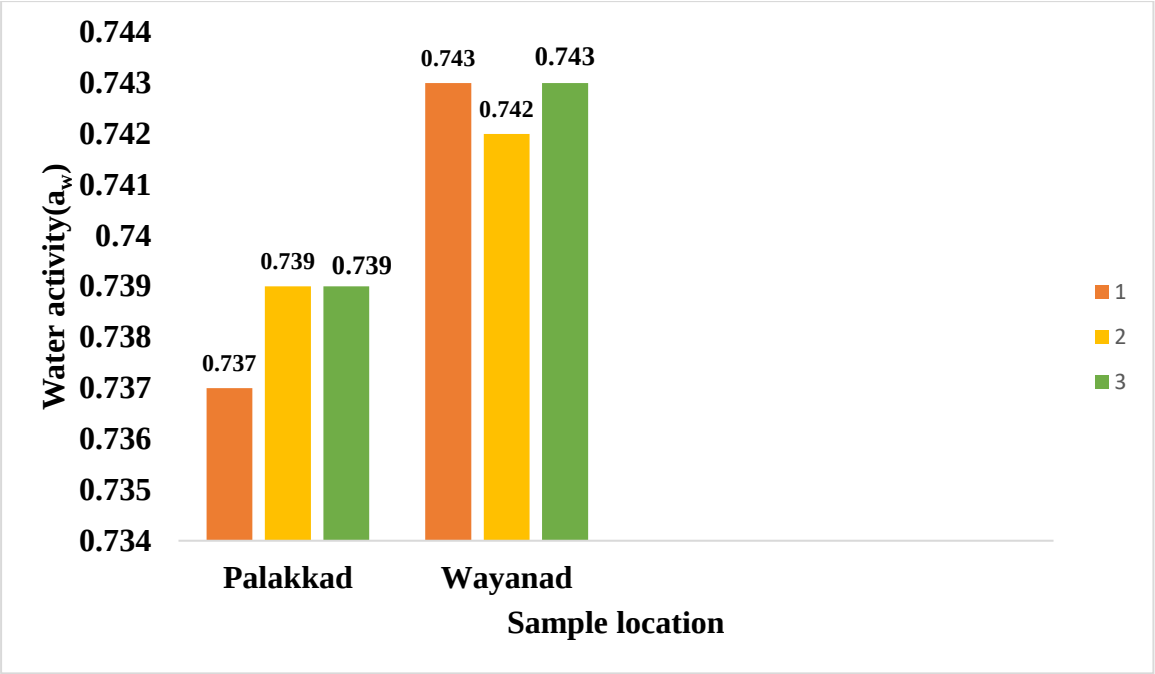


Fig 3. Variation in water activity (a_w) of hive stored pollen of *Apis cerana indica*

5.2.3 pH

The estimated pH of hive stored pollen samples collected from Palakkad varied between 3.81 to 3.84 with a mean pH of 3.83 whereas pH of samples collected from Wayanad varied between 3.74 to 3.8 with a mean pH of 3.77 (Fig 4). Usually, higher the pH value, chance of fungal contamination is more when compared with other micro-organisms as the fungi prefers acidic pH for their growth. Mateo and Bosch (1998) and Lopez *et al*. (1996) reported analysed the pH of pollen samples and it ranged between 3.61 and 4.97. Nanda *et al*. (2003) observed that that all pollen samples collected from different places in Northern India were acidic with a pH range of 3.49-4.43. The acidity of samples is due to the presence of organic acids, particularly the gluconic acid and inorganic ions such as phosphate and chloride. Anupama *et al*. (2003) reported that of pollen samples of Mysuru varied in pH between 3.62 to 4.00, similarly Esti *et al*. (1997) also reported pollen sample collected from Molise region and it ranged from 3.05 to 4.50. Downey *et al*. (2005) collected around fifty samples of pollen from Ireland and reported that pH ranged between 3.75 and 4.74. Acquarone *et al*. (2007) reported that pH of the pollen samples collected from Argentina varied from 3.003.88.

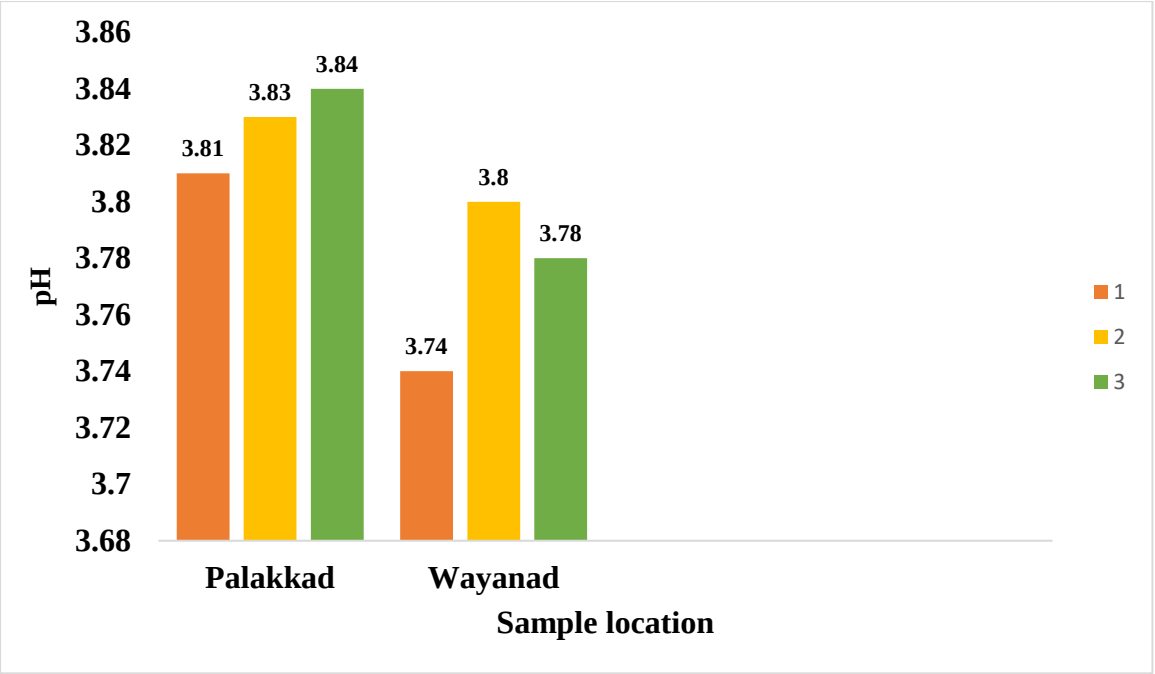


Fig 4. Variation in pH of hive stored pollen of *Apis cerana indica*

5.2.4 Ash content

The estimated ash content of the hive stored pollen samples collected from Palakkad ranged between 2.00 (g/100g) to 2.03 (g/100g) with a mean ash content of 2.01 (g/100g), whereas from Wayanad fluctuated between 1.87 (g/100g) and 1.88 (g/100g) with a mean ash content of 1.87 (g/100g) (Fig 5). Samples with more ash content has more minerals content like potassium, magnesium *etc.,* White *et al*. (1975) reported that ash content of pollen collected from hive stored pollen samples of *Apis cerana indica* was more than 1 per cent. Herbert and Shimanuki (1978), reported that ash content of pollen samples collected from Beltsville, Maryland ranged from 2 to 3.75 per cent. The mineral content in samples gave an indication of environmental pollution and hence also an indication of geographical origin (Anklam, 1998). Al-Khalifa and Al-Arify (1999) stated that ash content in general depends up on nectar composition of predominant plants in the collection of pollen from plants. Maria *et al*. (2008) reported that ash content in the hive stored pollen of *Apis cerana indica* ranged from 2 to 6 g/100g which was a safe ash content for the pollen with good amount of minerals. Kahraman *et al*. (2010) reported that ash content of pollen samples collected from Turkey were with mean value of 0.28 which was in the acceptable range. Anjos *et al*.

(2015) collected pollen samples from Uruguayan which were further analysed for ash content and reported results were in the range of 1.87-2.59. Feas *et al*. (2019) analysed the ash content in hive stored pollen from Spain were ranged from 2 to 4 per cent.

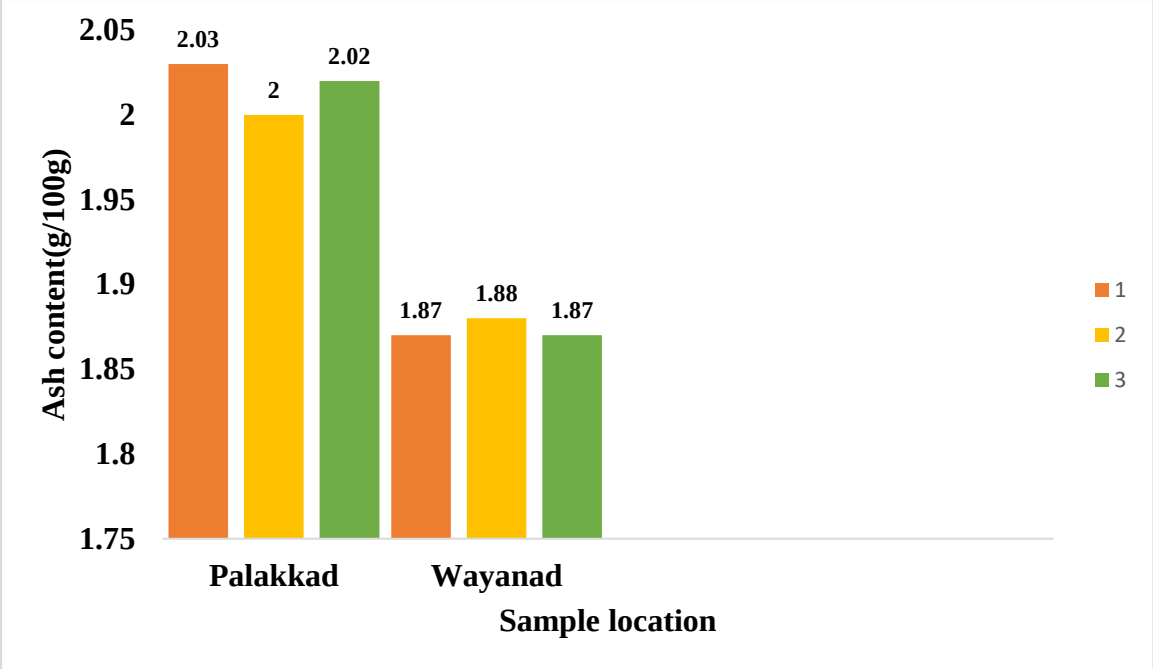


Fig 5. Variation in ash content(g/100g) of hive stored pollen of *Apis cerana indica*

5.2.5 Total acidity

The estimated total acidity of the hive stored pollen samples collected from Palakkad differed between 10.12 (mmol/g) and 10.17 (mmol/g) with a mean total acidity of 10.13 (mmol/g) whereas from Wayanad differed between 9.62 and 9.65 with a mean total acidity of 9.64 (mmol/g) (Fig 6). According to Al-Khalifa *et al*. (1999), mean total acidity of hive stored pollen collected from Saudi varied from 10-21.5. Costa *et al*. (1999) collected pollen samples from Brazil and analysed total acidity, which ranged between 8.20 and 50.0. Andrade *et al*. (1999) analysed total acidity of pollen sample collected from Portugal and it was recorded less than 40 indicates absence of undesirable fermentation. According to the study conducted by the acidity of pollen is directly related to the floral sources and the constituents *viz.*, amino acids, organic acids and aliphatic and aromatic acids (Jamnik *et al*., 2008). Higher acidity in the samples is an indicator fermentation of sugar into organic acids.

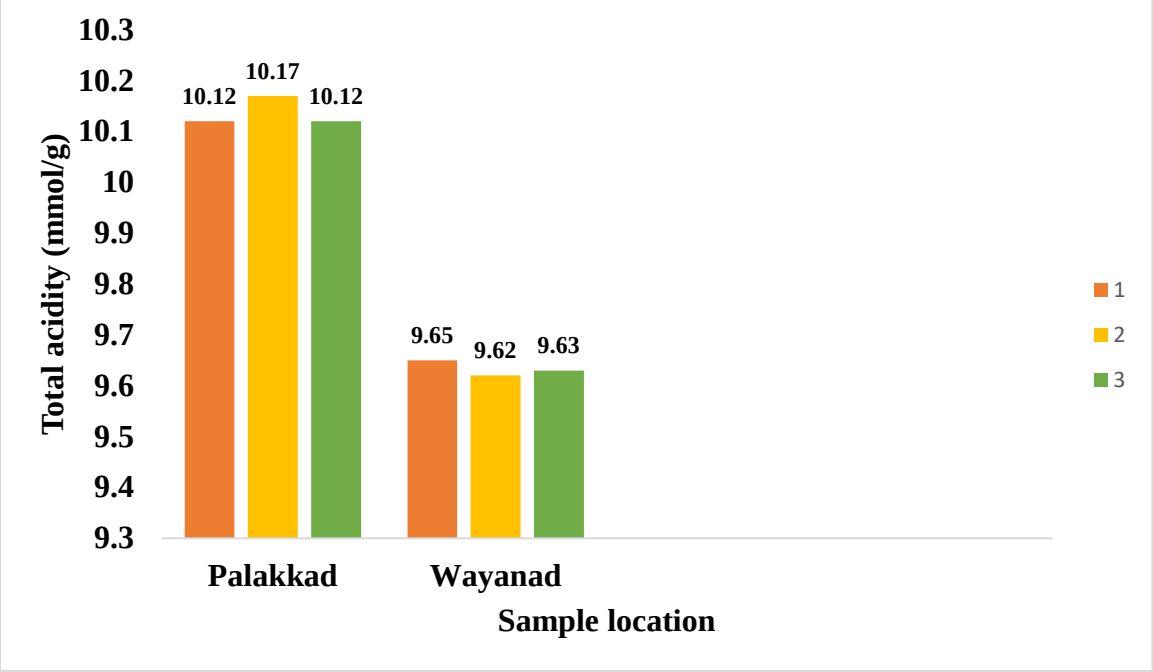


Fig 6. Variation in total acidity (mmol/g) of hive stored pollen of *Apis cerana indica*

5.2.6 Protein content

The estimated protein content of the hive stored pollen samples collected from Palakkad ranged between 3.45 (%) and 3.48 (%) with a mean protein content of 3.46 (%) whereas from Wayanad ranged between 3.69 (%) and 3.7 (%) with a mean protein content of 3.69 (%) (Fig 7). The majority of anemophilous species sampled came from three protein-poor clades, the conifers, the higher hamamelids (Sensu Manos and Steele, 1997) and the grasses. Anklam (1998) observed that the protein content of pollen is normally in the range of less than 5 mg/g. Roulston (2000) reported that bees collect pollen from Arizona ranged from 2.5-61 per cent protein, including the pollens of many anemophilous species. Pollen collected from sources like *Oenothera* and *Opuntia* contains less protein content. Animal pollinated (zoophilous) plants did not contain pollen richer in protein than anemophilous plants when phylogeny was factored into the analysis. Pollen of bee-pollinated species ranged from 12 to 14 per cent protein for some Asteraceae and Malvaceae to greater than 60 per cent which belongs to *Dodecatheon* and *Rhexia*. Proteins present in pollen were likely to be enzymes that aid

during pollen tube growth and subsequent fertilization. Almeida-Muradian *et al*. (2005) reported that protein content in beehive pollen collected from Brazil ranged from 10-40 per cent. Ouchemoukh *et al*. (2007) observed that the protein contents of analysed pollen samples collected from Algeria were ranged in between 3.7 and 9.4 mg/g. Higher the protein content infers that the sample is rich in amino acids.

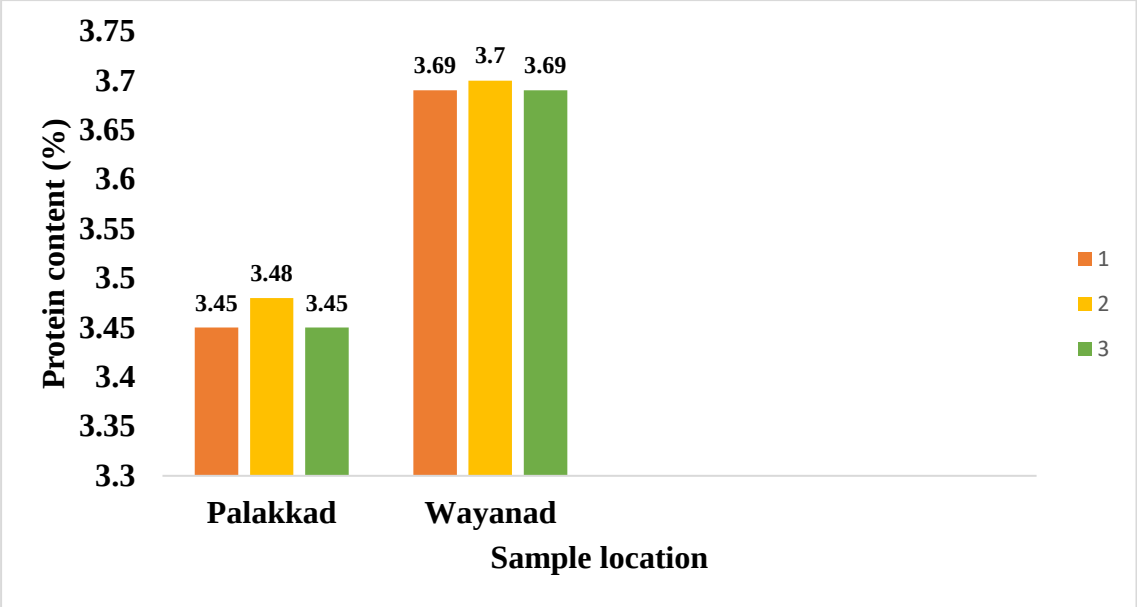


Fig 7. Variation in protein content (%) of hive stored pollen of *Apis cerana indica*

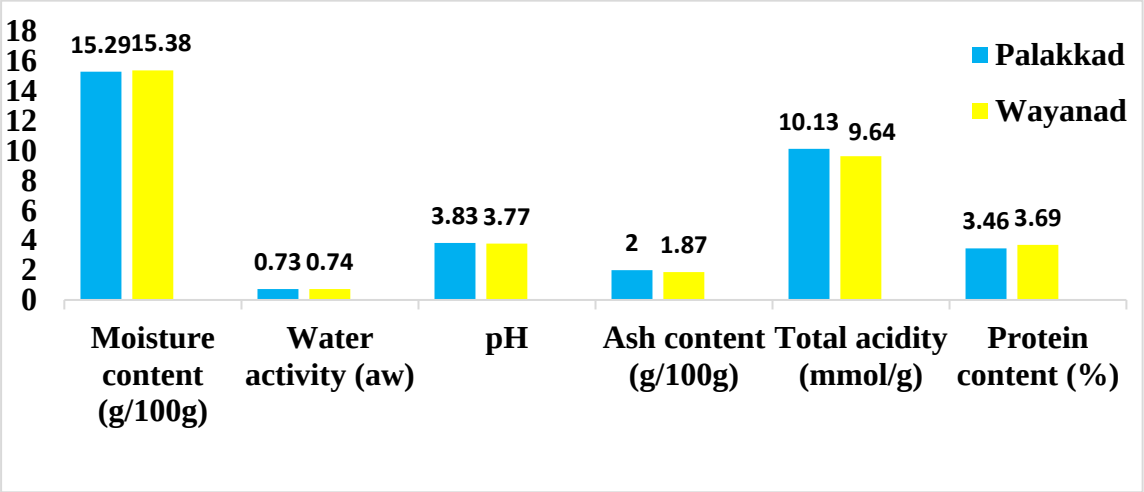


Fig 8. Over all comparison of mean values of the physicochemical properties of hive stored pollen of *Apis cerana indica* from Palakkad and Wayanad

Parameters like moisture content, water activity, protein content were more in Wayanad sample, it may be because of high humidity and temperature prevailing in the location. Parameters like pH, total acidity and ash content were more in Palakkad sample which might be due to higher mineral content in the pollen sources.

5.3 Microbiological determinations in hive stored pollen of *Apis cerana indica*

5.3.1 Microorganism identification

5.3.1.1 Bacterial isolate differentiation using staining technique from hive stored pollen of Palakkad and Wayanad districts

In the present study altogether four bacterial isolates *viz., Pseudomonas aeruginosa*, *Bacillus megaterium* and *Bacillus aryabhattai* were obtained from the hive stored pollen samples of *Apis cerana indica* collected from Palakkad and Wayand. Similar studies were conducted on other bee species and isolated bacteria belonged to the genus *Bacillus* and it was found to be prevalent in larval populations of two solitary bees (*Centris pallida* and *Anthophora* sp.) (Gilliam *et al*. 1984, 1990b) and some nests of stingless bees (*Melipona fasciata* and *Trigona hypogea*) (Gilliam *et al*. 1985, 1990a). Goerzen (1991) isolated bacteria viz., *Bacillus circulans, B. mycoides, Enterobacter agglomerans* and *Pseudomonas* sp. from hive stored pollen *Apis cerana indica*. Maria *et al*. (2008) reported four different types of bacterial isolates viz.,mention Name of bacteria in the pollen samples. were reported by Ceauşi *et al*. (2009) found *Escherichia coli* and coagulase-positive Staphylococci and *Bacillus cereus* in the pollen samples

In the present study, 16S rRNA sequencing from the hive stored pollen of *Apis cerana indica* revealed the presence of *Pseudomonas aeruginosa*, *Bacillus megaterium* and *Bacillus aryabhattai* in the hive stored pollens. Ceausi *et al*. (2009) identified that the bacteria present in pollen samples *viz., Escherichia coli*, coagulase-positive *Staphylococci and Bacillus cereus* based on 16S rRNA sequencing. Sinacori *et al*. (2013) also performed 16S rRNA sequencing for the identification of bacterial isolates and reported that *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus cereus, Bacillus thuringiensis, Bacillus licheniformis, Bacillus megaterium* and *B. pumilus* were the bacterial species found in pollen samples.

5.3.1.1.1 Construction of phylogenetic tree for individual bacterial isolates showing bacterial diversity of hive stored pollen of *Apis cerana indica*

In the present study, evolutionary analysis of B1, B2, B3 and B4 was conducted by constructing the phylogenetic trees. These were constructed for individual bacterial isolates by comparing with their closely related species in NCBI with the help of MAFFT software. Length of the phylogenetic tree has significance. Higher the length of phylogenetic tree, more the genetic divergence. It was constructed for individual isolates with their close species to find out the diversity among different species of the same genus. According to the study conducted by Ahn *et al*., (2012) phylogenetic trees were constructed for each Operational Taxonomic Unit (OTU). Representative sequences for each OTU and closely related sequences in GenBank were imported into the ARB software package (version 5.2) (Ludwig *et al.,* 2004) loaded with the SILVA 16S rRNA database (SSURef-108) (Pruesse *et al.,* 2007). Sequences were aligned using the SINA aligner (version 1.1) and the analysis showed the cluster is related to Pasteurellaceae.

5.3.1.2 Fungal strains isolated from hive stored pollen of *A. cerana indica*

In the present study, altogether three fungal isolates *viz., Trichoderma reesei, Westerdykella multispora and Neurospora crassa* were obtained from the hive stored pollen samples of *Apis cerana indica*. Felsociova *et al*. (2012) collected samples from Poland and obtained fungal colonies of *Alternaria* spp., *Aspergillus fumigatus, A. niger, Aspergillus* sp., *Cladosporium* spp., *Fusarium* spp., *Mycelium sterilium, Penicillium brevicompactum, P. commune, P. corylophilum, P. crustosum, P. expansum, P. griseofulvum, P. chrysogenum, P. polonicum, Rhizopus* spp. by following dilution plate method. Kacaniova *et al*. (2012) performed dilution plating analysis for the collected samples and isolated various fungal species like *Penicillium, Aspergillus* and *Cladosporium* respectively. Other fungal species like *Botrytis, Eurotium, Trichoderma, Phoma, Fusarium, Mucor, Acremonium, Alternaria* and *Epicoccum* were also identified from the samples. Sinacori *et al*. (2013) isolated and purified different isolates of fungi on PDA and they were checked for their colour, colony texture etc., Khan *et al*. (2020) isolated different isolates of fungi and sub cultured on PDA media which can be used for future references. All the purified fungal isolates belong to four

different families. Naseer (2004) performed dilution plating technique and isolated *Emericella nidulans, Fusarium oxysporum, Humicola grisea, Penicillium corylophilum, P. funiculosum* and *Trichoderma hamatum* which were rare in frequency whereas *Aspergillus* spp. was most commonly found fungal species in the sample. Kis *et al*. (2018) conducted plate count assay and isolated six different isolates of fungi.

The fungi identified by ITS sequencing from the hive stored pollen of *Apis cerana indica* were *Trichoderma reesei, Westerdykella multispora and Neurospora crassa.* Sinacori *et al*. (2013) performed ITS sequencing and identified *Aspergillus niger, Penicillium corylophilum* and *Z. mellis* from pollen samples. Kacaniova *et al*. (2012) identified fungi *viz., Mucor, Penicillium* and *Aspergillus flavus* from the samples based on ITS sequencing.

5.3.1.2.1 Construction of phylogenetic tree for individual fungal isolates showing bacterial diversity of hive stored pollen of *Apis cerana indica*

In the present study, evolutionary analysis of isolates T1, W1 and A1 was conducted by constructing the phylogenetic trees These were constructed for individual fungal isolates by comparing with their closely related species in NCBI with the help of MAFFT software. Length of the phylogenetic tree has significance. Higher the length of phylogenetic tree, more the genetic divergence. It was constructed for individual isolates with their close species to find the diversity among different species of the same genus. According to the study conducted by Mai *et al*., (2017), the phylogenetic tree was conducted based on DNA sequences of ITS 1, ITS 2 and 5.8 rRNA gene of fungal isolates was constructed and this found that the fungal species found were in conformity with the morphological identification. The identified fungal species were *Aspergillus, Cladosporium, Eupenicillium* and *Fusarium.*

5.3.1.3 Actinomycetes strains isolated from hive stored pollen of *A. cerana indica*

In the present study, no actinomycetes colonies were recorded on the plated media. Few reports revealed the presence of actinomycetes colonies in pollen samples of *Apis cerana indica*. *Streptomyces* were most commonly found microbes in pollen (Snowdon & Cliver, 1996) and honey related products, which possessed antimicrobial properties (Kwakman *et al*., 2008). Patil *et al*. (2010) isolated Actinomycetes,

Nocardiopsis alba from the pollen samples. Khan *et al*. (2020) isolated actinomycetes colonies belonged to six different families from pollen samples of *A. cerana indica.*

5.3.1.4 Yeast strains isolated from hive stored pollen of *A. cerana indica*

In the present study, no yeast colonies were recorded in the media. Few reports revealed the presence of yeast colonies in hive stored pollen samples of *Apis cerana indica.* Esteve-Zarzoso *et al*. (1999) isolated yeast species *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* from pollen samples. Martins *et al*. (2011) reported the yeast species *Candida humicola* and *Saccharomyces* in pollen samples, that that was responsible for high levels of contamination.

5.4 Microbial diversity and microbial load

5.4.1 Microbial diversity of hive stored pollen of *Apis cerana indica*

In the present study, two bacterial and one fungus were identified from Palakkad sample whereas two bacteria and two fungi were identified from Wayanad sample (Fig 9). Sinacori *et al*. (2013) reported that a total of seven bacteria and ten fungi were identified from thirty-eight samples.

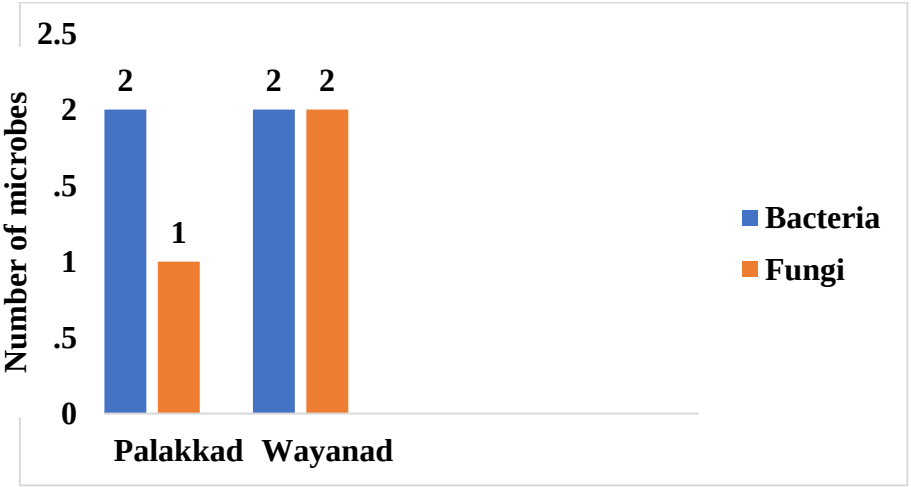


Fig 9. Microbial diversity of hive stored pollen of *Apis cerana indica*

5.4.2 Microbial load of hive stored pollen of *Apis cerana indica*

In the present study, total microbial load of $3.3x10^4$ cfu/g was recorded in samples collected from Palakkad, whereas for Wayanad sample it was $6.6x10^4$ cfu/g

(Fig 10-). Estevinho *et al*. (2012) reported that total microbial load in pollen samples ranged from $2.8x10^4$ to $7.6x10^4$ cfu/g. Similar type of studies were conducted by Sinacori *et al*. (2013) and reported that total microbial load ranged from 1 to 88. cfu/g. According to Zuluaga *et al*. (2013), total microbial load of hive pollen samples of bee ranged from 1.65±0.03 to 3.41±0.06 cfu/g. Jaya *et al*. (2020) reported that total microbial load of bee hive pollen collected from hives of *Apis cerana indica* was 5.58±0.38 cfu/g.

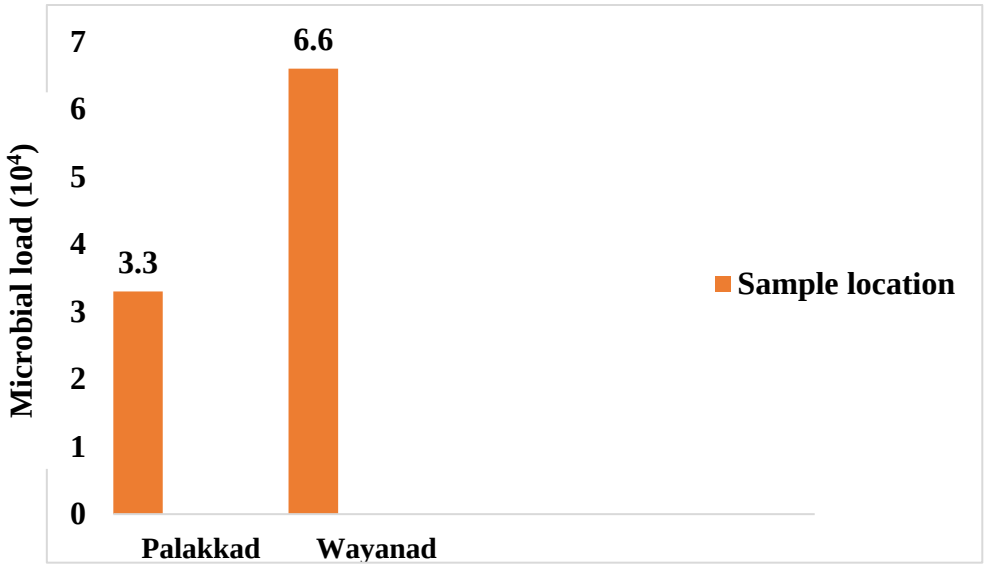


Fig 10. Microbial load of hive stored pollen of *Apis cerana indica* make the bars in the graph little broader as the major part of the graph appears empty

5.5 Microbial load to pollen grain ratio of hive stored pollen of *Apis cerana indica*

In the present study, total microbial load to pollen grain ratio of Palakkad sample was $3.3x10^4$ cfu/g, whereas for Wayanad sample it was $6.6x10^4$ cfu/g (Fig 11). Anderson *et al*. (2014) analysed total microbe to pollen grain ratio and obtained a total $3.6x10^4$ microbes per gram of stored pollen and inferred that at least one microbe will be present in every 2500 pollen grains. Higher the ratio, indicates that contamination of pollen with microbes is higher which might causes adulteration when consumed as human food.

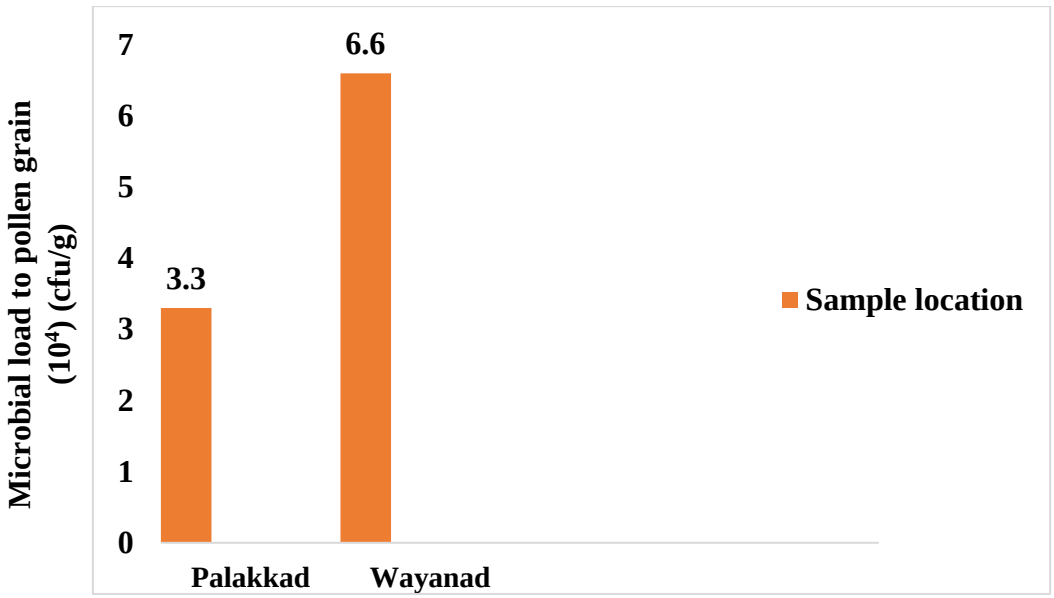


Fig 11. Microbial load to pollen grain ratio of hive stored pollen of *Apis cerana indica*

5.6 Total bacterial and fungal population of hive stored pollen of *Apis cerana indica*

In the present study, sample collected from Palakkad recorded bacterial population of $2.3x10^4$ cfu/g and whereas pollen samples collected from Wayanad recorded a high bacterial population of $3.3x10^4$ cfu/g (Fig 12). Bonvehı´ and Jorda (1997) reported that total coliform bacteria population present in the pollen sample ranged from 3 to 43 cfu/g. whereas, *Clostridum*, *Bacillus* and *Streptococcus* was in a range of 5-10 cfu/g, 5-12 cfu/g and 5-550 cfu/g respectively. Nogueira *et al*. (2012) reported a total bacterial population of $0.87x10^4$ cfu/g in pollen sample, whereas, Anderson *et al*. (2014) reported that bacterial population of $0.25x10^4$ cfu/g in hive stored pollen samples.

In the present study, the estimated fungal population from Palakkad sample was $1x10^4$ cfu/g and it was $3.3x10^4$ cfu/g for Wayanad sample (Fig 12). According to Bonvehi and Jorda (1997) total fungal microflora will remain constant in a number and which is lower than 5×10^4 cfu/g. Kacaniova *et al*. (2012) has reported that total fungal population varied from 2.00×10^2 cfu/g with an average value of 1.20×10^1 cfu/g when inoculated on czapek-Dox agar, whereas the fungal population varied from 1.58×10^2

cfu/g with an average 9.66 cfu/g when inoculated on Malt Extract Agar. Nagueira *et al*. (2013) reported the fungal population of 2.6x10^4 cfu/g in hive stored pollen sample. Sinacori *et al*. (2013) reported a higher that fungal population of 10 x10^4 cfu/g in hive stored pollen whereas Barbara *et al*. (2015) observed a low population of fungi at the rate of 1.5 × 10^4 cfu/g in pollen samples at 30°C.

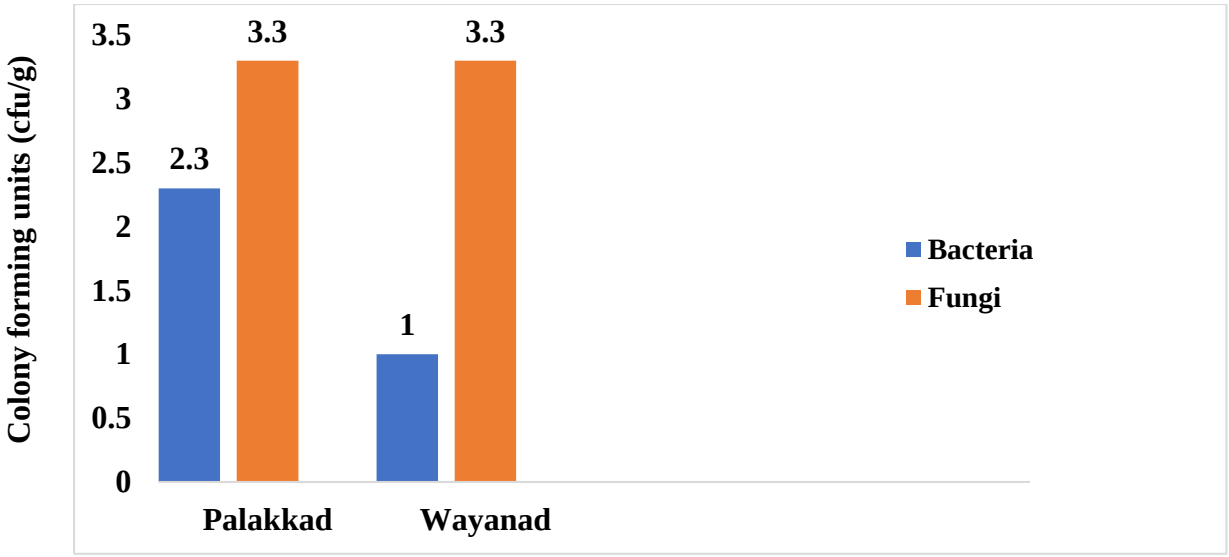


Fig 12. Total bacterial and fungal population of hive stored pollen of *Apis cerana indica*

SUMMARY

Chapter VI

Summary

Honeybees are beneficial insects for mankind as the most economically valuable insects which aid in pollination. Palynology is a branch of study which helps in identification of botanical and geographical origin of pollen by subjecting pollen sediment followed by SEM analysis which helps in pollen identification and characterization which plays a pivotal role in beekeeping industry. Physicochemical studies aid in analysing the conditions which are favourable to microbes therefore helps in preservation of pollen for future. Analysis of microbes associated with beehive stored pollen helps in gaining knowledge with respect to various microbes associated and their role for future mankind. From the point of above, the present study entitled 'Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius) was conducted at Insectary laboratory, Department of Agricultural Entomology, College of Agriculture, Kerala Agricultural University, Vellanikkara during the period 2020-2021.

Samples were collected from six locations *viz*., Mannarkkad, Nellipuzha, Nottanamala Kenichira, Kayakunnu and Cheengode which represented central midland (Palakkad) and high range (Wayanad) regions of Kerala.

Observations were recorded on different types of pollen, identification and characterization, percentage of families to which those pollen belongs, physicochemical properties like moisture content, water activity, pH, ash content, total acidity and protein content of hive stored pollen along with identification of microbes and diversity of microbes associated with the bee hive pollen in addition to microbial load, microbial load to pollen grain ration and total bacterial, fungal, actinomycetes and yeast populations. The data collected were analysed, presented in tables and figures were discussed in previous chapter V. The salient findings of the study are summarized below.

6.1 Characterization and identification of hive stored pollen of *Apis cerana indica*

- A total of 21 plants were recorded as a source of pollen by bees from the six sampling locations. All twenty-one pollen types were identified based on light microscopy.
- Identified pollen sources were *Caesalpinia pulcherrima, Terminalia arjuna, Albizia lebbeck, Citrus limon, Biophytum sensitivum, Bauhinia acuminata, Tecoma stans, Acacia* sp., *Borassus flabellifer, Pongamia pinnata, Cassia tora, Sphagneticola trilobata, Zinnia elegans, Ocimum sanctum, Capsicum frutescens, Cucumis sativus, Helianthus aspera, Amaranthus viridis, Portulaca grandiflora, Mimosa pudica* and *Clitoria ternatea.*
- Among twenty-one, ten dominant pollens were characterized and identified based on Scanning Electron Microscopy (SEM) analysis
- Characters of pollens of the plants *viz., Sphagneticola trilobata* (Asteraceae), *Caesalpinia pulcherrima* (Fabaceae), *Zinnia elegans* (Asteraceae), *Ocimum sanctum* (Lamiaceae), *Clitoria ternatea* (Fabaceae), *Biophytum sensitivum* (Oxalidaceae), *Bauhinia acuminate* (Fabaceae), *Mimosa pudica* (Fabaceae), *Tecoma stans* (Bignoniaceae), *Portulaca grandiflora* (Portulacaceae) were studied in details with SEM analysis.
- Pollen characters like nature, size, shape, aperture and exine pattern were studied for the ten samples collected from hive of *A. cerana indica*.
- Nature of pollen type varied from monad to tetrad, out of which monad was the dominant one. *Mimosa pudica* commonly known as shame plant of family Fabaceae was the only one source with tetrad type of pollen.
- Size of the pollen ranged from 7.08 to 56.62 µm which varied greatly within plant sources as well as with sapling locations.
- Smallest pollen size was of the pollen of *Mimosa pudica* (Shame plant) belongs to Fabaceae recorded the smallest size with 7.08 µm Whereas, pollen from *Portulaca grandiflora* (Moss-rose purslane) belongs to Portulacaceae recorded the largest pollen size with 56.62 µm.

- SEM analysis revealed that shapes of pollen were diversified from spherical, circular, triangular, spheroidal, cylindrical, prolate, elliptical. Out of which, spheroidal shaped pollens (50 %) were dominated.
- Aperture of pollen differed from tricolporate, hexacolpate, tricolpate, inaperturate, pantocolpate. Out of which, tricolpate aperture type pollens were dominant.
- Exine patterns of pollen were diversified from echinate, synaperturate, bireticulate, reticulate, microreticulate, pantoaperturate. Out of which, Echinate, sharp spines with conical base pollens were dominant.
- Among all families, Fabaceae occupied first position with a share of 38.09 per cent followed by Asteraceae with a share of 14.28 per cent.
- From the above study, high variation in pollen types were found in Wayanad sample due to rich and wide diversity of flora present in and around Kenichira, Kayakunnu, Cheengode.

6.2 Physicochemical properties of hive stored pollen of *Apis cerana indica*

- Moisture content of the hive stored pollen samples collected from Palakkad varied between 15.27 (g/100g) and 15.30 (g/100g) with a mean moisture content of 15.29 (g/100g) whereas moisture content of samples collected from Wayanad varied between 15.33 (g/100g) to 15.44 (g/100g) with a mean moisture content of 15.38 (g/100g).
- Water activity of the hive stored pollen samples collected from Palakkad ranged between 0.737 (a_w) and 0.739 (a_w)with a mean water activity of (0.73 a_w)whereas water activity of samples collected from Wayanad varied between (0.742 a_w) and (0.743 a_w) with a mean water activity of 0.74 (a_w).
- pH of hive stored pollen samples collected from Palakkad varied between 3.81 to 3.84 with a mean pH of 3.83 whereas pH of samples collected from Wayanad varied between 3.74 to 3.8 with a mean pH of 3.77.
- Ash content of the hive stored pollen samples collected from Palakkad fluctuated between 2.00 (g/100g) to 2.03 (g/100g) with a mean ash content of 2.01 (g/100g) whereas from Wayanad fluctuated between 1.87 (g/100g) and 1.88 (g/100g) with a mean ash content of 1.87 (g/100g).

- Total acidity of the hive stored pollen samples collected from Palakkad differed between 10.12 (mmol/g) and 10.17 (mmol/g) with a mean total acidity of 10.13 (mmol/g) whereas from Wayanad differed between 9.62 and 9.65 with a mean total acidity of 9.64 (mmol/g).
- Protein content of the hive stored pollen samples collected from Palakkad ranged between 3.45 (%) and 3.48 (%) with a mean protein content of 3.46(%) whereas from Wayanad ranged between 3.69 (%) and 3.7 (%) with a mean protein content of 3.69 (%).

6.3 Microbiological determination of hive stored pollen of *Apis cerana indica*

- Gram staining was performed for four bacterial isolates separately, out of which B1 (specify B1) as gram negative whereas remaining isolates B2, B3 and B4 were gram positive.
- B1 had rod shaped cells with slight yellowish large round colonies which were having irregular margin with a flat elevation.
- B2, B3 and B4 had rod shaped cells with creamy white small round colonies which were having entire smooth margin with a flat elevation.
- 16S rRNA sequencing of bacterial isolates revealed that B1 was *Pseudomonas aeruginosa*, B2 was *Bacillus megaterium*, B3 was *Bacillus aryabhattai* and B4 was *Bacillus megaterium.*
- Phylogenetic tree was constructed for each individual bacterial isolates in order to find diversity among different species of same genera.
- Slides were prepared for three fungal isolates T1, W1 and A1 separately and observations were recorded after observing under compound microscope.
- Isolate T1 had green colour large colonies which were having entire smooth margin with a flat elevation
- Isolate W1 had white colour small round colonies which were having entire smooth margin with a flat elevation
- Isolate A1 had orange colour large round colonies which were having entire smooth margin with a flat elevation

- ITS sequencing of fungal isolates revealed that fungal isolate T1 Was *Trichoderma reesei,* fungal isolate W1 was *Westerdykella multispora* and fungal isolate A1 was *Neurospora crassa.*
- Phylogenetic tree was constructed for individual fungal isolates with their close species to find the diversity among different species of the same genus fungi.
- Microbial diversity was more in Wayanad with two fungal and two bacterial colonies when compared to Palakkad with one fungal and two bacterial colonies.
- Microbial load was higher in Wayanad sample ($6.6x10^4$ cfu/g) when compared to Palakkad sample ($3.3x10^4$ cfu/g).
- Microbial load to pollen grain ratio was higher in Wayanad sample ($6.6x10^4$ cfu/g) when compared to Palakkad sample ($3.3x10^4$ cfu/g).
- Bacterial population of Palakkad ranges from 1.3-$3.3x10^4$ cfu/g whereas fungal population ranges from 0.6-$1.3x10^4$ cfu/g.
- Bacterial population of Wayanad ranges from 2.66-$4x10^4$ cfu/g whereas fungal population ranges from 3-$3.66x10^4$ cfu/g.
- Highest number of bacterial populations was observed at WYD-1 with mean value of 4 whereas lowest population of bacteria was observed at PKD-3 with mean value as 1.3.
- Highest number of fungal populations was observed at WYD-1 with mean value as 3.66 whereas lowest population of fungi was observed at PKD-1 with mean value as 0.6.
- From the present study, we can conclude that microbial diversity, microbial load and microbial population were more in Wayanad samples when compared with Palakkad samples.

REFERENCES

REFERENCES

Abramovic, H., Jamnik, M., Burkan, L. and Kac, M. 2008. Water activity and water content in Slovenian honeys. *Food Control.* 19(11): 1086-1090.

Abreu, M. 1992. Food use of pollen in relation to human nutrition. *Alimentaria.* 235: 45-46.

Abrol, D.P. and Bhat, A.A. 1985. Influence of atmospheric condition and floral rewards on diurnal activity pattern, flowers visitation rates and pollinating effectiveness of *Apis cerana indica* foragers. *Res. Dev. rep.* 4: 13-15.

Acquarone, C., Buera, P. and Elizalde, B. 2007. Pattern of pH and electrical conductivity upon honey dilution as a complementary tool for discriminating geographical origin of honeys. *Food chem.* 101(2): 695-703.

Agrawal, T.N. 2014. Beekeeping Industry in India: Future Potential. *Int. J. Res. Appl., Natural Soc. Sci.* 2(7): 133-140.

Ahmad, H. 2011. Role of Honey Bees in the Pollination of Guava (*Psidium Guajava* L.). M.Sc. (Ag.) in Apiculture Thesis. Department of Apiculture University of Agricultural Sciences. GKVK, Bangalore-560065, 2011.

Ahn, H In-Pyo Hong, Jeung-Im Bok, Byung-Yong Kim, Jaekyeong Song, and Hang-Yeon WeonPyrosequencing Analysis of the Bacterial Communities in the Guts of Honey Bees Apis cerana and Apis mellifera in Korea. The Journal of Microbiology (2012) Vol. 50, No. 5, pp. 735–745.

Alaux, C., Allier, F., Decourtye, A., Odoux, J.F., Tamic, T., Chabirand, M., Delestra, E., Decugis, F., Le Conte, Y. and Henry, M. 2017. A 'Landscape physiology' approach for assessing bee health highlights the benefits of floral landscape enrichment and semi-natural habitats. *Sci. Rep*. 7(1): 1-10.

Al-Khalifa, A.S. and Al-Arify, I.A. 1999. Physicochemical characteristics and pollen spectrum of some Saudi honeys. *Food chem.* 67(1): 21-25.

Almeida-Muradian, L.B., Pamplona, L.C., Coimbra, S. and Barth, O.M. 2005. Chemical composition and botanical evaluation of dried bee pollen pellets. *J. Food Compos. Anal.* 18: 105-111.

Anderson, K.E., Carroll, M.J., Sheehan, T., Mott, B.M., Maes, P. and Corby-Harris, V. 2014. Hive-stored pollen of honey Pollen diversity in semi-arid landscapes in Kenya 11 bees: Many lines of evidence are consistent with pollen preservation, not nutrient conversion. *Mol. Ecol*. 23(23): 5904-5917.

Anderson, K.E., Sheehan, T.H., Eckholm, B.J., Mott, B.M. and De Grandi Hoffman, G. 2011. An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*). *Insectes Sociaux*. 58: 431-444.

Andrade, P.B., Amaral, M.T., Isabel, P., Carvalho, J.C., Seabra, R.M. and da Cunha, A.P. 1999. Physicochemical attributes and pollen spectrum of Portuguese heather honeys. *Food Chem.* 66(4): 503-510.

Anjos, O., Iglesias, C., Peres, F., Garcia, A. and Javier, M. 2015. Neutral networks applied to discriminate botanical origin of honey. *Food Chem.* 175: 128-136.

Ankit, K., Kannanjia, Alok, Mahesh, and Prasad. 2014. Palynological investigation of honey from Khallagaon, Kanchanpur district, Westren Nepal. *Geophytol.* 44(1): 49-54.

Anklam, E. 1998. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* 63: 549-562.

Anonymous. 1983. Norma de calidad para la miel destinada al mercado interior. Spain. *Boletin Oficial del Estado.* 193: 22384-22386.

Anupama, D., Bhat, K. and Sapna, V. 2003. Sensory and physico-chemical properties of commercial samples of honey. *Food Res. Int.* 36(2): 183-191.

AOAC, Protein (Crude) in Animal Feed (AOAC, Gaithersburg, 2007)

AOAC. (1996). Official methods of analysis (16th ed.). Washington, DC: Association of Official Analytical Chemists. Methods: 969.38 B, 925.45.D, 920.181, 962.19 vol. II.

AOAC. 1990. In Official Methods of Analysis (15th ed.). K. Helrich (Ed.) Arlington, V.A. USA: Association of Official Analytical Chemists.

AOAC. 1995. Official methods of analysis (16th ed.). Association of Official Analytical Chemists. Washington, DC, USA.

Azeredo, L.D.C., Azeredo, M.A.A., De Souza, S.R. and Dutra, V.M.L. 2003. Protein content and physicochemical properties in honey samples of *Apis mellifera* of different floral origins. *Food Chem*. 80(2): 249-254.

Balasubramanyam, M.V. 2013. Vitamin Characteristics of Multifloral Honey of Indigenous Bee *Apis florea* and *Apis cerana indica* from Plains, Hills and Western Ghats of Karnataka. *J. Environ. Sci. Comput. Sci. Eng. Technol*. 2(3): 931-937.

Ballantyne, G., Baldock, K.C.R., Rendell, L. and Willmer. P.G. 2017. Pollinator importance networks illustrate and clinical values of bees in a highly speciose plant community. *Sci. Rep*. 7: 8389.

Bambara, S. B. 1991. Using pollen to identify honey. *Am. Bee J.* 131: 242-243.

Barajas, J., Misael, Cortes-Rodriguez and Eduardo Rodriguez-Sandoval. 2012. Effect of temperature on the drying process of bee pollen from two zones of Colombia. *J. Food Process Eng.* 35(1): 134-148.

Barajas, J., Misael, Cortes-Rodriguez, and Eduardo, Rodriguez-Sandoval. 2012. Effect of temperature on the drying process of bee pollen from two zones of Colombia. *J. Food Process Eng*. 35(1): 134-148.

Barbara, M.S., Machado, C.S., Sodre, G.D.S., Dias, L.G., Estevinho, L.M. and De Carvalho, C.A.L. 2015. Microbiological assessment, nutritional characterization and phenolic compounds of bee pollen from *Mellipona mandacaia* Smith, 1983. *Molecules*, 20(7): 12525-12544.

Barbattini, R.L. 1991. Ape insetto prodigioso, L. *Italia Agricola*. 128(1): 75-96.

Barth, O.M. 1990. Pollen in monofloral honeys from Brazil. *J. Apic. Res*. 29: 89-94.

Basualdo, M., Rodríguez, E.M., Bedascarrasbure, E. and De Jong, D. 2007. Selection and estimation of the heritability of sunflower (*Helianthus annuus*) pollen collection behaviour in *Apis mellifera* colonies. *Genet. Mol. Res*. 6(2): 374-381.

Bhargava, H.R., Jyothi, J.V.A., Bhushanam, M. and Surendra, N.S. 2009. Pollen analysis of *Apis* honey, Karnataka, India. *Apiacta*. 44(5): 14-19.

Block, G., Sinha, R. and Gridley, G. 1994. Collection of dietary supplement data and implication for analysis. *Am. J. Clinical Nutri*. 59:325-329.

Bogdanov, S. 1997. Nature and origin of the antibacterial substances in honey. *Lebensmittel Wissenchard Technol*. 30: 748-753.

Bogdanov, S. 2002. Harmonized methods of the International Honey Commission, Swiss Bee Research Centre, Switzerland.

Bolchi Serini, G. and Salvi, G. 1990. I mieli Lombardi: Risultati di analisi palinologiche. *Ape Nostra Amica*, 12: 6-9.

Borges, R.L.B., Lima, L.C.L., Oliveira, P.P., Silva, F.H.M., Novais, J.S. Dorea, M.C. and Santos, F.A.R. 2006. O polen no mel do Semi-Arido brasileiro. In: Santos, F.A.R. (ed.). *Apium plantae*. Recife, IMSEAR: 103-118.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding MARION. *Anal. Biochem.* 72(2): 248-254.

Braga, A.R., Gomes, D.G., Rogers, R., Hassler, E.E., Freitas, B.M. and Cazier, J.A., 2020. A method for mining combined data from in-hive sensors, weather and apiary inspections to forecast the health status of honey bee colonies. *Comput. Electr. Agric*. 169: 105161p.

Brodschneider, R. and Crailsheim, K. 2010. Nutrition and health in honey bees. *Apidologie*, 41(3): 278-294.

Buchmann, S.L. 1986. Vibratile pollination in Solanum and Lycopersicon: A look at pollen chemistry. in W. G. D'Arcy, editor. Solanaceae: biology and systematics. Columbia University Press, New York, USA. 237-252.

Carla, N., Antonio, I., Xesus, F. and Leticia M.E. 2012. Commercial bee pollen with different geographical origins: a comprehensive approach. *Int. J. Mol. Sci*. 13: 11173-11187.

Ceauşi, C., Iulian, Łogoe., Laurenniu, Tudor., Florin and Furnaris. 2009. The evaluation of microbiological quality of some bee honey sorts. *Vet. Med.* 66(1). 1843-5378.

Chandra, L. and Sharma, S.C. 2011. Melittopalynological investigation of honey bees from *Apis dorsata* Fabr and *Apis cerana indica* Fabr hives in Shahjahanpur district U.P. India. *Indian J. Applied Pure Bio*. 26(2): 339-346.

Chaudhary, R.K. 1978. Floral fidelity in the Indian honey bee (*Apis cerana indica* F.). *Indian Bee J*. 40: 239p.

Chauhan, M.S. and Bera, S.K. 1990. Pollen morphology of some important plants of tropical deciduous Sal (*Shorea robusta*) forests, district Sidhi, Madhya Pradesh. *Geophytology* 20(1): 30-36.

Chauhan, M.S. and Trivedi, A. 2011. Pollen analysis of honey from lucknow district, uttarpradesh. *J. Appl. Biosci*. 37(1):48-51.

Cherian, K.J. Bhowal, M. and Godghate. S.D. 2011. Pollen and physicochemical analysis of honey produced by *Apis cerana indica* of Nagpur, Maharashtra (India). *J. Envi. Res. Dev.* 5(3): 542p.

Chhuneja, P. K. 2009. Prospects and probserrlems of apiculture in India - A critical appraisal. Proceedings of Seminar-cum-Workshop on Bioresource Conservation and Utilization, CCS Universty of Meerut, India. 122-127.

Codex. Alimentarius Commission Standards, 1981/revised 1987/revised 2001. Codex Standards for honey. FAO, Rome. 1-7.

Conti, M.E. 2000. Lazio region (central Italy) honeys: a survey of mineral content and typical quality parameters. *Food Control* 11(6): 459-463.

Costa, L., Albuquerque, M., Trugo, L., Quinteiro, L., Barth, O., Ribeiro, M. and De Maria, C. 1999. Determination of non-volatile compounds of different botanical origin Brazilian honeys. *Food Chem*. 65: 347-352.

Crane, E. 1975. Honey. A comprehensive survey. Heinemann, London: International Bee Research Association. 608p.

Crane, E. 1990. Bees and beekeeping: science, practice and world resources. Ithaca, International Bee Research Association, New York.

Dalio, J.S. 2015. Foraging behaviour of *Apis mellifera* on *Trianthema portulacastrum*. *J. Entomol, Zool. Studies*. 3(2):105-108.

De Grandi-Hoffman, G., Eckholm, B.J. and Huang, M.H. 2013. A comparison of bee bread made by Africanized and European honey bees (*Apis mellifera*) and its effects on haemolymph protein titers. *Apidologie*. 44(1): 52-63.

Deepayan, P., Ramalakshmi, V., Dash, L. Sudhanshu, B.N. and Nihal, R. 2021. Pollen carrying capacity of *Apis cerana indica F*. in Gajapati District of Odisha. *Pharma Innov. J*. 10(6): 153-155.

Degrandi-Hoffman, G., Chen, Y., Huang, E. and Hua, M. 2010. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). *J. Insect Physiol*. 56(9): 1184-1191.

Deuri, A., Rahman, A., Gogoi, J., Borah, P. and Bathari, M. 2018. Pollinator diversity and effect of *Apis cerana* F. pollination on yield of mango (*Mangifera indica* L.). *J. Entomol. Zool. Stud*. 6(5): 957-961.

Dhaliwal, G.S. 1970. Observation in the pollen capacity carrying capacity of *Apis cerana* and *Apis mellifera* (Hym-Apidae). *Indian J. Entomol*. 32: 173-174.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L.P., Decourtye, A., Kretzschmar, A., Suchail, S., Brunet, J.L. and Alaux, C. 2013. Influence of pollen nutrition on honey bee health: Do pollen quality and diversity matter? *PLoS One* 8(8): 72016-13.

Diana Dominguez-Valhondo., Diego Bohoyo Gil., Maria Teresa, H. and David Gonzalez-Gomez. 2011. Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. *Int. J. Food Sci. Technol*. 46: 2204-2211.

Dorea, M.D.C., Novais, J.S.D. and Santos, F.D.A.R.D., 2010. Botanical profile of bee pollen from the southern coastal region of Bahia, Brazil. *Acta Botanica Brasilica*, 24: 862-867.

Downey, G., Hussey, K., Daniel Kelly, J., Walshe, T.F., and Martin, P.G. 2005. Preliminary contribution to the characterisation of artisanal honey produced on the island of Ireland by palynological and physico-chemical data. *Food Chem.* 91(2): 347-354.

Duman, A.B., Sezer, C., and Oral, N.B. 2008. Kars'ta Satısa Sunulan Süzme Balların Kalite Niteliklerinin Arastırılması. *Kafkas Universitesi Veteriner Fakültesi Dergisi*. 14(1): 89-94.

Eimanifar, A., Brooks, S.A., Bustamante, T. and Ellis, J.D. 2018. Population genomics and morphometric assignment of western honey bees (*Apis mellifera* L.) in the Republic of South Africa, BMC. *Genomics* 19: 615p.

El-kazafy abdou taha. 2015. A study on nectar and pollen sources for honeybee, *Apis mellifera* L. in Al-Ahsa Saudi Arabia. *J. Entomol. Zool. Studies*. 3(3): 272-277.

El-Sherbiny, G.A. and Rizk, S.S. 1979. Chemical of both clover and cotton honey produced in A.R.E. Egypt. *J. Food Sci.* 7: 69-75.

Elton, W., Herbert, Jr. H. and Shimanuki. 1978. Chemical composition and nutritive value of bee-collected and bee-stored pollen. *Apidologie*. 9(1): 33-40.

Erdtman, G. 1960. The Acetolysis method. Arevised description seven. *Bot. Tidskr*. 54: 561-564.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49: 329-337.

Estevinho, L.M., Rodrigues, S., Pereira, A.P. and Feas, X. 2012. Portuguese bee pollen: Palynological study, nutritional and microbiological evaluation. *Int. J. Food Sci. Technol.* 47: 429-435.

Esti, M., Panfili, G., Marconi, E. and Trivisonno, M.C. 1997. Valorization of the honeys from the Molise region through physicochemical, organoleptic and nutritional assessment. *Food Chem*. 58(1): 125-128.

Estupinon S, Sanjuan E. 1998. Quality parameters of honey II chemical composition. *Alimentaria.* 297: 117-122.

FAO [Food and Agriculture Organization]. 2017. Traditional Beekeeping in Uttarakhand Food and Agriculture Organization, Rome, Italy.

Feas, X., Vazquez-Tato, M.P., Estevinho, L., Seijas, J.A. and Iglesias, A., 2012. Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Mol.* 17(7): 8359-8377.

Feller-Demalsy, M.J. and Parent, J. 1989. Analyse polinique des miels de l' Ontario. Canada. *Apidologie*. 20: 127-138.

Felsociova Singh, N. and Bath, P.K. 1997. Quality evaluation of different types of Indian honey. *Food Chem*. 58(2): 129-133.

Felsociova, S., Kacaniova, M., Hleba, L., Petrova, J., Pavelkova, A., Dzugan, M. and Dorota Grabek-Lejko, G.D. 2012. Microscopic fungi isolated from polish honey. *J. Microbiol. Biotechnol. Food Sci.* 2(3): 1040-1049.

Finola, M.S., Lasagno, M.C. and Marioli, J.M. 2007. Microbiological and chemical characterization of honeys from central Argentina. *Food Chem*. 100: 1649-1653.

Foote, H.L. 1957. Possible use of microorganisms in synthetic bee bread production. *Am. Bee J*. 97: 476-478.

Gairola, A., Tiwari, P. and Tiwari, J.K. 2013. Physico-chemical properties of *Apis cerana-indica* F. honey from Uttarkashi district of Uttarakhand, *J. Global Biosci*. 2(1): 20-25.

Gallai, N., Salles, J.M., Settele, J. and Vaissiere, B.E. 2008. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol. Econ.* 68: 810-821.

Garg A., 1996. Palynocontent of bee-collected pollen loads of autumn season in Bhimal, India. *Taiwania*, 41 (3): 197-207.

Garg, A. 1996. Palynocontent of bee-collected pollen loads of autumn season in Bhimal, India. *Taiwania*, 41(3): 197-207.

Garg, A. and Nair, P.K. K. 1994. Seasonal analysis of honey bee pollen loads from a temperate zone in Kumaon Himalaya in Uttar Pradesh, India. *J. Palynol.* 30 (1-2): 1-34.

Gaur, R.D. and Nanwani, P.A. 1989. Melittopalynological analysis of apiary honeys from Pauri Garhwal, Uttar Pradesh, India. *Indian Bee J*. 51: 12-14.

Gilliam M., Prest D.B. and Lorenz B.J. Microbiology of pollen and bee bread: taxonomy and enzymology of molds. *Apidologie* 20: 53-68: 1989p.

Gilliam, M., Buchmann, S.L. and Lorenz, B.J. 1984. Microbial flora of the larval provisions of the solitary bees, *Centris pallida* and *Anthophora* sp. *Apidologie (Celle)* 15:1-10.

Gilliam, M., Buchmann, S.L., Lorenz, B.J. and Roubik, D.W. 1985. Microbiology of the larval provisions of the stingless bee, *Trigona hypogea*, an obligate necrophage. *Biotropica* 17: 28-31.

Gilliam, M., Buchmann, S.L., Lorenz, B.J. and Schmalzel R.J. 1990b. Bacteria belonging to the genus *Bacillus* associated with three species of solitary bees. *Apidologie (Celle)* 21: 99-105.

Gilliam, M., Roubik, D.W. and Lorenz, B.J. 1990a. Microorganism associated with pollen, honey and brood provisions in the nest of stingless bee, *Melipona fasciata*. *Apidologie (Celle)* 21: 89-97.

Gleiter, R.A., Horn, H. and Isengard, H.D. 2006. Influence of type and state of crystallization on the water activity of honey. *Food Chem.* 96, 441-445.

Goerzen D.W. 1991. Microflora associated with the alfalfa leaf cuting bee, *Egachile rotundata* (Fab) (Hymenoptera: Megachilidae) in Saskatchewan, Canada. *Apidologie.* 22: 553-561.

GOI [Government of India]. 2013. Status report on bee keeping and honey processing. Government of India, India.

Gomes, S., Dias, L., Moreira, L., Rodrigues, P. and Estevinho, L.M. 2010. Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. *Food and Chemi. Toxicol.* 48, 544-548.

Gonnet, M. 1986. Lanalyse des miels. Description de quelques methodes de controle de la qualite. *Bulletin Technique Apicole*. 13(1): 17-36.

Gonzalez, A.M., Gomez, A., Cordon, C., Garcıa-Villanova, R.F. and Sanchez, J. 2006. HPLC-fluorimetric method for analysis of amino acids in products of the hive (honey and bee-pollen). *Food Chem*. 95: 148-156.

Gonzalez-Gomez, D., Lozano, M., Fernandez-Leon, M. F., Bernalte, M.J., Ayuso, M.C. and Rodriguez, A. B. 2010. Sweet cherry phytochemicals: identification and characterization by HPLC DAD/ESI-MS in six sweet-cherry cultivars grown in 'Valle del Jerte' (Spain). *J. Food Composition Anal*. 23: 533-539.

Goodfellow, M. and Williams, S.T. 1983. Ecology of actinomycetes. *Ann. Rev. Microbiol.* 37:189-216

Hariprasad, Y., Nayagam, R.V. and Prabhu. 2006. Melittopalynological studies on Indian honey bee *Apis cerana indica* Fab. In: Proceedings of the International Workshop on Integrated Beekeeping Development in South Asian Countries, New Delhi, India, 71p. [13-14 Nov, 2006].

Haydak, M.H. 1958. Pollen-pollen substitutes-beebread. *Am. Bee J*. 98 (4): 145-146.

Hemalatha, D., Jayaraj, J., Murugan, M., Balamohan, T.N., Senthil, N., Chinniah, C. and Suresh, K. 2018. Foraging performance of Indian honey bee *Apis cerana indica* (F.) during winter in Madurai district of Tamil Nadu. *India. J. Entomol. Zool. Studies*. 6(3): 224-227.

Hemalatha, D., Jayaraj, J., Murugan, M., Balamohan, T.N., Senthil, N., Chinniah, C. and Suresh. K. 2018. Floral diversity for foraging of the Asiatic honey bee, *Apis cerana indica* (Hymenoptera: Apidae) in Madurai District of Tamil Nadu, India. *Int. J. Curr. Microbiol. App. Sci*. 7(10): 3452-3456.

Herbert, E.W. and Shimanuki, H. 1978. Chemical composition and nutritive value of bee-collected and bee-stored pollen. *Apidologie*. 9: 33-40.

Hervatin, H.L. 2009. Avaliac¸ao microbiologica e fisico-quimica do polen apicola in natura e desidratado sob diferentes temperaturas. 99p.

Hodges, D. 1955. Pollen loads, the way the worker bee gathers, packs and stores them for use. *Am. Bee J*. 95: 475-482.

Hosamani, V., Reddy, M.S., Venkateshalu, Hanumanthaswamy, B.C., Lingamurthi, K.R., Ashoka, N. and Ravikumar, B. 2019. Studies on foraging activity, pollen quantification and natural enemies of Indian honey bee, *Apis cerana* on mixed vegetation. *J. Ento mol. Zool. Studies*. 7(6): 162-167.

Hroncova, H., Killer, J., Hak, J., Titera, D. and Havlik, J. 2019. In-hive variation of the gut microbial composition of honey bee larvae and pupae from the same oviposition time, BMC. *Microbiol.* 19: 110p.

I'Anson Price, R. and Gruter, C. 2015. Why, when and where did honey bee dance communication evolve?. *Frontiers in Ecol. Evol*. 3: 125.

Iglesias, M.T., de Lorenzo, C., del Carmen Polo, M., Martin-Alvarez, P. J. and Pueyo, E. 2004. Usefulness of amino acid composition to discriminate between honeydew and floral honeys. Application to honeys from a small geographic area. *J. Agric. Food Chem.* 52: 84-89.

Instituto Portugues da Qualidade. 2002. Microbiologia alimentar-regras gerais para a contagem de microrganismos a 30°C. Caparica, Portugal. 42(41): NP-3788.

International Commission for Bee Botany (ICBB, 1970). Methods of melissopalynology. *Bee World* 51: 125-138.

International Standards Organization. 2008. Microbiology of food and animal feeding stuffs-horizontal method for the enumeration of yeasts and moulds-Part 2: International Standards Organization: Winterthur, Switzerland. 42(2): 21527p.

Jadhav, L.D. 1981. Role of insects in the pollination of onion (*Allium cepa* L.) in the Maharashtra state. *Indian Bee J*. 43: 61-63.

Jamwal, R. and Mattu, V.K. 2020. Pollen analysis of summer honeys from Kullu hills, Himachal Pradesh. *India. J. Entomol. Res*. 44(4): 581-588.

Jamwal, R. and Mattu, V.K. 2020. Pollen analysis of summer honeys from Kullu hills, Himachal Pradesh. *India. J. Entomol. Res*. 44(4): 581-588.

Jasim, A., Prabhu, S.T., Raghavan, G.S.V. and Ngadi, M. 2007. Physico-chemical, rheological, calorimetric and dielectric behavior of selected Indian honeys. *J. Food Eng*. 79(4): 1207-1213.

Jaya, F., Rosyidi, D., Radiati, L.E., Minarti, S., Susilo, A., Muslimah, R.H. and Husolli, M., 2020, April. Antioxidant activity and microbiological quality of bee bread collected from three different species honey bee. In *IOP Conf. Ser.: Earth Environ. Sci*. 145(1): 012033p.

Jhansi, P., Kalpana, T.P. and Ramanujam, C.G.K. 1991. Pollen analysis of rock bee summer honeys from the Prakasam district of the Andhra Pradesh. *India. J. Apiculture Res*. 30(1): 33-40.

Jivan, A. 2013. The impact of pesticides on honey bees and hence on humans. *Sci. Pap. Anim. Sci. Biotechnol*. 46(2): 272-277.

Jones, G.D. 2012. Pollen analyses for pollination research, unacetolyzed pollen. *J. Pollination Ecol*. 9: 96-107.

Joshi, M.A., Lakshmi, K., and Suryanarayana, M.C. 1998. Melittopolynogical investigations on *Apis* and *Trigona* honeys collected in and around Pune, Maharashtra. *Indian Bee J.* 60(2): 90-98.

Julika, W.N., Ajit, A. Sulaiman, A.Z. and Naila, A. 2019. Physicochemical and microbiological analysis of stingless bees honey collected from local market in Malaysia. *Indonesian J. Chem*. 19(2): 522-530.

Jyothi. 2006. Physico-chemical properties of honey from Bangalore region, Karnataka. *Indian Bee J.* 64(1-4): 18-22.

Kacaniova, M., Kacaniova, M., Knazovicka, V., Felsociova, S. and Rovna, K. 2012. Microscopic fungi recovered from honey and their toxinogenity, *J. Environ. Sci. Health*. Part A: Toxic/Hazardous Substances and Environmental Engineering, 47(11):1659-1664.

Kacaniova, M., Knazovicka, V., Felsociova, S. and Rovna, K. 2012. Microscopic fungi recovered from honey and their toxinogenety. *J. Environ. Sci. Health.* 47(11): 1659-1664.

Kahraman, T., Buyukunal, S.K., Vural, A. and Altunatmaz, S.S. 2010. Physico-chemical properties in honey from different regions of Turkey. *Food Chem.* 123(1): 41-44.

Kalpana, T.P. and Ramanujan, C.G.K. 1997. Melissopalynology bee plants and beekeeping potential in some coastal districts of Andhra Pradesh, India. *Indian Bee J*. 59: 1-8.

Kalpana, T.P., Fatima, K. and Ramanujam, C.G.K. 1990. Pollen analysis of *Apis cerana* and *Apis florea* honeys from Adikmet area, Hyderabad. *Proc. Indian Acad. Sci. Plant Sci*. 100(3):183-193.

Keller, I., Fluri, P. and Imdorf, A. 2005. Pollen nutrition and colony development in honey bees: Part I. *Bee World*. 86(1): 3-10.

Khan, K.A., Al-Ghamdi, A.A., Ghramh, H.A., Ansari, M.J., Ali, H., Alamri, S.A., Al-Kahtani, S.N., Adgaba, N., Qasim, M. and Hafeez, M., 2020. Structural diversity and functional variability of gut microbial communities associated with honey bees. *Microbial pathogenesis*, *138*, 103793p.

Kirk E.A., Mark, J., Carroll., Tim Sheehan., Brendon, M., Mott., Patrick Maes., Vane, S. Sa Corby. and Harr, I.S. 2014. Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. *Mol. Ecol*. 23: 5904-5917.

Kirtikar, K.R. and Basu, B.D., 1993. Indian Medicinal Plants. *Periodical Experts*. Delhi. 1-4.

Kis, M., Furmeg, S., Jaki Tkalec, V., Zadravec, M., Denzic Lugomer, M., Koncurat, A., Benic, M. and Pavlicek, D., 2018. Characterisation of Croatian honey by physicochemical and microbiological parameters with mold identification. *J. Food Safety* 38(5): 12492p.

Koetz, A.H. 2013. Ecology, behaviour and control of *Apis cerana* with a focus on relevance to the Australian incursion. *Insects.* 4(4): 558-592.

Kolhe, S., Shakh, M.A. and Khobragade, B. 2011. Melittopalynological study on rock bee honeys from Ahmednagar. *India. J. Palynology* 47: 89-96.

Krauze, A. and Zalewski, R.I. 1991. Classification of honey by principal component analysis on the basis of chemical and physical parameters. *Zeitschrift fuer Lebensmittel-Untersuchung und-Forschung.* 192: 19-23.

Krell, R. 1996. Value-added products from beekeeping. Rome: Food & Agriculture Org.

Krishna, A.G.S. and Rajashekhar, K. 2019. Foraging preferences of honey bees *Apis cerana* in Dakshina Kannada, Karnataka, India. 11(6): 13756-13764.

Krishna, S.A.G. and Patil, R.K. 2019. Foraging preferences of honey bees *Apis cerana* in Dakshina Kannada, Karnataka, India. *J. Threatened Taxa* 11(6): 13756-13764.

Krishna, S.A.G. and Patil.R.K. 2019. Foraging preferences of honey bees *Apis cerana* in Dakshina Kannada, Karnataka, India. *J. Threatened Taxa* 11(6): 13756-13764.

Kumar, D. and Sharma, V. 2016. Evaluation of *Acacia* species as honeybee forage potential. *Int. J. Sci. Res*. 5(1): 1726-1727.

Kuster, E. and Williams, S.T. 1964. Selection of media for isolation of *Streptomycetes*. *Nature* 202: 928-929.

Kwakman, P.H.S., te Velde, A.A. and de Boer, L. 2010. How honey kills bacteria. *Fed. Am. Soc. Exp. Biol.* 24: 2576-2582.

Lakshimi, K. 2012. Melissopalynology-A training manual prepared for a special training in Melissopalynology organized by Central Bee Research and Training Institute, Pune, 23pp.

Lakshmi, K. and Mohana Rao, G. 1998. Plants for bees Guava. *Bee World* 79 (3): 135-137.

Leonhardt, S.D. and Kaltenpoth. M. 2014. Microbial communities of three sympatric Australian stingless bee species. *PLoS One* 9(8): 105718p.

Lieux, M.H. 1980. Acetolysis applied to microscopical honey analysis. *Grana* 19(1): 57-61.

Lopez, B., Latorre, M.J., Fernandez, M.I., Garcia, M.A., Garcia, S. and Herrero, C. 1996. Chemometric classification of honeys according to their type based on quality control data. *Food Chem*. 55(3): 281-287.

Louveaux, J., Maurizio, A. and Vorwohol, G. 1978. Methods of melissopalynology. *Bee world* 59: 139-157.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., and et al. 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.

Lurlina, M.O. and Fritz, R. 2005. Characterization of microorganisms in Argentina honeys from different sources. Int. J. Food Microbiol. 105: 297-304.

Mai, C., Chainarong Sinpoo Geoffrey R. Williams, and Panuwan Chantawannakul. 2017. Dynamics of Fungal Communities in Corbicular Pollen and Bee Bread J. Sci. 2017; 44(4): 1244-1256.

Makkar, G.S., Chhuneja, P.K. and Gill, M.S., 2010. Beekeeping: The future growth engine for Indian farmers. *Bee world*, 87(3), 47-49.

Mamatha, K., Devender, R., Kailash, J. G., Niranja, S. and Ramakrishna, H. 2018. Comparative pollen analysis of *Apis cerana fabricius* and *A. mellifera* L. honey samples from Guntur, Sothern India. *Mellifera* 18(1): 1-14.

Manjunath, M.N.J. 1999. Carbohydrates of Indian honey. Ph.D. Thesis, University of Mysore, Mysore, 199p.

Maria G.R., Campos, S.B., Ligia, B., de Almeida-Muradian., Teresa, S., Yanina, M., Christian, F. and Francisco, F. 2008. Pollen composition and standardisation of analytical methods. *J. Apicultural Res*. 47(2): 154-161.

Martins, M.C., Morgano, M.A., Vicente, E., Baggio, S.R. and Rodriguez, A. 2011. Physicochemical composition of bee pollen from eleven Brazilian states. *J. Apicultural Sci*. 55(2).107-116.

Mateo, R. and Bosch, R.F. 1998. Classification of Spanish unifloral honeys by discriminant analysis of electrical conductivity, color, water content, sugars and pH. *J. Agric. Food Chem*. 46(2): 393-400.

Mattu, N., Verma, L.R., Mattu, V.K. and Verma, A.S. 1997. Palynological analysis of some autumn honeys from Himachal Pradesh. Paper presented at the National Conference on Apiculture and First Karnataka Honey Festival, Bangalore, 6-8 January, 1997.

Mayda, N., Özkök, A., Bayram, N.E., Gerçek, Y.C. and Sorkun, K., 2020. Bee bread and bee pollen of different plant sources: Determination of phenolic content, antioxidant activity, fatty acid and element profiles. *J. Food Measurement and Characterization*, *14*(4): 1795-1809.

Mendes, E., Proença, E.B., Ferreira, I.M.P.L.V.O. and Ferreira, M.A., 1998. Quality evaluation of Portuguese honey. *Carbohyd. Polym*. 37(3): 219-223.

Menezes, C., Vollet-Neto, A., Contrera, F.A.F.L., Venturieri, G.C. and Imperatriz Fonseca, V.L. 2013. The role of useful microorganisms to stingless bees and stingless beekeeping. In: Vit, P., Pedro, S.R.M. and Roubik, D.W. (eds.) Pot-Honey, *Springer* 153-171.

Michener, C.D. 1974. *The social behavior of the bees: a comparative study*. Harvard University Press. 395p.

Miroslava, K., Simona, P., Hapcik, G., Kociubinski., Vladimira, K.M., Sudzina., Janka, S. and Martina, F. 2009. Microbial communities in bees, pollen and honey from Slovakia. *Acta Microbiol. Immunol. Hung*. 56(3): 285-295.

Miroslava, K., Vladimíra, K., Sona, F. and Katarina, R. 2012. Microscopic fungi recovered from honey and their toxinogenity, *J. Environ. Sci. Health*. 47(11): 1659-1664.

Mishra, R.C., Kumar, J. and Gupta, J.K. 1998. The effect of mode of pollination on yield and oil potential of *Brassica campestris* L. Var. *sarson* with observations on insect pollinators. *J. Apicult. Res.* 27(2): 186-189.

Montovan, K. J., Karst, N., Jones, L.E. and Seeley, T.D. 2013. Local behavioral rules sustain the cell allocation pattern in the combs of honey bee colonies (*Apis mellifera*). *J. Theor. Biol.* 336: 75-86.

Muttoo, R.N. 1956. Facts about Beekeeping in India. *Bee World* 37(7): 125-133.

Nagamitsu, T. and Inoue, T. 1999. Differences in pollen sources of *Apis cerana* and *Apis mellifera* at a primary beech forest in central Japan, *J. Apiculture. Res.* 38(1-2): 71-78.

Nagamitsu. T. and Nagamasu. H. 1994. Keys for the pollen of Ashiu. Central Japan. Contribution from the Biological Laboratory. *Kyoto Univ.* 28: 261-355.

Nair, M.C. 2003. Apiculture resource biodiversity and management in southern Kerala. 186p.

Nair, P. K. K. 1960. A modification in the method of pollen preparation. *J. Sci. Industri.,* 19(C): 54-53.

Nair, P.K. and Singh, K.N. 1974. A study of two honey plants, *Antegonan leptopus* Hook and *Martinga pterigosperma Gaetrn*. *Indian J. Hortic*. 31: 375-379.

Nair, P.K.K. 1960. Amodification in the method of pollen preparation. *J. Sci. Ind.* 19(C): 54-53.

Nakajima, Y., Kitpreechavanich, V., Suzuki, K. and Kudo, T. 1999. *Microbispora coralline sp. nov.,* a new species of the genus *Microbispora* isolated from Thai soil. *Int. J. Syst. Bacteriol.* 49: 1761-1767.

Nakamura. J. 1980a. Diagnostic characters of pollen grains of Japan. II. Special publications from the Osaka Museum of Natural History. 12: 1-157.

Nakamura. J. 1980b. Diagnostic characters of pollen grains of Japan, I. Special publications from the Osaka Museum of Natural History. 13: 1-91.

Nanda, V., Sarkar, B. C., Sharma, H. K. and Bawa, A. S. 2003. Physico-chemical properties and estimation of mineral content in honey produced from different plants in Northern India. *J. Food Composition Anal.* 16(5): 613-619.

Nasser, L.A. 2004. Auces isolation and characterization of fungi contaminating packaged honey commonly consumed in Saudi Arabia. *Ass. Univ. Bull. Environ. Res*. 7(1). 1-7.

Natarajan, R. and Yesuvadian, M.S. 1978. Project report for honey concentration equipment. Indian Honey. 1: 15-21.

Naveen C Joshi, and Joshi, P.C. 2010. Foraging behaviour of *Apis* species on apple flowers in a Subtropical Environment. *New York Sci. J.* 3(3):71-76.

Naveen, C. and Joshi, P.C. 2010. Foraging behaviour of *Apis* species on apple flowers in a subtropical environment. New York. *Sci. J.* 3(3): 71-76.

Nayar, T.S. 1990. Pollen Flora of Maharashtra State, India. Today's & Tommorrow's Publishers & Printers, New Delhi.

Nogueira. C., Antonio, I., Xesus, F. and Leticia M.E. 2012. Commercial bee pollen with different geographical origins: a comprehensive approach. *Int. J. Mol. Sci*. 13: 11173-11187.

Nordin, A.N.Q.A., Sainik, S.V., Chowdhury, A.R., Saim, B. and Idrus, R.B.H. 2018. Physicochemical properties of stingless bee honey from around the globe: A comprehensive review. *J. Food Composition Anal.* 73: 91-102.

Oliveira, K., Moriya, M. and Azedo, R. 2009. Relationship between botanical origin and antioxidants vitamins of bee-collected pollen. *Quimica Nova*. 5: 1099-1102.

O'rourke, M.K. and Buchmann, S.L. 1991. Standardized analytical techniques for bee-collected pollen. *Environ. Entomol.* 20: 507-513.

Ouchemoukh, S., Louaileche, H. and Schweitzer, P. 2007. Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food control* 18(1): 52-58.

Ozcan, M., Arslan, D. and Ceylan, D.A. 2006. Effect of inverted saccharose on some properties of honey. *Food Chem.* 99: 24-29.

Pamela, Ochungo., Ruan, Veldtman., Rahab, Kinyanjui., Elfatih, M. Abdel-Rahman., Eliud, Muli., Michael, N.K. Muturi, H., Michael G. Lattorff. and Tobias, Landmann. 2021. Pollen diversity and protein content in differentially degraded semi-arid landscapes in Kenya. *J. Apicultural Res*. 60(5): 828-841.

Patil., Preeti, B. Yu, Z., Tami, C., Preston, H., Iain, M. and Shawn. C. 2010. Isolation and characterization of a *Nocardiopsis* sp. from honeybee guts. *FEMS Microbiol. Lett.* 312: 110-118.

Paz-Gonzalez, V. and Salazar-Olivo, L. A. 2005. Toxicol. *In vitro*. 19: 645-651.

Priti. 1998. Abundance and pollination efficiency of insect visitors of onion bloom. *Indian Bee J*. 60: 75-78.

Promnuan, Y., Kudo, T. and Chantawannakul, P. 2009. Actinomycetes isolated from beehives in Thailand. *World J. Microbiol. Biotechnol*. *25*(9), 1685-1689.

Promnuan, Y., Kudo, T. Ohkuma, M. and Chantawannakul, P. 2013. *Streptomyces chiangmaiensis sp. nov.* and *Streptomyces lannensis sp. nov.,* isolated from the South East Asian stingless bee (*Tetragonilla collina*). *Int. J. Syst. Evolutionary Microbiol*. 63(5): 1896-1901.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196.

Rabie, A.L., Wells, J.D. and Dent, L.K. 1983. The nitrogen content of pollen protein. *J. Apicultural Res*. 22(2): 119-123.

Ramalho, M., Guibu, L. S., Giannini, T. C., Kleinert, A. and Imperatriz, V. L. 1991, Characterization of some Southern Brazilian honey and bee plants through pollen analysis. *J. Apic. Res.* 30(2): 81-86.

Ramanujam, C.G.K., Fatima, K. and Kalpana, T.P. 1993. Nectar and pollen sources for dammer bee (*Trigona iridipennis* Smith) in Hyderabad (India). *Indian Bee J.* 55(1/2): 25-28.

Ransome, H. M. 1937. The sacred bee in ancient times and folklore. George Allen and Unwin: London, UK, 308pp.

Reddy, P.V.R., Rashmi, T., Verghese, A., and Info, P. 2015. Foraging activity of Indian honey bee, *Apis cerana* in relation to ambient climate variables under tropical conditions. *J. Environ. Biol.* 36: 577-581.

Rosa, C.A., Lachance, M.A., Silva, J.O.C., Teixeira, A.C.P., Marini, M.M., Antonini, Y. and Martins, R.P. 2003. "Yeast communities associated with stingless bees." *FEMS Yeast Res.* 4 (3):271-275.

Roulston, T.H., Cane, J.H. and Buchmann, S.L. 2000. What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny?. *Ecological Monogra*. 70(4): 617-643.

Rozanska, H., 2011. Microbiological quality of polish honey. *Bull. Vet. Inst. Puławy* 3(55). 24-100.

Ruttner, F., Tassencourt, L. and Louveaux, J. 1978. Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L. Material and methods. *Apidologie*. 9(4): 363-381.

Saa-Otero, M.P., Díaz-Losada, E. and Fernandez-Gomez, E. 2000. Analysis of fatty acids, proteins and ethereal extract in honeybee pollen-Considerations of their floral origin, *Grana* 39(4): 175-181.

Sadapotto, A., 2019. Study of bee forage based on bee bread from 4 species of honeybees in Teaching Forest Hasanuddin University. In *IOP Conf. Ser.: Mater. Sci. Eng.* 593(1): 012011p.

Sagona, S., Bozzicolonna, R., Nuvoloni, R., Cilia, G., Torracca, B. and Felicioli, A. 2017. Water activity of fresh bee pollen and mixtures of bee pollen-honey of different botanical origin. *Lwt*. 84: 595-600.

Said, F., Inayatullah, M., Ahmad, S., Iqbal, T. and Shah, R.A. 2015. Foraging behavior of the Himalayan Honeybee, *Apis cerana* (*Hymenoptera: Apidae*) associated with sunflower (*Helianthus annuus* L.) at Peshawar District of Khyber Pakhtunkhwa (KP). *J. Entomol. Zool. Stud*. 3(3): 203-207.

Samson, R.A. and Pitt, J.I. 1989. Modern concepts in *Penicillium* and *Aspergillus* classification. Ed. by Robert, A.S. and John, I. Pitt. *NATO ASI Ser. Ser. A*. 185. Plenum Press, New York, USA.

Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C. and Andersen, B. 2010. Food and indoor fungi. Utrecht: CBS-KNAW Fungal Biodiversity Centre, 390p.

Sarmento, T.M., Amorim, C. and da Silva, A.C. 2006. Chemical composition and free radical scavenging activity of pollen loads from stingless bee *Melipona subnitida* Ducke. *J. Food Composition Anal*. 19: 507-511.

Seeley, T.D. and Smith, M.L. 2015. Crowding honeybee colonies in apiaries can increase their vulnerability to the deadly ectoparasite *Varroa destructor*. *Apidologie* 46(6): 716-727.

Seethalakshmi, T.S. 1980. Melittopalynological investigations diverse on some Indian honeys. *Proc. 2nd Int. Conf. Apic. Trop. Clim.* New Delhi. 57(1): 119-132.

Sekhar. 2000. Melissopalynological studies for *Apis cerana indica* Fab. in bangalore region. M.Sc. Ag. Thesis. University of Agricultural sciences. GKVK, Bangalore, 143p.

Serra, B.J. and Jorda, E. 1997. Nutrient composition and microbial quality of honeybee collected pollen in Spain. *J. Agric. Food Chem*. 45: 725-732.

Serra-Bonvehi, J., Gonell-Galindo, J. and GoA mez-Pajuelo, A. 1986. Estudio de la composicioA n y caractero Asticas foAsico-quoAmicas del polen de abeja. *Alimentaria* 23(176): 63p.

Shamsudin, S., Selamat, J., Sanny, M., Razak, S.B.A., Jambari, N.N., Mian, Z. and Khatib, A. 2019. Influence of origins and bee species on physicochemical, antioxidant properties and botanical discrimination of stingless bee honey. *Int. J. Food Prop.* 22 (1): 239-264.

Sharma, H.K. and Nair, P.K.K. 1965. Pollen analysis of some honey from Uttar Pradesh. *Indian J. Hortic*. 22: 46-51.

Sharma, M. 1970. An analysis of pollen loads of honey bees from Kangra, India. *Grana* 10(1): 35-42.

Sharma, M. and Nair, P.K.K. 1965. Pollen analysis of some honeys from Uttar Pradesh. *Indian J. Hortic.* 22(1): 46-51.

Sharma, N. 1989. Melissopalynology and survey of foraging efficiency competition for pollen. Ph.D. Thesis. *Himachal Pradesh Univ.* Shimla, 185p.

Shubharani, R., Roopa, P. and Sivaram, V. 2013. Pollen morphology of selected bee forage plants. *Global J. Bio-Sci. Biotechnol.* 2(1): 82-90.

Sinacori, M., Francesca, A.N., Alfonzo, M., Cruciata, C., Sannino, L. Settanni. and G. Moschetti. 2013. Cultivable microorganisms associated with honeys of different geographical and botanical origin. *Food Microbiol*. 38: 284-294.

Singh, P., Verma, L.R., and Mattu, V.K. 1994. Pollen spectrum of some honey of the North East Himalayas as a determinant of honey bee forage. *Indian Bee J*. 56: 37-52.

Singh, T.S.M.S. and Suryanarayana, M.C. 2002. Melittopalynological investigations on the honeys of Tamil Nadu. *J. Palynol.* 37(2): 41-49.

Singh, Y.P. 1997. Studies on pollen gathering activity of Indian honey bees (*Apis cerana indica* Fab) under Saharanpur conditions. *Progressive Horti.* 12(4): 31-38.

Singh. P., Verma, L.R. and Mattu, V.K., 1994. Pollen spectrum of some honey of the North East Himalayas as a determinant of honey bee forage. *Indian Bee J.* 56: 37-52.

Sivaram, V. 2001. Honeybee flora and beekeeping in Karnataka State, India. In *Proc. 37th Int. Apicultural cong. Apimondia, Durban, South Africa*. October, 2001: 28.

Sivaram, V., Roopa, P., Shubharani, R., and Suwannapong. G. 2012. Pollen Analysis in Honeys Collected from Karnataka Region of Nilgiri Biosphere, South India. *J. Apicult.* 27(3): 223-231.

Smith, F. G. 1953. Beekeeping in the Tropics. *Bee World* 34(12): 233-245.

Smith, M.R., Singh, G.M., Mozafarian, D. and Myers, S.S. 2015. Efects of decreases of animal pollinators on human nutriton and global health: a modeling analysis. *The Lancet*. 386(10007): 1964-1972.

Snowdon, J.A. and Cliver, D.O. 1996. Microorganisms in honey. *Int. J. Food Microbiol.* 31(1-3): 1-26.

Sodre, G.D.S., Marchini, L.C., De Carvalho, C.A. and Moreti, A.C., 2007. Pollen analysis in honey samples from the two main producing regions in the Brazilian northeast. *Anais da Academia Brasileira de Ciencias*. 79(3): 381-388.

Sona, F., Miroslava, K., Lukas, H., Jana, P., Adriana, P., Molgorzata, D. and Dorota. 2012. Microscopic fungi isolated from polish honey. *J. Microbiol. Biotechnol. Food Sci*. 2(3): 1040-1049.

Subir, Bera, Mukhopadhyay, Samir Kumar., Joydeep Dhar, Das. and De, S. 1997. A melittopalynological investigation of *Apis cerana indica* F. summer honeys from Sikkim and Sub-Himalayan, West Bengal, India. *J. Palynology* 33: 209-218.

Sunita Saklani. and Mattu, V.K. 2018. Pollen analysis of honey samples from Hamirpur District, Himachal Pradesh, India. *Int. J. Plant Reproductive Biol.* 10(2): 145-150.

Suryanarayan, M.C. 1986. Honeybee – flower relationship. *Bull. of Botanical Survey of India,* 28(1- 4): 55-62.

Suryanarayan, M.C. 1986. Honeybee-flower relationship. *Bull. Bot. Surv. India* 28(1- 4): 55-62.

Suryanarayana, M.C., Rao, M.G. and Singh, T.S.M.S. 1991. Studies on pollen sources for *Apis cerana* Fabr and *Apis mellifera* L. bees at Muzaffarpur, Bihar, India. *Apidologie*. 23: 33-46.

Suryanarayana, M.C., Seethalakshmi, T.S. and Phadke, R.P. 1981. Pollen analysis of Indian honeys-1 Honeys from Litchi (*Nephelium Litchi*) and Jamun (*Syzygium cumini*). *IV Palynology Conf*. Lucknow. 491-498.

Tej, M.K., Aruna, R., Mishra, G. and nivasan, M.R. 2017. Beekeeping in India. In *Ind. Entomol*. Springer Singapore. 35-66.

Terrab, A., Di ez, M.J. and Heredia, F.J. 2002. Characterisation of Moroccan unifloral honeys by their physicochemical characteristics. *Food Chem*. 79: 373-379.

Terrab, A., Diez, M.J. and Heredia, F.J. 2003. Palynological, physico-chemical and colour characterization of Moroccan honeys: I. River red gum (*Eucalyptus camaldulensis* Dehnh) honey. *Int. J. Food sci. Technol.* 38(4): 379-386.

Thakur, R.K. 2014. Workshop on promotion of honeybee keeping in Haryana. *Res. Dev. beekeeping India*. June 24th, 2014.

Theisen-Jones, H. and Bienefeld, K. 2016. The Asian honey bee (*Apis cerana*) is significantly in decline. *Bee World*. 93(4): 90-97.

Thomas, D., Naresh Pal, K., and Subba Rao. 2001. Bee management and productivity of indian honey bees. *Proc. 37th Int. Apic. Cong.* 28th Oct-1st Nov 2001, Durban, South Africa.

Thomas, D., Pal, N. and Rao, K.S. 2002. Bee management and productivity of Indian honeybees. *Apiacta* 3: 1-15.

Tiwari, J.K., Gairola, A., Tiwari, P. and Ballabha, R. 2012. Pollen analysis of some honey samples from Kamad area of McGregor district Uttarakashi, in Garhwal Himalaya, India. *Asian J. Exp. Biol. Sci.* 3(4): 778-784.

Tiwari, J.K.A., Gairola, P., Tiwari. and Ballabha, R. 2012. Pollen Analysis of Some Honey Samples from Kamad Area of District Uttarakashi in Garhwal Himalaya, India. *Asian J. Exp. Biol. Sci.* 3(4): 778-784.

Tiwari, P., Tiwari, J.K., Dinesh Singh. and Dhanbir, Singh. 2013. Traditional beekeeping with the Indian honey bee (*Apis cerana* F.) in District Chamoli, Uttarakhand, India. *Int. J. Rural Stud*. 20(1): 1-6.

Todd, F.E. and Bretherick, O. 1942. The composition of pollens. *J. Econ. Entomol.* 35: 312-317.

Toumanoff, C. 1933. Documentation sur l'apiculture annamite. *Bull. econ. Indoch*. Jan-Apr.

Toumanoff, C. and Nanta, J., 1933. Enquete sur apiculture au Tonkin. *Bull. econ. Indochin*, 1015-1048.

Townsend, G.F. 1970. Preparation of honey for market. *Dept. of Agric.* 544p.

Vásquez, A. and Olofsson, T.C. 2009. The lactic acid bacteria involved in the production of bee pollen and bee bread. *J. Apicultural Res*. 48(3): 189-195.

Veer, S. and Jitender, N. 2017.Economics and Importance of Beekeeping. *Biomed. J. Sci. Tech. Res.* 1(7): 20p.

Velasquez Giraldo, A.M., Velez, Acosta., L.M. and Zuluaga, G.R. 2013. Physicochemical and microbiological characterization of *Apis mellifera* sp. honey from southwest of Antioquia in Columbia. *Ingenieria y Ciencia* 9(18): 61-74.

Velthius, H.H.W. 1992. Pollen digestion and the evolution of sociality in bees. *Bee world* 73(2): 77-89

Vijaya, A., Reddy. B. and Reddy, P.R. 2008. Occurrence of Medicinal Plant Pollen in *Apis cerana* Honeys of Khammam District, Andhra Pradesh, India. *Ethnobotanical Leaflets,* 12: 452-460.

Vijayakumar, K.T., Neethu, T., Bhat, N.S., Nayimabanu, T. and Varsharani, H. 2020. Physico-chemical property of different floral honeys of Bangalore region, Karnataka. *J. Entomol. Zool. Stud.* 8(5): 846-854.

Vikas, Nandaa., Sarkara, B.C., Sharmaa, H.K. and Bawab, A.S. 2003. Physico-chemical properties and estimation of mineral content in honey produced from different plants in Northern India. *J. Food Composition Anal.* 16: 613-619.

Von Der Ohe, W., Oddo, L.P., Piana, M.L., Morlot, M. and Martin, P. 2004. Harmonized methods of melissopalynology. *Apidologie* 35(1): 18-25.

Von der Ohe, W., Persano Oddo, L., Piana, M.L. Morlot, M. and Martin, P. 2004. Harmonized methods of melissopalynology. *Apidologie* 35(1): 18-25.

White, J.W. 1967. Honey, its composition and properties. U.S. *Dep. Agric*., 335: 147pp.

White, J.W., Riethof, M.L., Subers, M.H. and Icushner.1962. Composition of American honeys. *Tech. Bull. U.S. Dep. Agric*., 1261: 124 pp.

White, J.W.Jr. 1975. La miel. In Dadant e hijos, La colmena y la abeja melifera. *Montevideo: Hemisferio Sur.* 625-646.

White, J.W.Jr. 1978. Honey: *Adv. Food Res*. 24: 287-373.

Willmer, P.G., Cunnold, H. and Ballantyne, G. 2017. Insights from measuring pollen deposition: quantifying the pre-eminence of bees as flower visitors and effective pollinators. *Arthropod-Plant Interact*. 11(3): 411-425.

Winston, M.L. 1987. The Biology of the Honey Bee. Harvard University Press. Cambridge, MA, USA.144p.

Yaowanoot. P., Takuji, K., Moriya, O. and Panuwan, C. 2011. *Actinomadura apis* sp. *nov.*, isolated from a honey bee *(Apis mellifera*) hive, and the reclassification of *Actinomadura cremea subsp. rifamycini*. *Int. J. Systemat. Evol. Microbiol*. 61: 2271-2277.

Yilmaz, H. and Kufrevioglu, I. 2000. Composition of honeys collected from eastern and south-eastern Anatolia and effect of storage on hydroxymethyl furfural content and diastase activity. *Turkish J. Agric. For.* 25: 347-349.

MICROBIAL DIVERSITY IN HIVE-STORED POLLEN OF INDIAN HONEY BEE, *Apis cerana indica* (Fabricius)

by

ABHISHEK V.
(2019-11-009)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2021

ABSTRACT

Honey bees are eusocial flying insects. Among all insects that pollinate, honeybees are considered to be one of the crucial pollinators. Honey bees represent only a small fraction of the roughly 20,000 known species of bees. Honey bees obtain all of their nutritional requirements from a diverse combination of pollen and nectar. Pollen grains referred to as 'bee bread' or 'bee meat' are the natural sources of proteins, vitamins, fats, lipids and minerals, of which proteins are very much essential for building the body tissues of the bees, especially during the early embryonic growth. Worker bees of *Apis cerana indica* help in the fermentation of pollen which releases additional nutrients that are used in the production of antibiotics and fatty acids which inhibit spoilage.

Pollen analysis of samples provides the information regarding the plants preferred by bees for nectar, as the pollen grains dispersed are mostly collected by bees along with nectar. Honey has two sources of contamination, primary one being the hive stored pollen. Thus, it is very essential to study the microbial diversity of pollen, which may lead to spoilage of pollen and nectar.

The present investigation on “Microbial diversity in hive-stored pollen of Indian honey bee, *Apis cerana indica* (Fabricius)” was conducted at Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Kerala Agricultural University during 2020-2021 in order to study the diversity of hive-stored pollen and associated microbes in *A. cerana indica* (Fabricius) colonies.

Purposive surveys were conducted in six locations at Palakkad and Wayanad district *viz*., Mannarkkad, Nellipuzha, Nottaamala, Kenichira, Kayakunnu and Cheengode. Hive stored pollen collected were maintained in refrigerated conditions after assigning unique accession numbers as PKD-1, PKD-2, PKD-3, WYD-1, WYD-2, WYD-3. Microbial diversity, microbial load of hive stored pollen, identification and characterization of pollen with respect to its nature, size, shape, aperture, exine pattern along with physicochemical properties *viz*., moisture content, water activity, pH, ash content, total acidity and protein content were studied.

Palynological identification plays a crucial role in beekeeping industry. Pollen aids as a food supplement to honeybees for their growth and development. It assists in the identification of geographical and botanical origin of pollen. A total of 21 plants were recorded as pollen sources by bees from the study area (six locations) of two districts. Ten pollen types were identified based on its nature, size, shape, aperture, exine pattern using PalDAT software. Identified plant species were *Sphagneticola trilobata* (Asteraceae), *Caesalpinia pulcherrima* (Fabaceae), *Zinnia elegans* (Asteraceae), *Ocimum sanctum* (Lamiaceae), *Clitoria ternatea* (Fabaceae), *Biophytum sensitivum* (Oxalidaceae), *Bauhinia acuminata* (Fabaceae), *Mimosa pudica* (Fabaceae), *Tecoma stans* (Bignoniaceae), *Portulaca grandiflora* (Portulacaceae). Characterization of pollen revealed that ten plant species belonging to seven families serve as pollen sources in respective survey locations.

Physicochemical properties of hive stored pollen of *A. cerana indica* collected from six locations were investigated by estimating the moisture content, water activity, pH values, ash content, total acidity and protein content. Moisture content varied from 15.29-15.38 (g/100g). Water activity varied from 0.73 to 0.74 (a_w) and the pH varied between 3.77 and 3.83. Ash content varied from 1.87-2.01 (g/100g). Total acidity varied from 9.64-10.13 (mmol/g). Protein content varied from 3.46-3.69 (%). Parameters like moisture content, water activity, protein content were more in the Wayanad sample, it may be because of high humidity and temperature variations. Parameters like pH, total acidity and ash content were more in Palakkad sample, which infers that mineral content might be more.

Microbial diversity of hive stored pollen of *Apis cerana indica* collected from six locations was investigated by identifying the microbes through their morphological, cultural, molecular characterization. Other parameters like microbial diversity, microbial load, microbial load to pollen grain ratio, microbial population were also observed. In total, four bacteria and three fungal species were identified. However, colonies of actinomycetes and yeast were not obtained. Out of four bacteria, B2, B3, B4 – gram positive while B1- gram negative. The shape of all bacterial isolates was rod with change in their colony colour.16s rRNA sequencing of bacterial isolates revealed that B1 as *Pseudomonas aeruginosa*, B2 as Bacillus *megaterium*, B3 as *Bacillus aryabhattai* and B4 as *Bacillus megaterium* respectively. Out of the three fungi, colony

colour of T1 colony colour was green, W1 colony colour was white and A1 colony colour was orange. All isolates have flat elevation with an entire margin. ITS sequencing of fungal isolates revealed that T1 as *Trichoderma reesei*, W1 as *Westerdykella multispora* and A1 as *Neurospora crassa*. Microbial diversity was more in sample collected Wayanad with two fungal and two bacterial colonies whereas, one fungal and two bacterial colonies in sample collected from Palakkad. Microbial load was more in samples from Wayanad ($6.6x10^4$ cfu/g) when compared with Palakkad sample ($3.3x10^4$ cfu/g). Comparison of microbial load to pollen grain ratio revealed that Wayanad sample recorded highest with $6.6x10^4$ cfu/g to Palakkad sample with $3.3x10^4$ cfu/g. Bacterial and fungal populations were observed more in Wayanad sample when compared to Palakkad sample.

The study recorded the pollen sources of different localities in and around the Palakkad and Wayanad districts of Kerala. Moisture content plays a significant role which might be the reason for high microbial diversity and microbial load in Wayanad. The present study demands the investigations of other physicochemical properties like EC, starch content, amino acid composition, microbial spores present in pollen which could be useful for the food preservation industry or potentially hazardous in due course of time.