ETIOLOGY OF HONEY BEE BROOD DISEASE IN SOUTHERN KERALA

by

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DECLARATION

I, hereby declare that this thesis entitled **"ETIOLOGY OF HONEY BEE BROOD DISEASE IN SOUTHERN KERALA"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

%	Per cent
^0C	Degree celsius
CD	Critical difference
cm^2	Centimetre square
CRD	Completely Randomized Design
E	East
et al.	And other co workers
Fig.	Figure
g	Gram
H_2S	Hydrogen sulphide
hr	Hour
i.e.	That is
kg	Kilogram
L	Litre
mg	Milligram
mg mL^{-1}	Milligram per millilitre
min.	Minutes
min^{-1}	Per minute
mL	Millilitre
mL	Millilitre
MSL	Mean Sea Level
N	North
No.	Number
NS	Non-significant
ppm	Parts per million
sec	Second
sp.	Species
viz.	Namely
µg	Microgram
µgmL^{-1}	Microgram per millilitre

Introduction

1. INTRODUCTION

Honey bees and the fruits of its toil are familiar to man since the pre-historic times. Honey bees are social insects with remarkable degree of social instincts and division of labour. Beekeeping is an ideal, absorbing, instructive and economically profitable hobby that is practised worldwide. The scientific practise of beekeeping is known as Apiculture and it provides additional income for small and marginal farmers, landless labourers and other weaker sections of the society.

There are five species of honey bees indigenous to India: *Apis dorsata* Fabricius (Rock bee); *A. cerana indica* Fabricius (Indian bee); *A. florea* Fabricius (Little bee); *A. andreniformis* Smith (Black dwarf bee); *A. laboriosa* Smith (Himalayan bee); while *A. mellifera* Linnaeus (Italian bee) is an introduced species (Abrol, 1997).

In India, farmers are practising beekeeping with two important species of honey bees *viz. A. cerana indica* and *A. mellifera.* Honey bees are known for their products such as honey, bee wax, bee pollen, bee venom and propolis. Beekeeping in India is mainly practised by farmers in rural area and traditionally performed for honey harvesting. In 2018, the total honey production of the country was about 4000 metric tonnes (Statista, 2018).

Beekeeping is an age old practise in Kerala. According to Khadi and Village Industries Commission (KVIC), Kerala is considered as the most potential state for beekeeping where it produces largest quantity of honey in the country. Beekeeping in Kerala is mostly dependent on rubber and coconut for foraging. Apart from honey production, honey bees play an important role in crop production through pollination service. Pollination aided by *A. cerana indica* resulted an yield increase of 25 per cent in cucumber and also enhanced the quality as well as yield parameters of the crop (Premila *et al.,* 2014).

Though honey bees provide the bee keepers with diverse benefits, they are affected by a number of problems which include pollution due to the pesticides,

mobile tower radiation; climate change and problems due to pests and diseases. The diseases infecting honey bees are considered as the major constraint faced by the bee keepers worldwide (Perez-Sato *et al*, 2009).

Honey bee diseases are mainly caused by bacteria, virus, protozoa, mites and fungi, which lead to loss of health and vigour and thereby weaken the colonies. Honey bee diseases are generally classified into: brood diseases and adult diseases. Brood diseases are caused by bacteria (American Foul Brood and European Foul Brood); virus (Thai sac brood disease) and fungi (chalk brood and stone brood diseases). Adult diseases are mainly caused by protozoa (nosema disease and amoeba disease) (Abrol, 1997).

In order to control the bacterial diseases, the beekeepers are using various antibiotics which includes tetracycline, chloramphenicol, sulphonamides and glycosamides. These antibiotics are known to cause residue problem in honey, thus leading to quality reduction in the international market. Test reports conducted by Agricultural Processed Food Product Export Development Agency (APEDA) and Export Inspection Council (EIC) from 2005 onwards showed high levels of antibiotics and heavy metals in honey exported from India to European Union and United States.

During 1991-92, the beekeeping in the state was struck by the deadly viral disease, Thai sac brood disease which resulted in a loss of about 90 per cent of the then existed bee colonies. Amritha *et al.* (2014) reported that 45 per cent of the Indian bee colonies were infected with the brood disease, the symptoms observed were scattered egg laying, uncapped cells, loss of appetite in bee colonies and colour change in larvae. The adult bees were found more aggressive. The occurrence of the brood disease was severe during the brood rearing season.

Being a newly emerged problem faced by the bee keepers of Kerala, the disease incidence as well as the etiology is less explored. Due to the antibiotic residue problem in honey and other hive products, it's high time to move to the botanical means of management of the honey bee diseases.

In this context, the present study focuses on the following objectives:

- Survey on the diseased Indian bee colonies in the apiaries of Southern Kerala.
- To characterize, identify and confirm the infectivity of the microbial isolate from diseased Indian bee brood.
- To test the efficacy of botanicals in the diseased Indian bee colonies.

Review of Literature

2. REVIEW OF LITERATURE

Honey bees are social insects that are benefitted to man in many ways. They not only provide honey and other bee products but also, provide an important ecosystem service, pollination of crop plants. A healthy honey bee population is necessary for the agricultural sector as well as for the wild flora. Honey bees play a crucial role in the maintenance of biodiversity of nature (Le Conte and Navajas, 2008; Arbia and Babbay, 2011).

Klein *et al.* (2007) stated that diminishing honey bee populations would result in a homogenous diet for mankind. It reduces the agricultural production and hence become a threat to the world food security (Van-Engelsdorp and Meixner, 2010). Apiculture or beekeeping is the art and science of collecting or procuring honey bee colonies of desired species, hiving them in specified and standard boxes, installing at appropriate sites, managing optimum number of colonies scientifically round the year and harnessing both direct and indirect benefits of the activities (Thakur, 2014).

2.1 SCOPE OF BEE KEEPING

Kerala is having a tropical climate and is rich in its diversity of flora for successful beekeeping. Kerala is considered as the most potential state for bee keeping by KVIC. The state has been contributing 70 per cent of total honey production in India (Jacob *et al.* 1992). The state supports a green cover of wild and cultivated forest trees, conventional food crops, ornamental and medicinal plants, plantation crops such as coconut, tea, coffee, rubber, cardamom, etc. that has an immense potential for commercial bee keeping industry (Padmanabhan, 2003). Studies conducted by Nair (2005) reported Kerala as the largest producer of honey in the country. *Apis cerana indica* Fab., the Indian honey bee is the popular honey bee species used for commercial bee keeping in Kerala.

Apart from honey, bees provide the mankind with an important service, the pollination which is essential for crop production. Insect pollination has enhanced the seed quality parameters and thus improves the quality and market value of the crop produce. 75 per cent of the crop species benefit from insect pollinators that provide a global service worth $215 billion to food production (Bommarco *et al.,* 2012). Premila *et al.* (2014) reported that pollination by *A. cerana indica* resulted an yield increase of 25 per cent in cucumber and it also enhanced the quality parameters of the crop.

Scientific beekeeping was started in the state after the successful invention of movable frame hive by Rev. Newton in 1910. Detection of rubber (*Hevea brasiliensis* Muell. Arg.) as a rich source of extra floral nectar during seventies gave a big boost to the bee keeping industry in the state paving to the way for migratory bee keeping. The extra floral nectaries of rubber that is produced after the commencement of new flushes serves as rich source of honey during January to April (Padmanabhan, 2003). These nectaries present at the junction of the petiole produces the nectar, when the young leaves are half matured that is, they become light green in colour (Devanesan *et al.,* 2011).

There are three important seasons in beekeeping namely; brood rearing season, honey flow season and dearth season, mainly practised in our state. Brood rearing season is the active growth period of honey bees that is from October to December. There will be active egg laying by the queen bee and the bee population increases in this season. During honey flow season, foragers will be very active and thus have increased production of honey. This is during January to April as it coincides with the availability of extra floral nectaries of rubber. The availability of nectar and pollen will be scarce during dearth season, from May to September. Honey bee foraging will be less in this period (Aswini, 2013).

2.1.1 Honey Bee Diversity of India

Apiculture mainly deals with the insects belonging to the genus *Apis.* Among the honey bee species, only four species are common among the

beekeepers and only *A. cerana indica* and *A. mellifera* are widely used for commercial beekeeping in India. In India, all the bees can be seen foraging under natural environment (Thomas *et al.*, 2002).

2.1.1.1 Indian Honey bee (*Apis cerana indica* Fab.)

Indian honey bees are also known as Eastern honey bee or Asian hive bees. They are smaller than rock bee but larger than little bee. The bees are having a gentle temperament and thus can be easily domesticated. They build parallel combs in the cavities and hollows of trees, caves and other hidden sites. Hence, these bees can be domesticated in the movable frame hives (Ramachandra *et al.*, 2012). They have a strong tendency for swarming and absconding due to lack of flora. The absconding has also been observed during the dearth season as well as due to attack from diseases and pests. The annual yield of honey was recorded to be 3 to 5 kg per colony in plains. The Indian honey bees are domesticated in almost all parts of the country including the states of Himachal Pradesh, Jammu & Kashmir, Uttaranchal, Assam, Andhra Pradesh, Karnataka, Maharashtra, Tamil Nadu and Kerala (Yadav *et al.*, 2017).

2.1.1.2 Rock Bee (*A. dorsata* Fab.)

The rock bee is the largest of the honey bees. They are ferocious honey bees which builds single combs of huge size. Comb is fully exposed and hung from inaccessible branches of trees; along the sides of steep rocks in the forest and from the walls. They are migratory in nature. It is mainly distributed in the forests and plains of central India, from where the tribal people collect the honey. Also found in the Sunderban forests of West Bengal and Southern part of the India. Annual honey yield of rock bee is about 50-80 kg (Oldroyd and Wongsiri, 2006).

2.1.1.3 Little Bee (A. florea Fab.)

The Little bee is the smallest of the four species of genus *Apis.* It is seen only in plains where they build single combs on bushy plants and corners of roofs. It yields only very little honey that is about 0.5 to 1 kg per year (Abrol, 2010).

2.1.1.4 Italian Bee (A. mellifera L.*)*

The Western bee/ Italian bee, *A. mellifera* Linnaeus is an introduced species where the fertile queens of this species have been introduced to India in 1962 in Punjab and Himachal Pradesh by Prof. A.S. Atwal, with a view of improving the honey yield (Dhaliwal *et al.*, 2015). The bee is having all the good qualities for domestication that includes presence of prolific queen, less swarming tendency, gentle temperament thus easy to handle and foremost are good honey gatherers. They can protect their colonies against the attack from pests and diseases. Annual honey yield is 20-25 kg per colony (Yadav *et al.*, 2017).

2.2 PESTS AND DISEASES OF HONEY BEE

Beekeepers are facing the crisis of diminishing bee population due to pests and diseases along with other factors such as pesticide toxicity, mobile tower radiation, climate change, low price for the bee products, decreased resource diversity and loss and fragmentation of habitat, all over the world (Potts *et al.,* 2010).

About 10.50 per cent of beekeepers in Ethiopia are facing problem due to diseases and pests of honey bees along with other constraints such as lack of skilled man power, shortage of bee colony, marketing of bee products and absence of policy in apiculture (Ejigu *et al.,* 2009). Perez-Sato *et al.* (2009) revealed that the major constraint faced by the beekeepers all over the world is due to pests and diseases.

Honey bees are susceptible to a number of diseases and environmental problems. Numerous diseases have been reported on both brood and adult bees.

According to Genersch (2010), diseases and pests of honey bees play an important role in death of the honey bees as well as in the loss of the health and vigour of the colonies. Both biotic and abiotic factors of environment are contributing to the occurrence of the diseases of honey bees that may lead to premature colony mortality, affecting health and lifespan of the colony (Brar, 2016).

Honey bees are disposed to an array of natural enemies, from viral diseases to vertebrate predators, inspite of their highly evolved defensive strategies. According to Bansal *et al.* (2013), major challenges in the beekeeping in India are pests and diseases of honey bees, toxicity due to indiscriminate use of pesticides and also difficulty in the marketing of bees product.

A study conducted in Panchkula district of Haryana found that 86.70 per cent of the bee keepers in the district were facing pest and diseases as the major problem in bee keeping. It has caused total destruction of the colonies and replenishment of the colonies becomes difficult due to financial crisis. Thereby, caused a reduction in the number of colonies to a level where the activity became economically impractical (Monga and Manocha, 2011).

2.2.1 Pests of Honey Bees

Honey bees are attacked by a number of pests. The major pests include wax moths, mites, ants, wasps and other vertebrate pests like birds, bear, frogs and toads (Chantawannakul *et al.,* 2016). Among these, mites are causing major damage to honey bee population in a global level. *Varroa jacobsoni* Oudemans is the mite pest that is commonly found in association with Asian bees, *A. cerana* (De Jong *et al.,* 1982). Another important mite pest causing huge economic loss to the bee keepers of India is *Varroa destructor* Anderson. Chaudhary (2005) reported the loss due to *Varroa* in Haryana was in the tune of 3.9 to 29.3 per cent.

The yellow banded wasp, *Vespa cincta* Fabricius is described as one of the major predator of honey bees (Ramachandran, 1952). Studies conducted by Kolady (2017) recorded peak incidence of *Vespa tropica* Fabricius during

November (181 visits per month) in Kerala. The wasp attack may lead to absconding of the colonies in severe conditions.

Wax moth, *Galleria mellonella* Linnaeus infestation would results in total destruction of the colonies. The infestation mainly occurs in weaker colonies. The infestation was recorded very less during winter, summer and the autumn months (Jyothi, 2003) while, according to the findings of Varshneya *et al.* (2008), the infestation reaches its peak during the rainy season.

Hive beetle, *Aethina tumida* Murray is also a menace for bee keeping. It was reported as a parasite and scavenger of *A. mellifera scutellata* of Sub-Saharan Africa (Lundie, 1940). Bose (2017) has reported the incidence of hive beetle from the stingless bee hives of Kerala.

Apart from these, vertebrate pests also attack the honey bees. The bee eater birds namely, *Merops orientalis, Dicrurus adsimilis, Cypselus* sp., *Appus* sp., *Lanius* sp. *and Picus* sp., prey on honey bees (Mishra, 1998).

2.2.2 Diseases Infecting Honey Bees

Bees naturally suffer from a broad range of pathogens that include bacteria, fungi, protozoa and viruses. They are known to cause annual colony loss for beekeepers. Major diseases are of the brood diseases which includes Bacterial diseases (American foul brood (AFB) and European foul brood (EFB)), fungal diseases (chalk brood and stone brood disease) and viral disease (thai sac brood disease) and also the nosema disease caused by protozoa infecting the adult bees (Dzierzon, 1882; Arbia and Babbay, 2011).

Though many diseases were reported in honey bee brood, American Foul Brood (AFB) and European Foul Brood (EFB) are considered as global threat to honey bees (Smith *et al.*, 2014; Goulson *et al.*, 2015).

2.2.2.1 Fungal Diseases

The two important fungal diseases infecting the brood of honey bees include chalk brood and stone brood disease. The chalk brood disease of honey bee was found to be caused by *Ascosphaera apis* while the stone brood disease of honey bees reported to be caused by the fungus *Aspergillus flavus*. The disease has been reported as a rare disease (Jensen *et al.,* 2013).

The chalk brood disease has spread throughout the globe (Zaghloul *et al.,* 2005). The disease has known to affect the brood of the honey bees through the ingestion of the infective spores of the pathogen. It may not be causing total destruction of the colonies but would lead to a reduction in the bee number and thereby reduces the colony strength. In this way, the disease would result in an economic loss to the bee keepers across the globe (Aronstein *et al.,* 2007).

2.2.2.2 Viral Diseases

In honey bees, 18 viral diseases have been reported, in which six were found to cause serious damage to the apiaries across the globe. These include Thai sac brood virus (TSBV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), deformed wing virus (DWV) and Kashmir bee virus (KBV). Among these, the Thai sacbrood virus was reported to cause severe threat to the bee keeping in Asia and particularly India (Berenyi *et al.,* 2006).

The virus was first detected from Thailand in Asia hence it is named as Thai sac brood virus (Bailey *et al.,* 1982). As the name suggests, the disease is affecting the brood of honey bees. The disease is reported from all parts of Asia including India, Thailand, South Korea, Japan, Nepal, China and Vietnam. It has resulted in the destruction of about 90 per cent *A. cerana indica* colonies in Kashmir (Chantawannakul *et al*., 2016).

The disease has also caused a serious stroke for the bee keepers of Kerala, as it destroyed almost 90 per cent of the honey bee colonies during 1991-92. This lead to a harsh drop in honey production and thereby affected the bee keepers of the state horribly (Devanesan and Jacob, 2001).

2.2.2.3 Protozoan Disease

Protozoan or microsporidial disease was reported to be caused by two species, *Nosema apis* and *Nosema ceranae*. They generally affect the adults of honey bees and would not cause any damage to the brood. *N. ceranae* infect Indian honey bees while *Nosema apis* was known to infect the European honey bees. *N. apis* has been reported from the bees of Taiwan, Vietnam, China, Indonesia and Jordan (Chaimanee *et al.,* 2017). *N. ceranae* was reported from China, Vietnam, Indonesia, Solomon Islands, and Thailand (Botías *et al*., 2012).

2.2.2.4 Bacterial Diseases

Two economically important bacterial diseases of honey bees are American foul brood and European foul brood.

2.2.2.4.1 Occurrence and distribution

The bacterial diseases are observed world-wide throughout the continents. Earliest report of the bacterial disease was given by Aristotle (384-322 B.C.) in his Book IX of History of Animals. Schirach (1769) characterised the disease by the foul smell and coined the term 'Foul Brood'. In 1885, the cause of disease was ascribed to *Bacillus alvei* (Cheshire and Cheyne, 1885). Philips (1906) coined the terms American and European foul brood to distinguish the diseases and White (1907) differentiated the bacterial disease as two different diseases.

American Foul Brood is more prevalent in tropic and sub-tropic areas. It was reported for the first time by White in 1907. Later, it was reported to be serious disease from all parts of the world (Bailey and Ball, 1991).

The European foulbrood disease has long been reported from United Kingdom (Cheshire and Cheyne, 1885) and later from United States of America (White, 1912) in *A. mellifera*. It was then known to cause serious problems all over the world, resulted an economic loss to the bee keepers of North and South America, Europe, Japan, Australia, India and South Africa (Matheson, 1993).

The EFB disease was reported to affect about 4.30 to 7.20 per cent colonies in Colorado (Moffett, 1952), 7.00 per cent in Italy (Gaiavarini, 1956) and 26.00 per cent in Hungary (Buza and Kovacs, 1969) in *A. mellifera* colonies.

The AFB disease was reported from *A. mellifera* in Taiwan (Yen and Chyn, 1971). From the Asian subcontinent, the EFB disease was found in the colonies of *A. mellifera* from China, Vietnam, Japan and Thailand (Forsgren *et al.,* 2015). In China, during 1972 to 1976 the EFB disease has reported to cause serious damage to the bee keeping industry and it is becoming more serious in Vietnam and Japan recently (Chantawannakul *et al.,* 2016).

From India, there is only a single report of American foulbrood disease in *A. cerana indica* F. from Nainital, Uttar Pradesh (now in Uttarakhand) during 1960 (Singh, 1961). In India, European foul brood disease was first reported in *A. cerana indica* during 1970 (Diwan *et al.,* 1971) from Maharashtra and in *A. mellifera* during 1998 from Himachal Pradesh (Viraktamath, 1998).

The EFB affected 25-30 per cent of the *A. cerana indica* colonies during 1970 in Maharashtra (Diwan *et al.*, 1971) and also killed 25 - 40 per cent *A. mellifera* colonies in Karnataka (Viraktamath, 1998). The disease infected 60 per cent of the colonies in Himachal Pradesh during 2002 (Rana *et al.*, 2004) causing severe loss to the bee keepers of the state.

2.2.2.4.2 Symptomatology

Both the bacterial diseases are affecting the brood of honey bees. Brood combs from healthy colonies have a solid and compact brood pattern. Cappings

are uniform in colour and are convex, higher in centre than at margins. Larvae are glistening and pearly white. Healthy pupae are also white in colour (Abrol, 1997).

The symptoms of AFB can be observed during the initial stage of infection as isolated capped cells with no brood emergence or with dead larvae. The caps of the infected cells were darker than that of healthy cells. The cells were sunken and punctured. As the severity increases, the disease spreads throughout the colony and there occur scattered, irregular pattern of sealed and unsealed brood cells. The characteristic symptom of the disease was the presence of a protruding tongue, also called 'pupal tongue', a protrusion from the pupal mouthparts that traverses the cell and adheres to the roof, with rest of the body decayed. The colour of the larvae changed from pearly white to light brown then coffee brown and finally black. The resulting cadaver dried down to black-brown scale and sticks tightly to the cell wall. It exhibits a stinking odour, thus disease is also known as "Stinking disease" (Ritter and Akratanakul, 2006).

The bacteria causing EFB infects 4-5 day old larvae that are younger than those killed by AFB. Rana *et al.* (2004) recorded the death of brood at pre-pupal and pupal stages in *A. cerana indica*, which exhibits typical symptoms like those of Thai sac brood disease and mite infestations. The colour of the larvae changes from white to yellow and finally to brown. The cell capping of the dead brood were perforated, sunken and convex. The brood pattern becomes erratic and larvae were twisted with creamy-white guts visible through the body wall. The disease was noticed during dearth period. The larvae died while still in coiled state. Infected larvae were first soft and watery, afterwards became pasty, but not ropy. Dead and dried scales were tough, rubbery rather than brittle and can be easily removed. Sudden weakening of the colonies was observed as a general symptom (Abrol and Ball, 2006).

According to Amritha *et al.* (2014), 45 per cent of the bee colonies in Kerala were severely infected with the brood disease where the symptoms observed include scattered egg laying; uncapped cells; infected colonies exhibited

loss of appetite; and colour change in larvae which are similar to that of bacterial diseases. The adult bees become more aggressive. The disease is severe during the brood rearing season. Reduction in the bee population occurs, finally resulting a decline in the honey yield and huge economic loss to the bee keepers.

2.2.2.4.3 Spread of the disease

The spread of the bacterial disease can occur within a colony and between the colonies. Within the colony the spread mainly occurs by the action of nurse bees as the disease mostly affects the brood of the honey bees. Gilliam (1997) stated that the honey bee larvae obtain the microbes, which are found associated with the adult worker bees, pollen and combs by the ingestion of contaminated food.

Belloy *et al.* (2007) observed that workers act as the carriers of bacterial diseases, it carries the disease not only within the colony but also from one colony to another even without any characteristic symptoms. The bacteria are reported to be present in the workers bees of those colonies without the clinical symptoms.

Apart from the action of worker bees, other beekeeping practices such as exchange of hive materials, robbing and drifting. Studies show that poor beekeeping practise is one of the most important causes for the transmission of AFB disease between the colonies (Human *et al.*, 2011).

Also, young larvae reared in already infected cells become infected themselves. Allowing bees to rob honey from contaminated colonies or stored honey combs; and in a minor way by drifting bees also contribute to the spread of the disease (Matheson and Reid, 1992).

2.3 ETIOLOGY OF HONEY BEE BACTERIAL DISEASES

2.3.1 Epidemiology

In AFB, 4-5 day old larvae are more susceptible. They ingest the bacteria while feeding the food contaminated with the bacterial endospores. The spore

germinates in the larval midgut after 24 hr of ingestion. The bacterium multiplies and proliferates in larval midgut and destroys the peritrophic membrane. It penetrates to the haemolymph through the gut wall and causes the death of the insect by bacterial septicaemia (Yue *et al.,* 2008).

In case of EFB, as it is non-spore forming the bacteria itself has to be ingested by the young larvae less than 48 hr old. Similar to AFB, the bacteria enters the midgut and proliferates there. The death of the larvae is due to the competition for food by the bacteria. In EFB, the larvae starve to death (Bailey, 1956). The reinfection occurs through the deposition of the bacteria in the cell wall of the infected cell by faecal discharge from the infected larvae (Shimanuki, 1990).

Bacterial diseases of the honey bees are highly contagious. AFB bacteria have spores and hence it is very difficult to control the disease. The spores can survive in the extreme conditions like high and low temperature, UV radiation, desiccation and even the harsh chemicals. The spores can be carried by honey, pollen, bees' wax and worker bees (Ebeling *et al.,* 2016). AFB is more problematic than EFB and once occurred in an apiary, the best remedial method in initial stage is to destroy the infected colonies (Adjlane and Haddad, 2017).

EFB caused by *Melissococcus plutonius* is more related to the colony stress conditions as well as the environmental conditions. The study conducted by Ruossenova and Parvanov (2005) states that the EFB disease triggers when the bee larvae weakens its resistance. Also, climatic conditions, diseases of adult bees and pesticide toxicity can become predisposing factors for the occurrence of the disease.

2.3.2 Morphological Characters of Causal Organism

American foul brood disease is caused by *Paenibacillus larvae* as it was first described by White in 1907. The bacterium is gram positive, rod shaped and

flagellated. It produces endospores under unfavourable conditions (Antunez *et al.*, 2011).

P. larvae exist as either dormant spore or as vegetative cell depending on environmental conditions. These spores are resistant to high temperature, desiccation, UV irradiation, and harsh chemicals, and can remain viable for as long as 35 years (Forsgren *et al.*, 2008). The endospores were reported to be the infectious form of the bacteria and the only known host of these bacteria was found to be honey bees (Genersch, 2010).The morphology of the bacteria varies according to the media used for culturing.

Genersch *et al.* (2005) studied the morphology of *P. larvae* in different media and reported different morphology for the culture. On *Paenibacillus larvae* agar (PLA) media the colonies appeared small, pale green to yellow in colour with slightly opaque and rough texture. While, on MYPGP agar media, the colonies were small, whitish in colour with a rough texture and may be raised.

European foul brood is primarily caused by *M. plutonius.* The bacterium is gram positive, lancet shaped and non-spore forming. It was reported that the bacterium is pleomorphic in nature and attains rod shape in culture (Bailey, 1956). It requires carbon dioxide for its growth and development thus it is micro-aerophilic to anaerobic in nature. Young honey bee larvae are more susceptible to the disease.

According to the studies of Bailey and Ball (1991), *M. plutonius* would be deposited in the cell wall of weakly infected larvae and remains in the colonies for several years. The disease is reported to be severe under the colony stress conditions like, lack of food, water and space (Roetschi *et al.*, 2008).

The disease becomes more dangerous due to the infection by secondary bacteria such as *Achromobacter eurydice, Bacillus pumilus, Paenibacillus alvei, Bacillus laterosporus* and *Enterococcus faecalis* (Shimanuki, 1990). The

secondary invaders do not cause disease but affects the odour and consistency of the dead brood.

2.3.2.1 Morphology of the Associated Micro-Organisms

Bacteria that are found associated with the EFB disease were not the actual cause but they could help in the assessment of the disease (Shimanuki and Knox, 2000).

Bailey (1957a) reported *Achromobacter eurydice,* which was found to be associated with *Melissococcus pluton,* as a gram negative bacterium that was first identified and described by White. The bacterium has been found to cause a reduction in oxygen during the infection maintaining anaerobic condition that is favourable for the growth of *M. pluton.* When the diseased larvae were cultured, the colonies of *Bacterium eurydice* was the first to appear along with the colonies of *M. pluton* (Bailey, 1957b).

Paenibacillus alvei was found as a secondary bacterium often allied with the primary pathogen, *M. plutonius*. It is a rod shaped, aerobic and spore forming bacterium. It multiplies in the decomposing remains of the affected larvae. The bacteria produce a characteristic stale odour in the affected colonies. They were not able to affect the healthy larvae but could establish very easily in affected bee colonies. Bacterium produces spore that could ensure its survival in the bee colonies (Forsgren, 2010). Characteristic roppiness that helps in the diagnosis of the bacterial disease was due to *P. alvei*.

Erler *et al.* (2014) isolated *Bacillus pumilus* from the larvae infected with EFB disease. They identified the bacterium as a secondary pathogen along with *M. plutonis*. They also reported that the bacterium was able to grow on 25 per cent sunflower honey while the growth was partially inhibited by 25 per cent sugar solution.

Bacillus laterosporus is found occasionally associated with the EFB affected bee colonies. Buchanan and Gibbons (1974) first reported the association of this bacterium with the EFB affected colonies. A characteristic feature of the bacterium is the presence of canoe-shaped para-sporal body (Shimanuki and Knox, 2000).

Enterococcus faecalis morphologically resembles *M. plutonius* and is often confused. The bacteria have ovoid cells that are in pairs or in chains. The bacterium is a facultative anaerobe and is brought to the hives by the foraging honey bees (Tendolkar *et al.,* 2003). The sour smell associated with the disease was mainly due to this bacterium. *E. faecalis* could not multiply in the bee colonies in the absence of *M. plutonius* (Forsgren, 2010).

2.3.3 Pathogenicity of Isolated Bacteria

The pathogenicity of the bacteria was studied by artificially inoculating the healthy colony and observing for the symptom expression. The test for pathogenicity has been done in different ways. Krasikova and Naumova (1957) mixed the diseased larvae in the sugar syrup and fed this to three day old healthy larvae till the time of sealing. Another study conducted by Bailey (1957c) employed spraying the suspension of *M. pluton* on the healthy colonies. Studies were also done by inserting a piece of diseased comb into the healthy colonies of *A. mellifera* to study the pathogenicity (Tomasec and Fizan, 1963).

The pathogenicity study in the *A. mellifera* colonies of Himachal Pradesh was done by feeding the pure culture of the bacteria in 500 mL sugar syrup (Rao *et al.,* 2011). Rana *et al.* (2012) studied the pathogenicity in the healthy colonies of *A. cerana indica* by mixing the diseased brood suspension and pure culture of the isolates in the sugar syrup fed to the bees. As a control, the crude extract of healthy brood from *A. cerana indica* was fed to healthy colonies.

2.3.4 Molecular Characterisation of Bacteria

The molecular characterisation of the bacteria causing honey bee disease was first done by Govan *et al.* (1998). The molecular tool used was PCR assay based on 16S rRNA gene sequence. Lauro *et al.* (2003) had done direct analysis of honey as well as the hive samples by developing the protocol of nested PCR assay. Later, several efforts were made to enhance the specificity to understand the sub-species level of the bacterium using the PCR based on 16S rRNA gene.

Apart from PCR technique, other molecular tools were also used by different scientists for the characterisation of the bacterium. These include SDS-PAGE method that diagnoses the whole cell protein of the bacterium (Hornitzky and Wilson, 1989). Djordjevic *et al.* (1998) developed hemi-nested PCR assay for the detection of *M. plutonius.* DNA finger printing techniques like RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) were performed for taxonomic or epidemiological studies.

The molecular characterisation of *M. plutonius* infecting the *A. mellifera* colonies in Himachal Pradesh was done using the PCR assay. The study also employed biochemical, cultural and serological methods for the confirmation and identification of the pathogen causing the disease in the *A. mellifera* colonies of Himachal Pradesh (Rao *et al.,* 2011).

2.4 MANAGEMENT OF THE BACTERIAL DISEASES

As honey bees are social insects, the pathogens can be easily distributed within the colony. Honey bees have developed their own defence mechanism to manage the spread of the disease causing pathogens within the colony. The significant method used was the hygienic behaviour that includes the identification and removal of the diseased brood from the colony. Worker bees inspect each brood cell and find diseased larvae in the sealed brood cells. The identified sealed cells were opened to remove the diseased brood from the colony

and thereby prevent the spread of the disease within the colony. The scattered brood surface will help the beekeeper to recognise the disease incidence. Despite these defence mechanisms, the bees are frequently facing the problems of diseases, parasites and pests (Ritter and Akratanakul, 2006).

The bacterial diseases were difficult to manage and earlier the bee keepers used to burn the infected colonies in order to manage the spread of the disease within the apiary (Waite *et al.,* 2003). As it leads to the destruction of the whole colony, several other prophylactic methods were developed and practised by the bee keepers across the globe. Some of these methods include selection of good site for the apiary that is dry with shade. The healthy colonies should not be shifted to apiaries with infection. Re-queening of the infected colonies could be used as a management strategy in the early stage of infection. Do not leave honey or honey combs accessible to bees and avoid drifting or robbing between colonies as these would help in the spread of the pathogen between the colonies (Russenova and Paranov, 2005).

2.4.1 Use of Antibiotics for Disease Management

The most effective method followed by bee keepers as a curative measure was treatment using antibiotics. Among the antibiotics used, tetracycline became more popular as it was cheap and easily available in the market. Beekeepers started to use antibiotics for the control of the disease even though it was banned in several countries (Matheson and Reid, 1992). The only approved chemical for the management of bacterial diseases of honey bees in India was Oxytetracycline (Johnson *et al.,* 2010).

Oxytetracycline has been available in the trade name terramycin or TM25. It has bacteriostatic function with a broad range of activity. The chemical affects the 16S rRNA of the bacterium (Lodesani and Costa, 2005). These antibiotics were effective only in suppressing the clinical symptoms but were not able to eliminate the infective spores of the bacteria.

According to the study conducted by Bahman and Rana (2002), application of oxytetracycline at the rate of 200 mg per 300 mL of sugar syrup per colony could effectively manage the EFB disease after 8 days of application via feeding. A new chemical, Ciprofloxacin (98 % a.i.) was tested against the bacterial diseases of honey bees. It was tested in 3 different doses *viz.*, 10, 20 and 40 mg in 500 mL sugar syrup per colony. Result showed 40 mg of chemical as better compared to other two doses used. There was 100 per cent control of the disease in 9 days (Rao *et al.*, 2011).

2.4.1.1 Contaminants in Honey

Honey is having two types of contaminants; environmental contaminants which includes, heavy metals, pesticides and radioactive materials, and beekeeping contaminants that are, acaricides, antibiotics and other chemicals that are used by bee keepers for disease and pest control in the colonies (Al-Waili *et al.*, 2012).

Due to the continuous use of the antibiotics, there occurred residue problems of the chemicals in the honey as well as other bee products. About 20 μgL^{-1} of tetracycline residue was reported from the honey samples of China (Chen *et al.*, 2001). In France, Belgium and Switzerland about 20 to 50 per cent of the imported honey was reported to have the antibiotic residues of streptomycin and tetracycline (Martel *et al.*, 2006).

According to the tests done by Agricultural Processed Food Product Export Development Agency (APEDA) and Export Inspection Council (EIC) on honey exported from India to European Union and United States has reported high levels of antibiotic residues from 2005 onwards (Johnson *et al.*, 2010).

These antibiotics can persist in the beehive also. Solomon *et al.* (2006) reported the presence of about 11-29 μgkg^{-1} of streptomycin, 3-44 μgkg^{-1} of ampicillin and 26-48 μgkg^{-1} of kanamycin in the samples of nectar and honey

obtained from the beehives of southern parts of Tamil Nadu during the peak flowering seasons of rubber and banana.

Another important problem related to the use of antibiotics in honey is the resistance development in the strains of the bacteria causing the disease (Miyagi *et al.,* 2000). Resistance development may be due to the continuous use of the antibiotics for the control of diseases. This problem has been reported in many countries namely USA, Canada and Argentina (Alippi *et al.,* 2007).

No resistance is observed in *M. plutonius* by using the antibiotics so far, but studies observed the chances for transfer of resistance from the OTC resistant strains of *P. larvae* (Lodesani and Costa., 2005).

2.4.1.2 Health Hazards due to Antibiotics

Honey is a natural product. It is known as the 'food of God'. Honey is a viscous fluid that is rich in carbohydrate. It is included in the diet and is directly consumed by man. Honey can provide about 5500 calories per kg and thus is a rich source of heat and energy. Honey is used in ayurveda and sidha medicine systems followed in India. It is used as a carrier to enhance the properties of drugs. Honey is the best tonic for infants and athletes (Boukraa, 2015).

Antibiotics in honey can affect the health of the consumers. The antibiotic residue in honey can cause chronic and acute problems in human beings. Some antibiotics were responsible to cause toxic reactions in the consumers while others cause allergic and hypersensitivity reactions (Velicer *et al.*, 2004). They also reported that at very low dose, the antibiotic β-lactam causes dermatitis, gastro-intestinal problems, cutaneous eruptions and anaphyalaxis. Acute symptoms included carcinogenicity, microbiological hazards, reproductive problems and teratogenicity. Among these, microbiological hazards were reported to cause major problems.

Chronic exposure to oxytetracycline causes blood changes, liver injury and delayed blood coagulation. This antibiotic can damage teeth and bones. Other chronic effects include increased sensitivity to sun, wheezing and asthmatic attack (Johnson *et al.,* 2010).

2.4.2 Management of Diseases using Botanicals

Due to the problems of antibiotics usage, researchers and beekeepers across the globe has been searching for alternative management strategies for the control of bacterial diseases. Many were supporting botanical means of management and conducted studies using several botanicals. The antibacterial property of 11 essential oils were tested for the control of AFB in which the essential oils of cinnamon, thyme, clove and lemongrass has reported to be more effective against the bacteria (Russenova, 2011). Organic extracts of three different bryophyte plants were tested for effectiveness against the EFB disease. The study observed that acetone extract of *Marchantia polymorpha* L. (umbrella liverwort) and chloroform extract of *Dicranum undulatum* Brid. (dicranum moss) has given effective control of the disease comparable to the standard drug used (Gahtori *et al.,* 2011). Another study conducted by Chaimanee *et al.* (2017) reported that the plant extract of *Chromolaena odorata* showed high bioactivity against *P. larvae* among the 36 plants selected.

A study was conducted by Chand and Tiwari (2012) in Uttarakhand, about the efficacy of cow urine against the European Foul Brood disease in *A. mellifera*. Pure cow urine was sprayed on the naturally infected colonies. Terramycin was used as the check treatment. They observed that the disease was reduced to below detectable level in 10 to 12 days. The treatment with cow urine also enhanced the growth of the brood.

2.3.2.1 Garlic (*Allium sativum* L.)

Allicin, one of the active principles of freshly crushed garlic homogenates, has a variety of antimicrobial activities. Allicin in its pure form was

found to exhibit antibacterial activity against a wide range of Gram- negative and Gram-positive bacteria. The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes (Ankri and Mirleman, 1999).

The study conducted by Aronstein and Hayes (2004), reported that both bacterial and fungal diseases of honey bees could be managed by allicin that is obtained from garlic. Also, allicin does not cause any known harmful effects on bees and residue problems were not observed in the bee products. Garlic extract when incorporated with candy feed had stimulated the egg laying by the queen bee, caused reduction in the population of *Bacillus* sp. and increased the number of brood cells in the colonies of *A. mellifera carpatica* (Patruica *et al.,* 2017).

2.3.2.2 Kodangal (*Centella asiatica* L.)

It is well-known that many spices especially those belonging to the Lamiaceae family possess a wide range of biological and pharmacological activities. *C. asiatica* belonging to family Umbeliferae popularly known as "Kodangal" is a very useful medicinal plant having anti-bacterial property (Jagtap *et al.,* 2009). Dash *et al* (2011) reported anti-bacterial property of *Centella asiatica* against *Staphylococcus aureus* and *Bacillus subtilis*.

2.3.2.3 Tulsi (Ocimum sp.)

Ocimum sanctum is a small herb plant that exists all over the country. The plant belongs to family Labiatae in which the members were well-known for their therapeutic potential. *O. sanctum* is commonly known as tulsi plant and has been traditionally used for the treatment of several diseases including chronic fever, dysentery, hemorrhage and dyspepsia etc. The therapeutic potential of tulsi plant is due to the presence of the phenolic compound, Eugenol (Prakash and Gupta, 2004). *O. sanctum* has found to possess antibacterial property against most of the gram positive bacteria (Joshi *et al.,* 2009).

O. basilicum has shown both polyphenolic content and the antimicrobial activity at higher levels against the bacteria responsible for AFB disease, *P. larvae* (Marghitas *et al.,* 2011). The study conducted by Goswami and Khan (2013) in *A. mellifera* colonies of Uttarakhand has shown that treatment with tulsi oil showed 56.85 to 75.03 per cent mite (*Varroa destructor*) mortality after three weeks of treatment.

2.3.2.4 Turmeric (*Curcuma longa* L.)

Turmeric is a spice that is widely exploited for medicinal purposes in the Indian sub-continent. Curcumin is the bioactive principle that imparts the medicinal and anti-microbial property to turmeric (Chattopadhyay *et al.,* 2004). Studies report antibacterial property of turmeric against gram positive bacteria like *Staphylococcus, Bacillus* etc (Tajbakhsh *et al.,* 2008). Gokul and Geetha (2017) have reported the inhibitory action of curcumin against *Streptococcus mutans.*

Curcumin has reported to increase the lifespan of worker bees and also reduced the *Nosema* infecton in the colonies of *A. mellifera.* It has also shown increased key enzyme activity, metabolic compound concentration and decreased the percentage of global DNA methylation in the honey bees. Curcumin has improved the honey bee health and vitality (Strachecka *et al.,* 2015).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Etiology of honey bee brood disease in Southern Kerala" was conducted in AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani during 2016-18. The study was conducted with the objective to characterize, identify and confirm the infectivity of the microbial isolate from diseased Indian honey bee brood and also to test the efficacy of botanicals against it.

The research had three experimental parts *viz.,* a survey on the disease incidence in the Indian bee apiaries of Southern Kerala; isolation, identification and characterisation of the pathogen, and the field evaluation of botanicals for the management of the disease.

3.1 ESTIMATION OF DISEASE INTENSITY

3.1.1 Selection of Apiaries in Southern Kerala

The survey was conducted in three southern districts of Kerala *viz.* Pathanamthitta, Kollam and Thiruvananthapuram for a period of one year from May 2017 to May 2018. Five apiaries with a minimum of ten hives were selected randomly from each district for assessing the disease intensity (Plate 1).

3.1.2. Disease Incidence in the Apiaries of Southern Kerala

Monthly observations were made on the disease symptoms, number of diseased and healthy colonies, bee strength and infected brood area in the Indian bee apiaries. The study location of each district is detailed in Table 1.

3.1.2.1. Symptoms of the Disease

The disease symptoms were observed and recorded from the selected Indian bee colonies in the apiaries of Southern Kerala, during the period of survey. The necessary observations like change in colour of the larvae, shape and position of larvae in the brood as well as the odour and texture of larvae, condition

Table 1. Geographical details of apiaries from Southern Kerala

Districts	Sl. No	Location	Latitude	Longitude	Altitude (m) above MSL
Thiruvananthapuram	1	Nedumangad	8^0 36' 27.93'' N	77^0 0' 11.64'' E	60
	2	Peyad	8° 30' 51.54" N	77° 1' 11.138" E	42
	3	Ooroottambalam	8° 27' 24.27" N	77° 3' 33.568" E	72
	4	Kalliyoor	8^0 25' 57.09''N	77^0 1' 0.030'' E	56
	5	Karakonam	8° 23' 26.17" N	77° 10' 7.129" E	83
Kollam	1	Puthoor	9° 2' 29.803" N	76° 42' 54.49" E	26
	2	Edamon	9° 0' 22.392" N	76° 58' 53.715" E	66
	3	Pathanapuram	9° 5' 30.058" N	76° 51' 21.48" E	36
	4	Ottakkal	8° 58' 31.718" N	77° 2' 26.319" E	100
	5	Thenmala	8° 57' 39.793" N	77° 3' 42.461" E	91
Pathanamthitta	1	Neervilakom	9° 18' 58.016" N	76° 38' 59.765" E	17
	2	Aranmula	9° 19' 35.387" N	76° 41' 2.364" E	17
	3	Elanthoor	9° 17' 21.271" N	76° 43' 40.369" E	48
	4	Konni	9° 13' 36.142" N	76° 50' 58.840" E	34
	5	Ranni	9° 23' 11.649" N	76° 47' 7.998" E	13

Plate 1a. An apiary at Thiruvananthapuram

Plate 1b. An apiary at Kollam

Plate 1c. An apiary at Pathanamthitta

Plate 1. Apiaries of Southern Kerala selected for survey

of the cell cap at the pupal stage were recorded with help of the beekeepers. Apart from the brood, the symptoms exhibited by the adult bees as well as the deviation in colony characters, if any were also observed during the survey from the selected apiaries.

3.1.2.2. Percentage infection of the disease

Apart from monthly observations, the three seasons in the apiculture practise of Kerala was also taken into consideration, *viz.* honey flow season (January to April), dearth season (May to August) and brood rearing season (September to December).

The total number of colonies and those infected with disease were recorded from the apiaries of the respective districts. The percentage infection of the disease in the Indian bee apiaries in each month was calculated using the following equation:

$$\text{Percentage infection in the Apiary} = \frac{\text{Number of diseased colonies}}{\text{Total number of colonies}} \times 100$$

3.1.2.3. Bee Strength of the Colony

Colony strength is the number of worker bees in a colony. It was assessed by visual observation i.e., by assessing the bees covering the frames in the colony. Visual scoring was used to analyse the bee strength of the healthy as well as the infected colonies. The score was given as follows:

6 – All the six combs were covered with bees

5 – At least five combs covered with bees

4 – At least four combs covered with bees

3 – At least three combs were covered with the bees

2 – At least two combs were covered with the bees

1 – Very low bee population

3.1.2.4. Infected Brood Area

The infected brood area of the diseased Indian bee colonies was measured. Area of single cell was calculated with the help of equation:

$$\text{Area of a hexagon} = \frac{3\sqrt{3}}{2} \times a^2$$

Where, 'a' is the side length of the hexagon. The side length was measured by computing the diameter of the cell, as the side length was equal to the radius of the cell. The side length was 0.30 cm and thus, area of a bee cell was determined as 0.47 cm^2. The infected brood area was calculated by multiplying the number of cells infected with the area of a single cell.

Infected brood area = No. of cells infected x area of a single cell

3.2 ISOLATION, CHARACTERISATION AND IDENTIFICATION OF THE PATHOGEN

The honey bee brood combs showing the disease symptoms were brought from the apiaries for the laboratory studies. The laboratory studies were conducted in Department of Microbiology, College of Agriculture, Vellayani.

3.2.1 Isolation of the Microbes

The infected larvae and the pupae were taken out from the comb in Laminar Air Flow Chamber (LAF) and were sterilised using the standard procedure.

The infected larvae and pupae were first washed in water, and then kept in alcohol (70 %) for one minute. They were washed in sterile water. Then, the specimens were kept in sodium hypochlorite (4 %) for one minute for surface sterilisation. They were washed thoroughly in sterile water thrice and the excess water in the infected larvae and pupae was removed by keeping on a sterile blotting paper. The midgut of the larvae and pupae were dissected and a smear was prepared in a sterile Petriplate. The smear was then streaked on the Nutrient Agar medium.

Nutrient Agar medium was prepared using the following constituents: Peptone (0.5 %); beef extract (0.3 %) and agar (2 %). The pH of the medium was adjusted to 6.5. The medium was autoclaved at a temperature of 121^{0}C for 30 minutes. It was then poured to sterile Petriplates and kept at room temperature for 24 hrs, for checking contamination. These plates were streaked with the smear from the larval midgut. The streaked plates were incubated at room temperature (28 ^{0}C) for 2 days and observed for the growth of the microbes.

3.2.2 Pathogenicity Study of the Isolated Microbes

The microbes grown in the medium were subjected to pathogenicity studies. The pathogenicity study of the isolated organisms was done in the Indian bee colonies maintained at AICRP on Honey Bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani. Four healthy *A. cerana indica* hives were selected that were having equal bee strength, brood and having active queen bee. From each colony, three frames each with equal brood strength were selected. From each frame, three cells with 1-2 days old larvae were selected at random from either side of the brood comb and inoculated cells were marked using a marker pen.

The bacterial suspension was prepared by mixing a loopful of culture in 2 mL sterile water taken in a 2 mL suspension tube. It was prepared freshly on the day of inoculation. Inoculation was done with 5 mL sterile syringe.

Inoculation was carried out during the morning hours around 10 am. The freshly prepared bacterial suspension was taken to the field and injected into the selected cells @ 0.5 to 1 mL per cell. Three different colonies were inoculated with the three bacterial isolates while a separate colony was inoculated with sterile water and was kept as control (Plate 2).

Observations on disease symptoms, if any were taken on 1st, 3rd and 5th day after inoculation. The diseased brood was collected for the re-isolation of the pathogen.

The isolates showing pathogenicity was subjected to morphological, molecular and biochemical characterisation.

3.2.3 Identification of the Pathogen

3.2.3.1 Morphological Characterisation of the Microbes

Morphology of the isolated microbes was studied by staining method and microscopy. Thin bacterial smear was prepared on a clean glass slide. After heat fixing, the smear was flooded with crystal violet stain for one minute and gently washed under running tap water. Again flooded with gram's iodine for one minute and washed gently under running tap water. It was decolourised using grams decolouriser and washed with water. Finally counter stained using safranine stain for about one minute and observed under the microscope in low and high power for bacterial characters.

Apart from the Gram staining procedure, the bacterial colony characters like colour, shape, colony margin, opacity and texture were also studied by visual as well as microscopic techniques (Rao, 2009). The size of the bacterium was measured using micrometry study as well as by using the Zen lite software.

Plate 2. Injecting the bacterial suspension into the Indian bee cells

3.2.3.2 Molecular Detection of Pathogenic Microbes

The molecular detection of the samples was done using the 16S rRNA technique.

3.2.3.2.1 Genomic DNA isolation from bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of the culture was taken in a micro-centrifuge tube. 180 µL of T_1 buffer and 25 µL of proteinase K was added and incubated at 56°C in a water bath until, it were completely lysed. After lysis, 5 µL of RNase A (100 mg mL^{-1}) was added and incubated at room temperature for 5 min. 200 µL of B_3 buffer was added and incubated at 70°C for 10 min. 210 µL of 100 per cent ethanol was added and mixed thoroughly by vortexing. The mixture was then pipetted into NucleoSpin® Tissue column placed in a 2 mL collection tube and centrifuged at 11000 x g for 1 min. The NucleoSpin® Tissue column was transferred to a new 2 mL tube and washed with 500 µL of BW buffer. Washing 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.

3.2.3.2.2 Agarose gel electrophoresis for DNA quality and quantity check

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 to 0.8 % agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg mL^{-1} Ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has 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 Gel documentation system (Bio-Rad).

3.2.3.2.3 PCR analysis

PCR amplification reactions were carried out in a 20 µL reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH - 8.3; 500 mM KCl), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg mL^{-1} BSA, 4 % DMSO, 5 pM of forward and reverse primers and template DNA.

3.2.3.2.3.1 Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.2.3.2.3.2 PCR amplification profile

16S rRNA

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

3.2.3.2.4 Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.2 % agarose gel prepared in 0.5 X TBE buffer containing 0.5 μg mL^{-1} ethidium bromide. 1 μL of 6X loading dye was mixed with 5 μL of PCR products and was loaded. Electrophoresis was performed at 75V power supply with 0.5 X TBE as electrophoresis buffer for about 1-2 hr 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 Gel documentation system (Bio-Rad).

3.2.3.2.5 ExoSAP-IT treatment

ExoSAP-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.

Five micro litres of PCR product is mixed with 2 μL of ExoSAP-IT and incubated at 37°C for 15 min followed by enzyme inactivation at 80°C for 15 min.

3.2.3.2.6 Sequencing using big dye terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	- 10-20 ng
Primer	- 3.2 pM (either Forward or Reverse)
Sequencing Mix	- 0.28 μL
Reaction buffer	- 1.86 μL

Sterile distilled water - make up to 10µL

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 min followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 min.

3.2.3.2.7 Post sequencing PCR clean up

1. Master mix I of 10 µL milli Q and 2 µL 125 mM EDTA per reaction and master mix II of 2 µL of 3M sodium acetate pH 4.6 and 50 µL of ethanol were prepared.

2. 12µL of master mix I was added to each reaction containing 10µL of reaction contents and was properly mixed.

3. 52 µL of master mix II was added to each reaction.

4. Contents were mixed by inverting and incubated at room temperature for 30 min.

5. Spun at 14,000 rpm for 30 min.

6. Decanted the supernatant and added 100 µL of 70 % ethanol

7. Spun at 14,000 rpm for 20 min.

8. Decanted the supernatant and repeated 70 % ethanol wash

9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.2.3.2.8 Sequence analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

The 16S rRNA nucleotide sequence obtained was compared with the available sequences in the NCBI database using the BLAST tool provided by National Centre for Biotechnology Information (NCBI) in the website http://blast.ncbi.nlm.nih.gov/Blast.cgi.

The dendrogram for analysing the Phylogenetic relationship between the isolates and other bacterial pathogens of honey bees was drawn with CLC sequence viewer 7.

3.2.3.4 Biochemical Identification of the Microbe

The biochemical characterisation of the bacteria which proved pathogenicity was done by conducting various biochemical tests using Himedia© kits (Hi-Bacillus kit). The culture suspensions of the isolates were spot inoculated in test strips and kept for 24 h incubation. After incubation, the colour change was observed in the amended media of the kit and this revealed the reaction to be positive or negative with respect to the different biochemical tests utilised. The biochemical tests conducted include Malonate utilisation, Voges Proskauer, Citrate utilisation, ONPG, Nitrate reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose and H_2S production. The results obtained was utilised for arriving at a tentative identification of the microbial isolate.

3.3 FIELD EVALUATION OF BOTANICALS

A field experiment was conducted in the naturally infected *A. cerana indica* colonies maintained by AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani. The design used was CRD, with 10 treatments and 3 replications. The treatments were as follows:

T_1 : Crushed garlic 0.25 %

T_2 : Crushed garlic 0.5 %

T_3 : Crushed leaves of *Centella asiatica* 0.05 %

T_4 : Crushed leaves of *Centella asiatica* 0.1 %

T_5 : Crushed leaves of *Ocimum* sp 0.05 %

T_6 : Crushed leaves of *Ocimum* sp 0.1 %

T_7 : Turmeric powder 0.2 %

T_8 : $CaSO_4$ (Homeo medicine) 8 tablets per 250 mL

T_9 : Oxytetracycline hydrochloride 40 ppm

T_{10} : Untreated

3.3.1 Preparation of Feed

Sugar syrup was prepared by dissolving sugar in water in the ratio 1:1(w/v) and boiling. The syrup was cooled and fed to the bee colonies.

3.3.2 Preparation of Treatments

Plant leaves for the treatments were freshly collected from the field on the day of experiment. The other materials were purchased from the local market and were stored separately. The required amounts were weighed and taken to the field in separate polythene covers.

3.3.2.1 Preparation of Garlic Extract

Garlic was freshly purchased from the local market. The required quantity was weighed using a weighing balance. 625 mg and 1250 mg per 250 mL of sugar syrup were taken respectively. These were taken to the field in separate polythene covers.

3.3.2.2 Preparation of Centella asiatica Extract

Young leaves of *Centella asiatica* was collected from the field during the morning hours on the day of experiment. 125 mg and 250 mg per 250 mL of sugar

syrup were weighed respectively, using a weighing balance. These were taken to field in separate polythene covers.

3.3.2.3 Preparation of Ocimum sp. *Extract*

The *Ocimum* leaves were freshly collected from the field in the morning on the day of the experiment. The required amount was weighed using a weighing balance. 125 mg and 250 mg per 250 mL of sugar syrup was weighed respectively and taken to the field in separate polythene covers.

3.3.2.4 Preparation of Turmeric Powder

Fresh turmeric powder was purchased from the local market. 500 mg of turmeric powder was mixed with 250 mL of sugar syrup was weighed using a weighing balance and was stored in polybags.

3.3.2.5 Preparation of Homeo Medicine

The homeo medicine (Hepar sulf 200 x) was purchased from the homeo medical store. 8 tablets were counted and powdered using a pestle and mortar. Then it was dissolved in 250 mL sugar syrup and given to the selected Indian bee colonies.

3.3.2.6 Preparation of Antibiotic treatment

Oxytetracycline hydrochloride (Terramycin 650 mg) tablets were purchased from the local medical shop. Tablets were powdered using a pestle and mortar and 10 mg of this powder was dissolved in 250 mL sugar syrup. It was then taken to the field.

3.3.2.7 Preparation of Treatment Feed

In the field, the garlic and the leaves were crushed using pestle and mortar. These were mixed with the sugar syrup at the required quantity. The required

amount of turmeric powder was mixed with the sugar syrup. The tablets were powdered using pestle and mortar before mixing with the feed.

Indian bee hives taken as control were fed with sugar syrup only.

3.3.3 Application of Feed to the Indian Bee Colonies

The selected colonies were marked or labelled as per treatments imposed for future observation. The feed mixed with the respective treatments were given to the respective colonies at the rate of 250 mL per colony. The feed was given in coconut shells to the bee colonies.

3.3.4 Observations of Field Evaluation

The number of cells with infected larvae and pupae were counted before giving the treatments as pre-count. After the treatment, the observation on the number of infected cells was done on weekly intervals for subsequent four weeks. The observations on the development of symptoms were also noted. The percentage reduction in the brood disease over pre-count was calculated using the formula:

$$\% \text{ reduction} = \frac{(\text{Initial no. of infected cells} - \text{final no. of infected cells})}{\text{Initial no. of infected cells}} \times 100$$

The observations on infected brood area (cm^2), sealed brood area (cm^2), unsealed brood area (cm^2), pollen storage (cm^2), honey storage (cm^2) and number of returning foragers min^{-1} were also recorded to evaluate the effect of treatments on the normal activities of the colony. The brood area was calculated following the method mentioned in 3.1.2.4.

3.3.5 Statistical Analysis

In the field evaluation, the relation between the pre-count and the weekly observations were found using the Analysis of Co-Variance (ANACOVA), in case the pre-count observation was found significant. Otherwise, the analysis used was ANOVA with CRD design.

Results

4. RESULTS

The study entitled "Etiology of honey bee brood disease of Southern Kerala" was conducted during 2016 - 2018 in the All India Co-ordinated Research Project (AICRP) on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani. The results of the study are detailed below.

4.1 ESTIMATION OF DISEASE INTENSITY

4.1.1 Disease Incidence in the Apiaries of Southern Kerala

4.1.1.1. Symptomatology of Disease

Observations on Indian bee brood in the apiaries of Southern districts revealed that the infection occurred in the early instars of the honey bee larvae.

In the healthy honey bee colonies, the brood was observed to be uniformly capped and was light brown in colour. The larvae were pearly white in colour with a characteristic 'C' shape towards the base of comb cells (Plates 3a and 3b). In healthy colonies, the adult bees' strength was uniform and was having a gentle temperament.

The symptoms observed in the diseased Indian bee colonies during the survey were scattered presence of sealed and unsealed cells giving "pepper box symptom" (Plate 4). The colour of the larvae changed from pearly white to yellow and then to yellowish brown. The cells were not capped even in the pre-pupal stage. The larvae and pupae were found dead in the infected cells. Perforated cell cappings were also observed with dead pupae (Plates 5 and 6).

The larvae were found dead and decayed in the comb. The decayed mass of the larvae get dried and transformed into scales that adhere to the bottom of the cell. The pollen and honey storage of the infected colonies were highly reduced or

Plate 3a. Uniformly capped cells of Indian bee combs

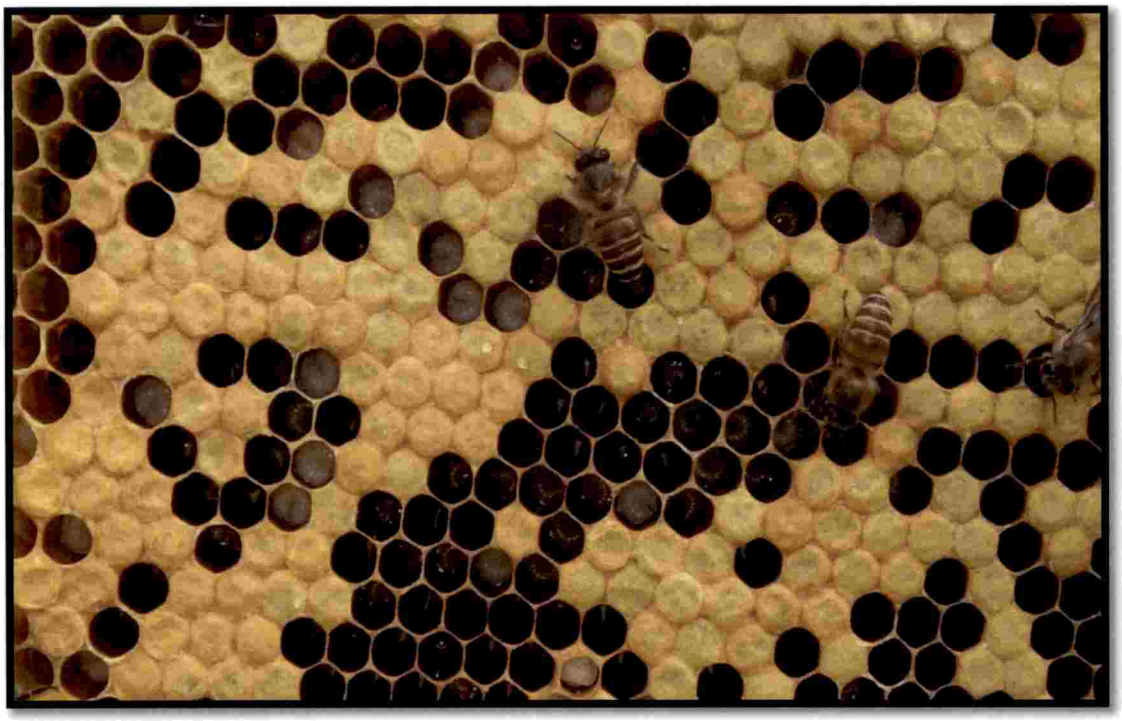

Plate 3b. Pearly white larvae of Indian bee

Plate 3. Healthy brood of Indian bee

Plate 4. Pepper box symptom in the Indian bee brood

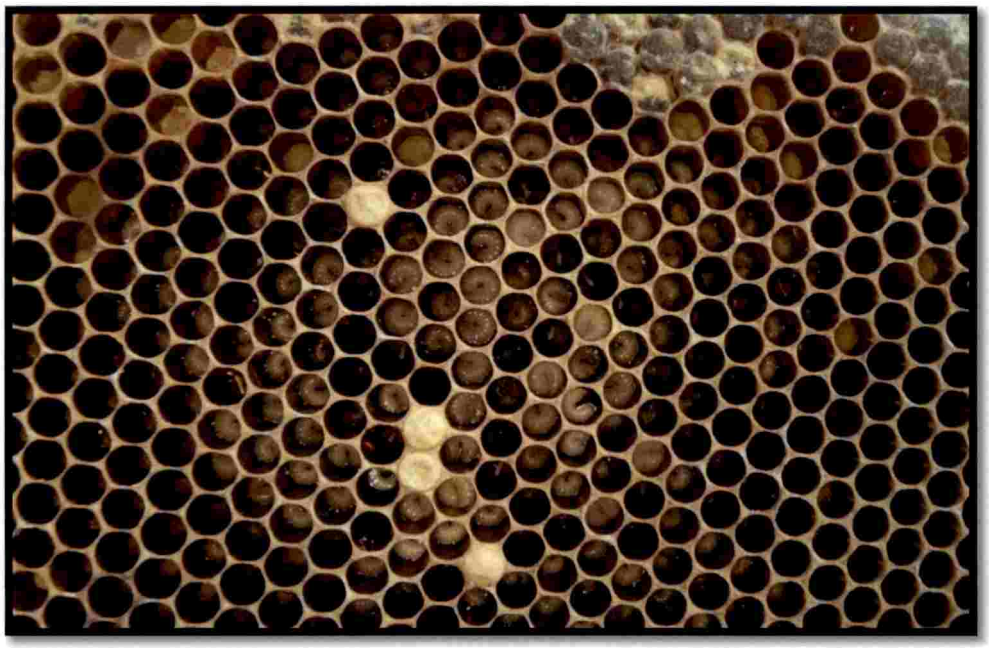

Plate 5. Change in colour of the infected larvae

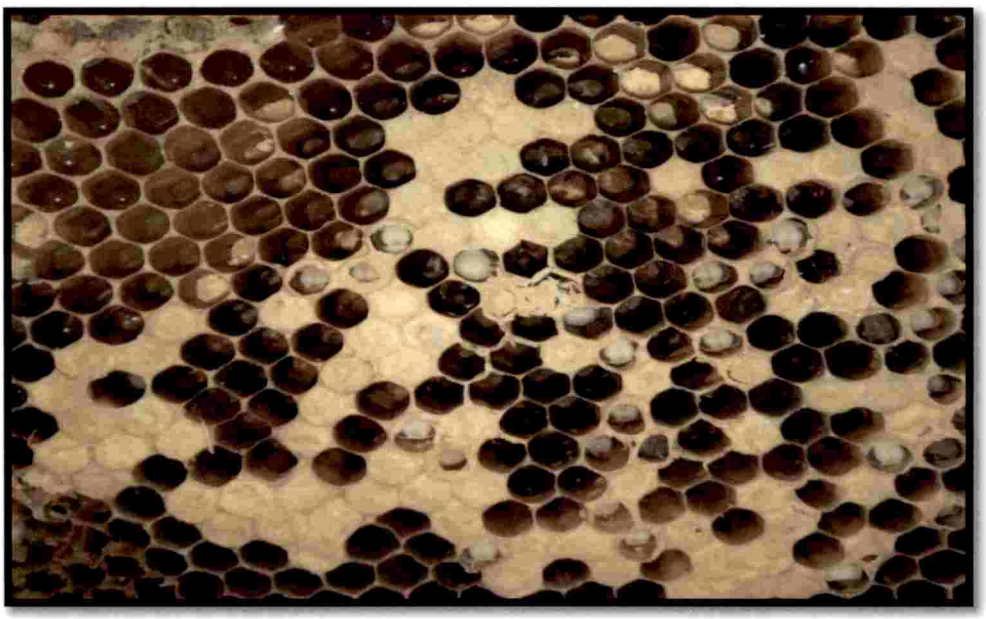

Plate 6. Perforated cell cappings with dead pupae

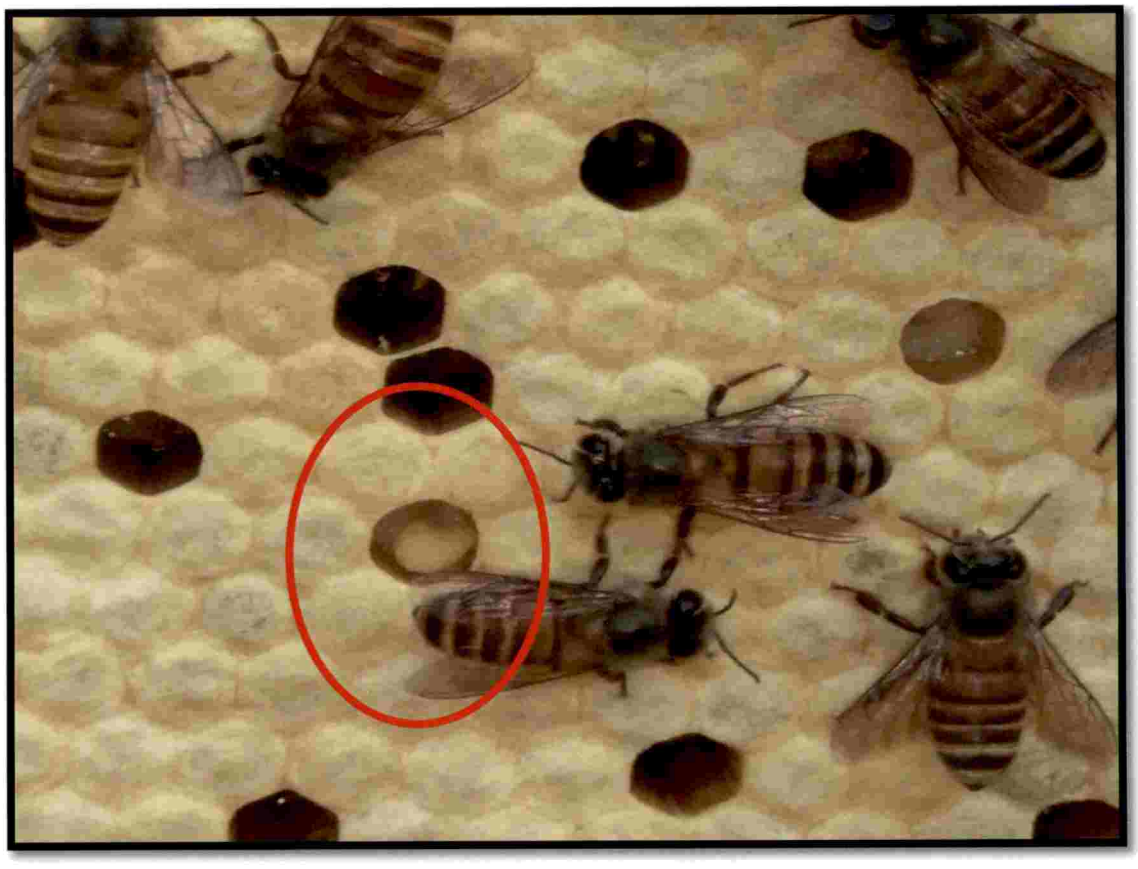

Plate 7. Decaying infected larvae

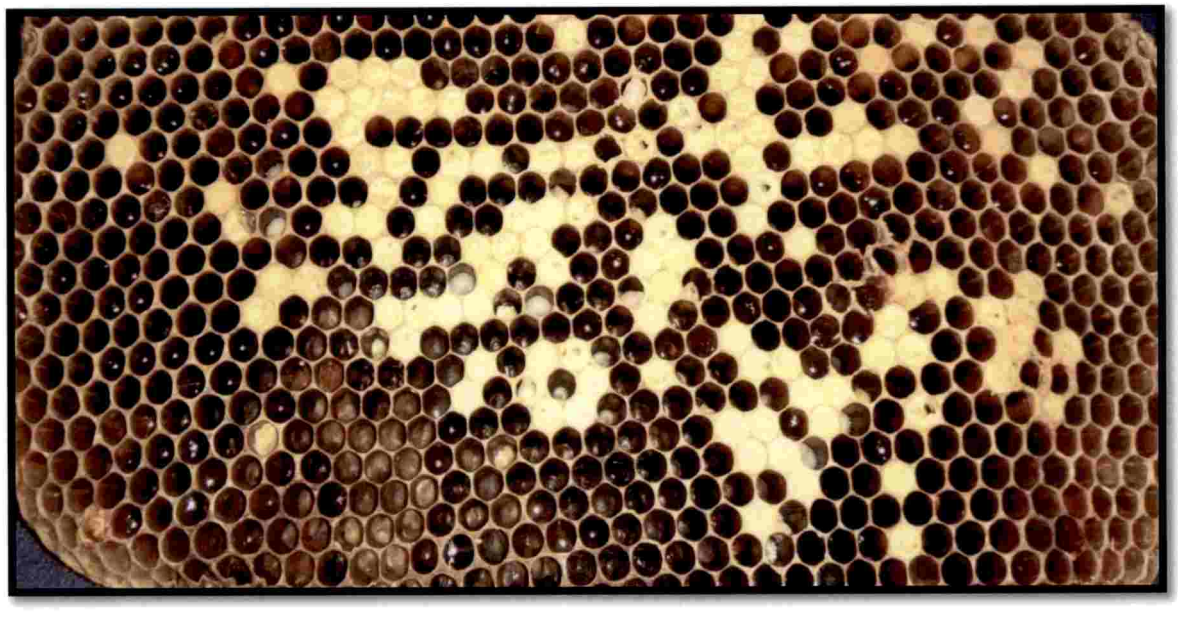

Plate 8. Brown scales adhering to cells

found negligible. Finally, the colour of the infected comb became dark brown or black (Plates 7 and 8).

Apart from these, the adult bee strength was greatly reduced. Adult bees were very aggressive and appeared more restless in the infected colonies. Reduced foraging activity and higher tendency for absconding were also observed from the diseased Indian bee colonies.

4.1.1.2. Monthly Incidence of the Disease

The monthly incidence of Indian bee brood disease in the apiaries of three southern districts of Kerala *viz.,* Thiruvananthapuram, Kollam and Pathanamthitta for one year from May 2017 to April 2018 is shown in Table 2 to 4.

The month wise disease incidence varied among the apiaries of southern districts of Kerala. In Thiruvananthapuram district, maximum brood disease incidence was observed during the dearth season (May to September) with maximum disease intensity in the apiaries of Nedumangad, Ooroottambalam and Kalliyoor. In the apiaries of Karakonam, the disease incidence was recorded from September to December, while from the apiaries of Oorottambalam disease was documented in March and April also. In Peyad, the disease incidence was recorded only during March and April (Table 2).

In Kollam district, the brood disease incidence was recorded throughout the year in the apiary at Edamon (Table 3). The disease incidence was prevalent in the apiaries of Pathanapuram, Ottakkal and Thenmala, both during the dearth season (May to September) and brood rearing season (September to January). In the Indian bee apiaries of Puthoor, the disease incidence was recorded only from October to January.

The brood disease incidence in the Pathanamthitta district was observed only in two apiaries, Neervilakom and Aranmula in March and April (Table 4).

Table 2. Month wise incidence of Indian bee brood disease in Thiruvananthapuram district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)			
		May	June	July	Aug.	Sept.	Oct	Nov	Dec.	Jan.	Feb.	March	April
1	Nedumangad	+	+	+	+	+	-	-	-	-	-	-	-
2	Peyadu	-	-	-	-	-	-	-	-	-	-	+	+
3	Ooroottambalam	+	+	+	+	-	-	-	-	-	-	+	+
4	Kalliyoor	+	+	+	+	+	+	-	-	-	-	-	-
5	Karakonam	-	-	-	-	+	+	+	+	-	-	-	-

\+ : disease present
\- : disease absent

Table 3. Month wise incidence of Indian bee brood disease in Kollam district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)			
		May	June	July	Aug.	Sept.	Oct	Nov	Dec.	Jan.	Feb.	March	April
1	Puthoor	-	-	-	-	-	+	+	+	+	-	-	-
2	Edamon	+	+	+	+	+	+	+	+	+	+	+	+
3	Pathanapuram	+	+	+	+	+	+	+	+	+	-	-	-
4	Ottakkal	+	+	+	+	+	+	+	+	+	-	-	-
5	Thenmala	+	+	+	+	+	+	+	+	+	-	-	-

+ : disease present
- : disease absent

Table 4 – Month wise Incidence of Indian bee brood disease in Pathanamthitta district

Sl. No.	Location	Dearth Season (2017)				Brood Rearing Season (2017)				Honey Flow Season (2018)			
		May	June	July	Aug.	Sept.	Oct	Nov	Dec.	Jan.	Feb.	March	April
1	Neervilakam	-	-	-	-	-	-	-	-	-	-	+	+
2	Aranmula	-	-	-	-	-	-	-	-	-	-	+	+
3	Elanthoor	-	-	-	-	-	-	-	-	-	-	-	-
4	Konni	-	-	-	-	-	-	-	-	-	-	-	-
5	Ranni	-	-	-	-	-	-	-	-	-	-	-	-

+ : disease present

- :disease absent

4.1.1.2.1. Percentage infection of disease in the apiaries of Southern Kerala

The number of infected Indian bee colonies in the selected apiaries of three districts *viz.,* Thiruvananthapuram, Kollam and Pathanamthitta were recorded and estimated as percentage infected at monthly intervals in Table 5 to 7.

Maximum disease incidence was detected in Ooroottambalam (44.19 %) in August, followed by Nedumangad (30.77 %) in May in the Thiruvananthapuram district. Peak infection of 11.28 per cent was recorded from the apiary of Nedumangad followed by 9.92 per cent from Ooroottambalam. The least mean percentage infection was observed from Peyad (0.75). A mean percentage infection of 17.26 was observed in August followed by 13.45 in July over the five locations of Thiruvananthapuram district (Table 5).

In Kollam district, the disease infection was severely reported from Edamon (95.15 %) in the month of December, followed by September with a mean percentage infection of 93.41 (Table 6). The colonies in the apiaries of Ottakkal were severely infected with a percentage incidence of 82.00 in October. Location wise disease incidence revealed that Edamon recorded maximum disease incidence of 76.69 per cent which was followed by Ottakkal (42.16 %) and Thenmala (35.05 %). Of the five locations in the Kollam district, maximum incidence was observed in November (64.18 %).

The percentage disease incidence in the Pathanamthitta district was very low compared to other two districts with maximum incidence of 22.22 per cent recorded from the apiary, Aranmula in April. The mean disease incidence was also highest in Aranmula (3.10 %) over the 12 months. Maximum disease incidence was in April (6.02 %) over the five locations (Table 7).

4.1.1.3. Bee Strength of the Colony

The mean month wise bee strength of the colonies analyzed by visual scoring method from the southern districts of Kerala is depicted in Table 8.

Table 5. Percentage infection of brood disease in the Indian bee apiaries of Thiruvananthapuram district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)				Mean
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	
1	Nedumangad	*30.77	32.31	29.69	26.23	16.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.28
2	Peyad	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.67	2.38	0.75
3	Ooroottambalam	20.00	18.37	26.53	44.19	0.00	0.00	0.00	0.00	0.00	0.00	7.50	2.50	9.92
4	Kalliyoor	15.00	13.20	11.05	7.37	5.15	3.59	0.00	0.00	0.00	0.00	0.00	0.00	4.61
5	Karakonam	0.00	0.00	0.00	8.50	5.00	4.50	3.03	0.00	0.00	0.00	0.00	0.00	1.75
	Mean	13.15	12.77	13.45	17.26	5.31	1.62	0.61	0.00	0.00	0.00	2.83	0.98	

Table 6. Percentage infection of brood disease in the Indian bee apiaries of Kollam district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)				Mean
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	
1	Puthoor	0.00	0.00	0.00	0.00	0.00	22.50	27.50	41.67	36.11	13.16	0.00	0.00	11.75
2	Edamon	62.00	68.71	78.29	88.24	93.41	91.43	92.78	95.15	85.29	70.00	65.00	30.00	76.69
3	Pathanapuram	38.00	41.50	45.60	34.10	37.28	43.95	43.41	51.46	24.21	0.00	0.00	0.00	29.96
4	Ottakkal	28.75	49.32	56.52	71.93	81.13	82.00	72.09	38.24	25.93	0.00	0.00	0.00	42.16
5	Thenmala	13.00	18.28	27.06	31.65	52.46	57.38	85.11	87.23	48.48	0.00	0.00	0.00	35.05
Mean		28.35	35.56	41.49	45.18	52.86	59.45	64.18	62.75	44.00	16.63	13.00	6.00	

Table 7. Percentage infection of brood disease in the Indian bee apiaries of Pathanamthitta district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)				Mean
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	
1	Neervilakom	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.50	7.89	1.70
2	Aranmula	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.00	22.22	3.10
3	Elanthoor	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	Konni	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	Ranni	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mean		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.50	6.02	

In Thiruvananthapuram district, a decline in the bee strength was observed in the Indian bee colonies from May to October with score 5 in May to July and score 3 in August and September. Thereafter, bee strength increased and by February it reached the maximum score of 6.

In Kollam district, the colonies severely infected by the disease showed a steep decline in the bee strength. The bee strength was observed to be highly reduced in the brood rearing season *i.e.* September to December, with score 2 in October to December and score 1 in January. The colony strength has shown a slight increase in the honey flow season (January to April).

The infected Indian bee colonies of Pathanamthitta district have shown a slight reduction in colony strength in March and April.

4.1.1.4 Infected Brood Area

The infected brood area of Indian bee colonies in the Southern Kerala is shown in Table 9 to 11.

In Thiruvananthapuram district, severe infection of brood area was observed during the dearth season (May to August) with maximum infection in July (106.59 cm^2) followed by August (80.62 cm^2) and the least infection was observed in November with 2.63 cm^2. Of the five locations, maximum infected brood area was recorded from the apiary of Karakonam in July (197.71 cm^2) (Table 9).

The brood area infection in Kollam district was severe during the brood rearing season (September to December) with maximum infection in the month of November (132.56 cm^2) followed by a mean area of 131.39 cm^2 in October. Among the five locations, the colonies of the apiary Ottakkal (210.32 cm^2) recorded maximum infected brood area in September and the least infected area was recorded from Pathanapuram with 41.23 cm^2 in January (Table 10).

Table 8. Bee Strength of the Indian Bee colonies of Southern Kerala

Sl. No.	Location	Dearth season (2017)								Brood rearing season (2017)								Honey flow season (2018)							
		May		June		July		Aug.		Sept.		Oct.		Nov.		Dec.		Jan.		Feb.		March		April	
		H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D
1	Thiruvananthapuram	6	5	6	5	6	5	6	3	6	3	6	4	6	4	6	5	6	5	6	6	6	6	6	6
2	Kollam	6	3	6	3	6	3	6	3	6	3	5	2	6	2	6	2	6	1	6	2	6	3	6	3
3	Pathanamthitta	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	4	6	5

H : Healthy Colonies

D : Diseased Colonies

Table 9. Infected brood area (cm^2) of the Indian bee colonies of Thiruvananthapuram district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)			
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April
1	Nedumangad	*126.39	158.65	112.32	78.27	26.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	Peyad	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	84.01	94.82
3	Ooroottambalam	33.13	84.48	96.35	128.54	0.00	0.00	0.00	0.00	0.00	0.00	95.68	69.21
4	Kalliyoor	96.35	151.22	126.55	92.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	Karakonam	0.00	0.00	197.71	103.61	60.69	34.09	13.16	0.00	0.00	0.00	0.00	0.00
	Mean	51.17	78.87	106.59	80.62	17.41	6.82	2.63	0.00	0.00	0.00	35.94	32.81

*Mean of three replication

Table 10. Infected brood area (cm^2) of the Indian bee colonies of Kollam district

Sl. No.	Location	Dearth season (2017)				Brood rearing season (2017)				Honey flow season (2018)			
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April
1	Puthoor	*0.00	0.00	0.00	0.00	0.00	99.72	84.01	47.89	63.01	51.47	0.00	0.00
2	Edamon	62.86	124.20	117.73	173.43	145.23	120.15	144.52	149.36	113.30	85.39	98.12	121.32
3	Pathanapuram	62.86	66.32	109.33	112.44	127.67	131.23	96.36	75.32	41.23	0.00	0.00	0.00
4	Ottakkal	152.75	153.22	172.02	193.71	210.32	165.31	172.65	135.84	83.65	0.00	0.00	0.00
5	Thenmala	112.35	104.87	125.36	152.75	143.00	140.53	165.28	141.00	117.50	0.00	0.00	0.00
	Mean	78.16	89.72	104.89	126.47	125.24	131.39	132.56	109.88	83.74	27.37	19.62	24.26

*Mean of three replication

Table 11. Infected brood area (cm^2) of the Indian bee colonies of Pathanamthitta district

Sl. No.	Location	Dearth Season (2017)				Brood Rearing Season (2017)				Honey Flow Season (2018)			
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April
1	Neervilakom	*0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	66.32	32.94
2	Aranmula	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	96.28	43.67
3	Elanthoor	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	Konni	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	Ranni	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mean	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	32.52	15.32

*Mean of three replication

The infected brood area of the Pathanamthitta district had maximum brood infection in March (32.52 cm^2). Within the five locations, maximum brood area infection was observed from the apiary of Aranmula with 96.28 cm^2 followed by Neervilakom with 66.32 cm^2 in March (Table 11).

4.2 ISOLATION, CHARACTERISATION AND IDENTIFICATION OF THE PATHOGEN

4.2.1 Isolation of Microbes

Three bacteria (J_1, J_2 and J_3) were isolated from the infected brood samples of Indian bee colonies in the Nutrient Agar medium (Plate 9).

4.2.2 Identification of the Pathogen

4.2.2.1 Pathogenicity Study of the Isolated Microbes

Out of the three bacteria isolated, only two *viz.* J_1 and J_3 isolates were found pathogenic to the honey bee larvae at five days after inoculation (Table 12). The larvae in the inoculated cells were found dead and the cells were found uncapped or punctured by the adult bees, similar to that of the bacterial disease reported in Indian honey bees (Plate 10). These isolates were taken for further characterisation studies.

4.2.2.2 Morphological Characterisation of the Microbes

The morphological characters of the bacterial isolates which proved pathogenicity are represented in Table 13.

a. J_1 isolate

The colonies were yellow in colour, round shaped and the bacteria were gram positive. They had irregular margins and were found spreading in appearance. The colonies were opaque in nature and were having a rough texture. The bacteria were rod shaped and average size of the bacteria was 0.311µm.

Table 12. Symptom appearance in the Indian bee brood inoculated with isolates

Isolate	Incidence of disease		
	1st day	3rd day	5th day
J1	-	-	+
J2	-	-	-
J3	-	-	+
Control	-	-	-

+ : Presence of disease

- : Absence of disease

Table 13. Morphology of pathogenic isolates

Isolate	Colony morphology	Colour	Appearance	Size	Opacity	Gram stain	Texture
J1	Round	Yellow	Spreading	0.311 µm	opaque	Gram +	Rough
J3	Round and glassy	Cream	Slightly raised	0.213 µm	Transparent	Gram -	Smooth

Plate 9a. J_1 Isolate

Plate 9b. J_2 Isolate

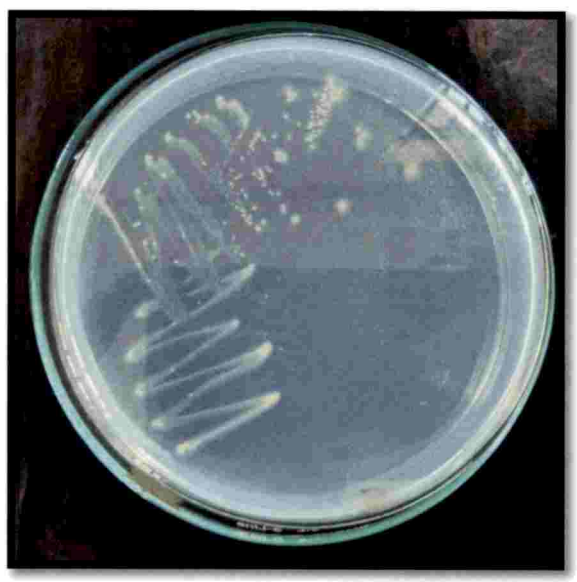

Plate 9c. J_3 Isolate

Plate 9. Bacterial colonies isolated in the NA medium

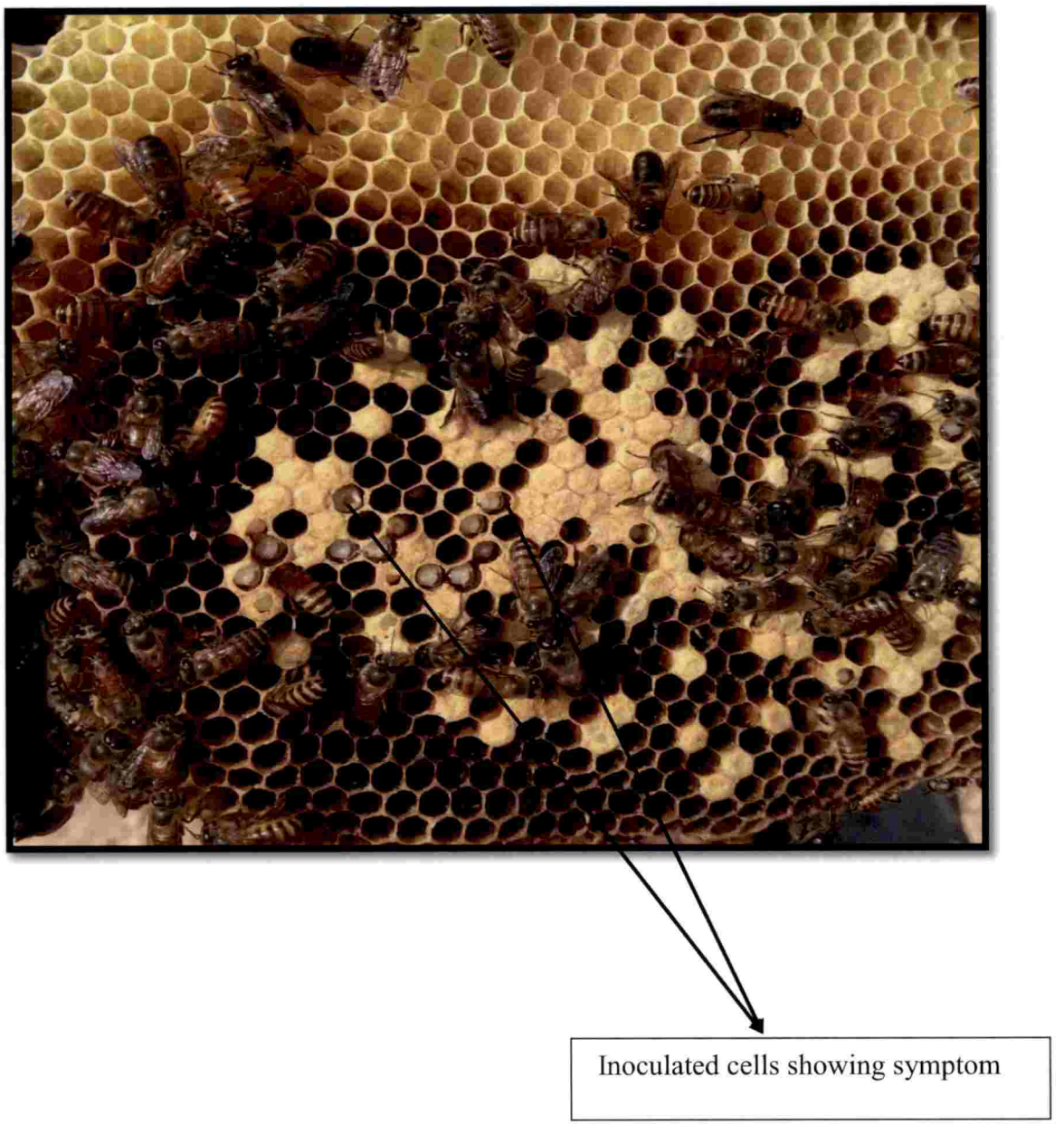

Plate 10. Re-occurrence of the symptom in the inoculated cells and spread of the disease to the he neighbouring cells

b. J_3 isolate

The colonies were cream in colour that was round and glassy shaped. The bacteria were gram negative. The colonies were found transparent and were slightly raised. The colony was having a smooth texture. The bacterium was round shaped and the average size was measured to be 0.212 µm.

4.2.2.3 Molecular Characterisation of Pathogenic Microbes

The results of the 16S rRNA sequencing of the pathogenic microbial isolates are presented in the Table 14. Amplification was done by using CAGGCCTAACACATGCAAGTC as the forward primer and GGGCGGWGTGTACAAGGC as the reverse primer.

The sequences producing significant alignment of the bacterial isolates obtained by the BLAST search are given in the Table 15. The J_1 isolate showed 99 per cent identity with *Bacillus pumilus* strain MB7 NIOT 16S ribosomal RNA gene partial sequence while the J_3 isolate presented 91 per cent identity with *Achromobacter* sp. strain HZ17 16S ribosomal RNA gene partial sequence.

The phylogenetic tree was drawn to analyse the similarity of the isolated bacteria with other bacterial pathogens infecting the honey bee. The Phylogenetic tree also revealed that J_1 isolate was showing higher similarity to *Bacillus* sp. and it was showing relation to *M. plutonius,* pathogen of EFB. While the J_3 isolate was showing higher similarity to *Achromobacter* sp. (Fig. 1).

4.2.2.4 Biochemical Characterisation

The results of the biochemical tests conducted for the bacterial isolates which proved the pathogenicity (Plate 11) are presented in Table 16.

The J_1 isolate exhibited positive reaction to the following biochemical tests *viz.* Voges proskauer, ONPG, catalase, sucrose, mannitol, glucose, arabinose

Table 14. Sequence homology (16S rRNA) of the pathogenic isolates

Isolate	Sequence
J1	>SR1186-J1-RSF1_A01.ab1 GCAAAGGTGCCTTTGGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGA CTGGGATA ACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGG CTGTCACT TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC CTGAGAGG GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA ATGGACGAA AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA AGTGCAAG AGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT ACGTAGGT GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCC CGGCTCAA CCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG AAATGCGTA GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG GGGGAGCGA ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCCGCCCCCC TTAATTGC TGCAGCTAACGCATTAAGCCACTCCGCCC

J3	>SR1186-J3-RSF1_C01.ab1 GGGGGGGGGGAGGCTTGCTTTCCTGCCGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCTGTA GTGGGGGA TAACTAGTCGAAAGATTAGCTAATACCGCATACGACCTGAGGGTGAAAGTGGGGGACCGCAAGGCCTCATG CTATAGGAG CGGCCGATGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAG GACGATCA GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGG CAACCCTGA TCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCGCACTG GTTAATAC CTGGTGTGGATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG GGTCCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGGGCTTA ACCTGGGA ATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGT AGAGATGT GGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCA AACAGGATT AGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAACT ACGCGTG AAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAATTGACGGGGACCCGCACAAGCC GGTGGATGA TGTGCATTAATTCGAATGCCATCGGCGGAAAAAAA

Table 15. Blast results from NCBI Data base for 16S rRNA sequence

Isolates	Description	Max. score	Total score	Query cover	E value	Identity	Accession no.
J1	*Bacillus pumilus* strain MB7 NIOT 16S ribosomal RNA gene partial sequence	1463	1463	98%	0.0	99%	HQ858063.1
J3	*Achromobacter* sp. Strain HZ17 16S ribosomal RNA gene partial sequence	1181	1181	94%	0.0	91%	KY064081.1

Table 16. Biochemical characters of the pathogenic isolates

Sl. No.	**Biochemical Reaction**	**J_1 Isolate**	**J_3 Isolate**
1	Malonate Utilization	-	-
2	Voges Proskauer	+	-
3	Citrate Utilization	-	+
4	ONPG	+	-
5	Nitrate reduction	-	+
6	Catalase	+	-
7	Arginine	-	-
8	Sucrose	+	+
9	Mannitol	+	-
10	Glucose	+	-
11	Arabinose	+	-
12	Trehalose	+	-
13	H_2S Production	-	-
	Tentative Identification of Isolates	*Bacillus pumilis*	*Achromobacter* sp

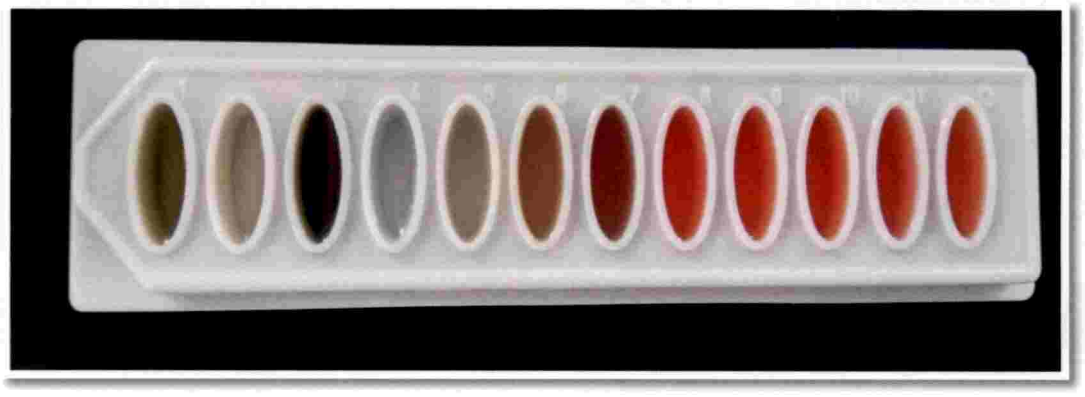

Plate 11a. Before inoculation

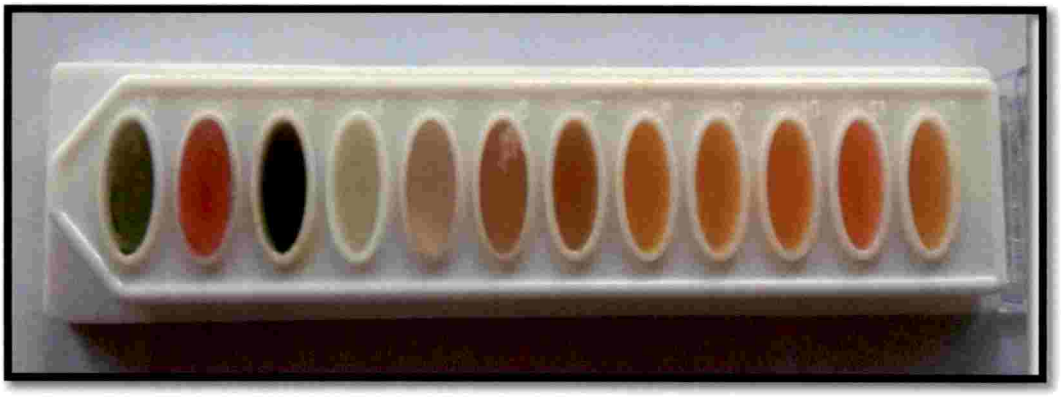

Plate 11b. After inoculation

Plate 11. Biochemical characterisation of pathogenic isolatees

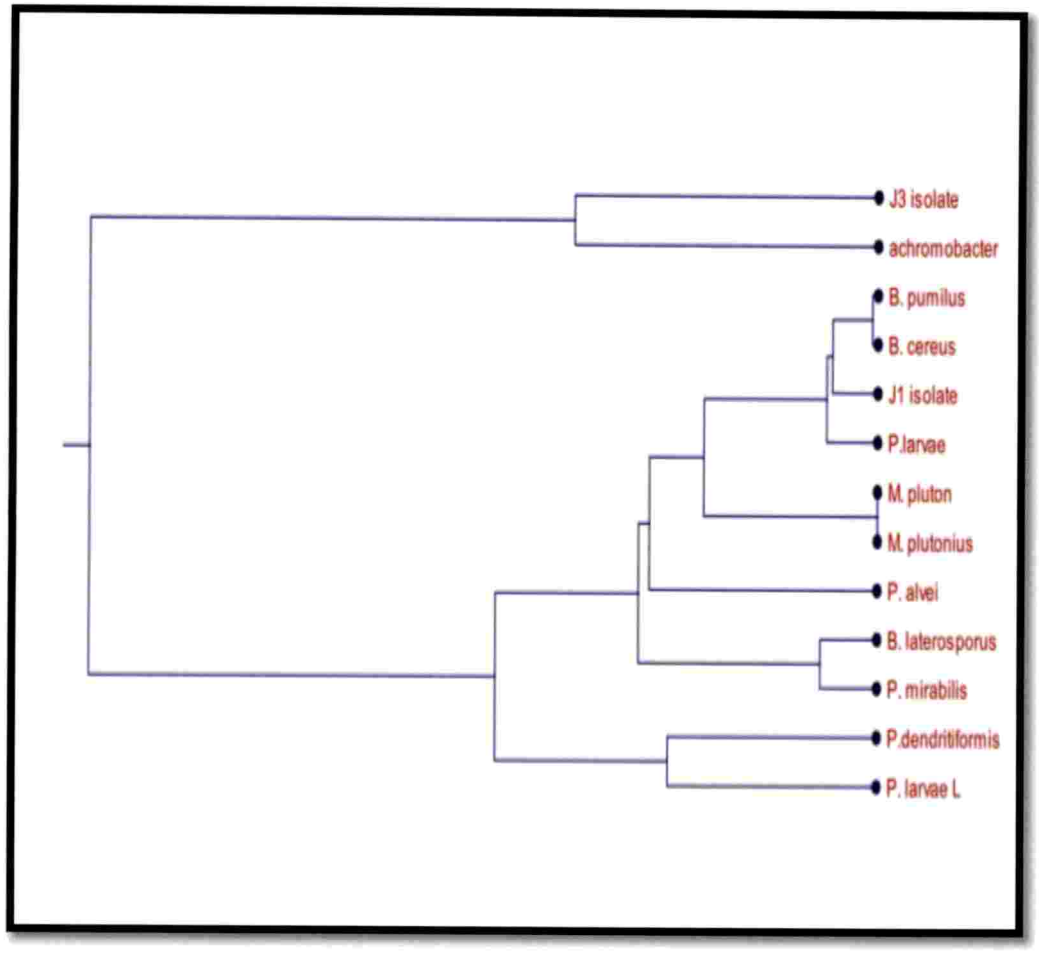

Fig. 1 Dendrogram of Phylogenetic relationship of the isolates with the honey bee bacterial pathogens

and trehalose. The J_3 isolate has exhibited positive reaction for the biochemical tests including, citrate utilization, nitrate reduction and sucrose.

The result of biochemical test was used for the tentative identification of the bacteria. The J_1 isolate was identified as *Bacillus pumilus* and the J_3 isolate as *Achromobacter* sp and these bacteria were reported to be found associated with the pathogen of European foul brood disease of honey bees, *M. plutonius*.

3.3 FIELD EVALUATION OF BOTANICALS

3.3.1 Effect of Botanicals on the Number of Cells of Treated Indian Bee Colonies

The effect of the botanicals in terms of mean number of infected cells in the diseased Indian bee colonies is depicted in Table 17.

Significant reduction in the mean number of infected cells (7.61) was recorded from the Indian bee colonies treated with crushed leaves of *Ocimum* sp. 0.5 % at one week after treatment (WAT). This was on par with that of crushed garlic 0.25 %, turmeric powder 0.2 % and crushed leaves of *C. asiatica* 0.1 % which had a mean number of infected cells of 10.50, 11.39 and 15.94 respectively.

The colonies treated with crushed leaves of *Ocimum* sp. 0.1 %, crushed leaves of *C. asiatica* 0.05 %, crushed garlic 0.5 % and $CaSO_4$ (homeo medicine) recorded mean of 17.94, 26.72, 28.50 and 29.83 infected cells respectively which were on par with the control treatment (23.83). The colonies treated with oxytetracycline hydrochloride (40 ppm) had 28.06 infected cells which were found on par with the control treatment in one WAT.

The results obtained in two week after treatment indicated that the treatment with crushed garlic 0.25 % has recorded a significant reduction in the mean infected cells (9.14). This was found on par with that of turmeric powder 0.2 %, crushed leaves of *Ocimum* sp. 0.05 %, crushed leaves of *C. asiatica* 0.1 % and crushed leaves of *Ocimum* sp. 0.1 % with an average of 11.49, 11.74, 12.93

Table 17 - Infected brood cells of Indian bee colonies

Treatment	Week 1	Week 2	Week 3	Week 4
Crushed garlic 0.25%	10.50	9.14	7.37	5.79
Crushed garlic 0.5%	28.50	22.57	18.79	15.26
Crushed leaves of *Centella asiatica* 0.05%	26.72	24.22	25.74	16.58
Crushed leaves of *Centella asiatica* 0.1%	15.94	12.93	13.85	9.73
Crushed leaves of *Ocimum* sp. 0.05%	7.61	11.74	9.78	8.04
Crushed leaves of *Ocimum* sp. 0.1%	17.94	19.38	20.64	15.77
Turmeric powder 0.2%	11.39	11.49	7.46	12.04
$CaSO_4$ (homeo medicine)	29.83	30.83	23.16	21.27
Oxytetracycline hydrochloride (40 ppm)	28.06	17.42	12.49	9.39
Control	23.83	22.29	19.07	18.13
CD (0.05)	12.607	9.854	11.549	7.904

and 19.38 infected cells respectively. The crushed garlic 0.25 % was also found on par with the check treatment, oxytetracycline hydrochloride (40 ppm) with 17.42 mean infected cells.

The Indian bee colonies treated with $CaSO_4$ with a mean infected cells of 30.83 was found on par with crushed leaves of *C. asiatica* 0.05 % and crushed garlic 0.5 % with mean of 26.72 and 28.50 infected cells respectively. These treatments were found on par with the mean infected cells of control treatment (22.29) in two WAT.

In three week after treatment, significant reduction in the mean number of infected cells was observed in the colonies treated with crushed garlic 0.25 % (7.37) which was found on par with turmeric powder 0.2% (7.46), crushed leaves of *Ocimum* sp. 0.05 % (9.78), crushed leaves of *C. asiatica* 0.1 % (13.85) and check treatment, oxytetracycline hydrochloride (12.49).

The infection in the colonies treated with crushed leaves of *C. asiatica* 0.05 % with mean infected cells of 25.74 was found on par with crushed garlic 0.5 %, crushed leaves of *Ocimum* sp. 0.1 % and $CaSO_4$ the mean number of infected cells being 18.79, 20.64 and 23.16 respectively. These were on par with the control treatment (19.07 infected cells) in three WAT.

A significant reduction in the infected cells was observed in treatment with crushed garlic 0.5 % (5.79) in four week after treatment. This was on par with crushed leaves of *Ocimum* sp. 0.05 %, crushed leaves of *C. asiatica* 0.1 %, turmeric powder 0.2 % and also with the check oxytetracycline hydrochloride (40 ppm) which had the mean infected cells of 8.04, 9.73, 12.04 and 9.39 respectively. The colonies treated with control treatment had 18.13 infected cells and was found on par with colonies treated with $CaSO_4$ (21.27). It was also on par with crushed garlic 0.5 %, crushed leaves of *Ocimum* sp. 0.1 % and crushed leaves of *C. asiatica* 0.05 % with average infected cells being 15.26, 15.77 and 6.58 respectively in four WAT.

3.3.1.1 Percentage Reduction of Infected Cells in Indian Bee Colonies over Pre-count

The percentage reduction in the brood disease of the Indian bee colonies over the pre-count observation in the four subsequent weeks after treatment is presented in the Table 18.

The percentage reduction observed in one WAT with respect to the pre-count was found maximum for crushed leaves of *Ocimum* sp. 0.05 % with 72.97 followed by crushed garlic 0.25 % with 59.26. The minimum value was obtained for the check treatment with oxytetracycline hydrochloride (40 ppm) with mean percentage of (-14.29).

The colonies treated with crushed garlic 0.25 % has reported maximum reduction in the brood cell with a mean of 62.96 per cent followed by turmeric powder 0.2 % (56.16 %) and crushed leaves of *Ocimum* sp. 0.05 % (55.41 %). For the colonies treated with $CaSO_4$ (homeo medicine), no significant reduction was observed in the mean percentage of the brood disease in two WAT.

The mean percentage reduction in the infected brood cell recorded maximum for the colonies treated with turmeric powder 0.2 % in three WAT (69.86 %) followed by crushed leaves of *Ocimum* sp. 0.05 % and crushed garlic 0.25 % with mean percentage reduction of 60.81 and 61.11 respectively.

In four WAT, the maximum reduction in the infected brood cell of the Indian bee colonies was observed for the treatment with crushed garlic 0.25 % with a mean percentage reduction of 68.52 followed by the treatment with crushed leaves of *Ocimum* sp. 0.05 % (67.57).

3.3.2 Effect of Botanicals on the Infected Brood Area of Diseased Indian Bee Colonies

The infected brood area (cm^2) calculated for the diseased Indian bee colonies are presented in the Table 19.

Table 18. Percentage reduction in the infected brood cells of Indian bee colonies

Treatment	Week 1	Week 2	Week 3	Week 4
Crushed garlic 0.25%	59.26	62.96	61.11	68.52
Crushed garlic 0.5%	8.08	27.27	42.42	57.58
Crushed leaves of *Centella asiatica* 0.05%	15.53	24.27	24.27	21.46
Crushed leaves of *Centella asiatica* 0.1%	36.76	48.53	39.71	57.35
Crushed leaves of *Ocimum* sp. 0.05%	72.97	55.41	60.81	67.57
Crushed leaves of *Ocimum* sp. 0.1%	22.03	11.86	-3.39	20.34
Turmeric powder 0.2%	57.53	56.16	69.86	50.68
$CaSO_4$ (homeo medicine)	2.08	0.00	27.08	33.33
Oxytetracycline hydrochloride (40 ppm)	-14.29	30	47.14	60
Control	32.56	38.76	34.26	27.36

Significant reduction in the infected brood area (3.58 cm^2) was recorded in diseased Indian bee colonies treated with crushed leaves of *Ocimum* sp. 0.05 %. This was on par with crushed garlic 0.25 %, turmeric powder 0.2 %, crushed leaves of *C. asiatica* 0.1 % and crushed leaves of *Ocimum* sp. 0.1 % with a mean infected area of 4.94 cm^2, 5.36 cm^2, 7.50 cm^2 and 8.44 cm^2 respectively in one WAT.

Highest mean infected brood area was recorded in colonies treated with $CaSO_4$ (homeo medicine) with 14.02 cm^2 which was found on par with crushed garlic 0.5 % and crushed leaves of *C. asiatica* 0.05 % with a mean infected brood area of 13.40 cm^2 and 12.56 cm^2 respectively. The treatment with $CaSO_4$ was also on par with the check oxytetracycline hydrochloride with a mean infected area of 13.19 cm^2. These were found on par with the control treatment also with 11.20 cm^2 infected brood area in one WAT.

In two WAT, colonies treated with crushed garlic 0.25 % recorded significant reduction in the infected brood area (4.30 cm^2) compared to the pre-count observation. This was on par with turmeric powder 0.2 %, crushed leaves of *Ocimum* sp. 0.05 %, crushed leaves of *C. asiatica* 0.1 % and crushed leaves of *Ocimum* sp. 0.1 % with the mean infected brood area of 5.40 cm^2, 5.52 cm^2, 6.08 cm^2 and 9.11 cm^2 respectively. The treatment with crushed garlic 0.25 % was found on par with the check treatment, oxytetracycline hydrochloride (40 ppm) also with mean a infected area of 8.19 cm^2.

Similar to that of one WAT, CaSO4 treated colonies recorded maximum infected brood area with a mean of 14.49 cm^2 which was on par with crushed leaves of *C. asiatica* 0.05 % and crushed garlic 0.5 % with a mean infected brood area of 11.39 cm^2 and 10.61 cm^2 respectively. These were on par with the control treatment with a mean infected area of 11.20 cm^2.

Least brood infected area was recorded in colonies treated with crushed garlic 0.25 % with a mean of 3.46 cm^2 which was on par with turmeric powder 0.2 %, crushed leaves of *Ocimum* sp. 0.05 %, crushed leaves of *C. asiatica* 0.1 %

Table 19. Infected brood area (cm^2) of the treated Indian bee colonies

Treatments	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	8.46	4.94	4.30	3.46	2.78
Crushed garlic 0.5%	15.51	13.40	10.61	8.83	6.52
Crushed leaves of *Centella asiatica* 0.05%	16.14	12.56	11.39	12.10	7.76
Crushed leaves of *Centella asiatica* 0.1%	10.65	7.50	6.08	6.51	4.60
Crushed leaves of *Ocimum* sp. 0.05%	11.59	3.58	5.52	4.60	3.76
Crushed leaves of *Ocimum* sp. 0.1%	9.24	8.44	9.11	9.70	7.45
Turmeric powder 0.2%	11.44	5.36	5.40	3.50	5.68
$CaSO_4$ (homeo medicine)	15.13	14.02	14.49	10.89	9.98
Oxytetracycline hydrochloride (40 ppm)	10.97	13.19	8.19	5.87	4.44
Control	10.21	11.20	10.48	8.97	8.44
CD (0.05)	-	5.924	4.631	5.428	3.656

and crushed garlic 0.5 % with mean infected brood area of 3.50 cm^2, 4.60 cm^2, 6.51 cm^2 and 8.83 cm^2 respectively in three WAT. While colonies treated with crushed leaves of *C. asiatica* 0.05 % had a mean infected brood area of 12.10 cm^2 which was on par with $CaSO_4$ (10.89 cm^2) and crushed leaves of *Ocimum* sp. 0.1 % (9.70 cm^2).

In four WAT, the Indian bee colonies treated with crushed garlic 0.25 % recorded significant reduction in the infected brood area with a mean of 2.78 cm^2. This was on par with crushed leaves of *Ocimum* sp. 0.05 %, crushed leaves of *C. asiatica* 0.1 % and turmeric powder 0.2 % with the mean infected brood area of 3.76 cm^2, 4.60 cm^2 and 5.68 cm^2 respectively. The treatment with crushed garlic 0.25 % was found on par with the check treatment oxytetracycline hydrochloride (40 ppm) also, with the mean infected brood area 4.44 cm^2. Whereas, maximum mean infected brood area of 9.98 cm^2 was recorded in colonies treated with $CaSO_4$ in four WAT which was on par with crushed garlic 0.5%, crushed leaves of *Ocimum* sp. 0.1% and crushed leaves of *C. asiatica* 0.05%, the mean infected brood area being 6.52 cm^2, 7.45 cm^2 and 7.76 cm^2 respectively. These were found on par with the control treatment with a mean area of 8.44 cm^2.

3.3.3 Effect of Botanicals on the Sealed Brood Area of the Treated Indian Bee Colonies

The results on the sealed brood area in the diseased Indian bee colonies in the four subsequent weeks after treatment are detailed in Table 20.

In one WAT, highest sealed brood area was observed in the colonies treated with crushed garlic 0.25 % with a mean area of 188.00 cm^2 which was on par with crushed leaves of *C. asiatica* 0.05 %, crushed garlic 0.5 % and crushed leaves of *Ocimum* sp. 0.1 % the mean sealed brood area being 167.82 cm^2, 168.26 cm^2 and 178.76 cm^2 respectively. The lowest mean sealed brood area of 105.44 cm^2 was recorded for the control treatment that was significantly different from all the treatments. No significant difference among the treatments was observed in the sealed brood area of the colonies before treatment.

Table 20. Sealed brood area (cm^2) of the treated Indian bee colonies

Treatments	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	173.59	188..00	197.26	243.04	286.07
Crushed garlic 0.5%	161.68	167.82	180.32	209.90	255.12
Crushed leaves of *Centella asiatica* 0.05%	154.91	168.26	145.54	132.70	122.98
Crushed leaves of *Centella asiatica* 0.1%	160.58	149.60	143.82	141.90	145.40
Crushed leaves of *Ocimum* sp. 0.05%	162.15	156.40	171.08	229.15	261.32
Crushed leaves of *Ocimum* sp. 0.1%	161.05	178.76	188.43	206.50	221.10
Turmeric powder 0.2%	180.95	132.90	100.27	107.00	120.95
$CaSO_4$ (homeo medicine)	167.48	140.90	125.18	118.95	99.95
Oxytetracycline hydrochloride (40 ppm)	135.05	149.00	166.78	172.50	185.00
Control	145.70	105.44	78.65	45.12	34.62
CD (0.05)	NS	21.05	17.077	17.886	30.609

Colonies treated with crushed garlic 0.25 % recorded highest sealed brood area (197.26 cm^2) two WAT. This was found on par with crushed garlic 0.5 % and crushed leaves of *Ocimum* sp. 0.1 % with a mean sealed brood area of 180.32 cm^2 and 188.43 cm^2 respectively. The colonies treated with crushed leaves of *Ocimum* sp. 0.05 % (171.08 cm^2) was on par with the check oxytetracycline hydrochloride (40 ppm) with a mean area of 166.78 cm^2. The sealed brood area (132.90 cm^2) in colonies treated with turmeric powder 0.2 % was significantly different from other treatments.

In three WAT, the sealed brood area of the colonies treated with crushed garlic 0.25 % (243.04 cm^2) was on par with crushed leaves of *Ocimum* sp. 0.05 % with mean area of 229.15 cm^2. Least sealed brood area (118.95 cm^2) was recorded from colonies treated with $CaSO_4$ which was on par with crushed leaves of *C. asiatica* 0.05 % and turmeric powder 0.2 % with mean sealed brood area of 132.70 cm^2 and 107.00 cm^2 respectively.

Highest mean sealed brood area was obtained for the Indian bee colonies treated with crushed garlic 0.25 % with a mean sealed brood area of 286.07 cm^2 four WAT. This was on par with crushed garlic 0.5 % and crushed leaves of *Ocimum* sp. 0.05 %, the mean sealed brood area being 255.12 cm^2 and 261.32 cm^2 respectively. Colonies treated with $CaSO_4$ recorded a mean sealed brood area of 99.95 cm^2 which was on par with crushed leaves of *C. asiatica* 0.05 % and turmeric powder 0.2 % with a mean area of 122.98 cm^2 and 120.95 cm^2 respectively. The control treatment was significantly different from other treatments with a mean sealed brood area of 34.62 cm^2.

3.3.4 Effect of Botanicals on the Unsealed Brood Area of the Treated Indian Bee Colonies

The results of unsealed brood area of the diseased Indian bee colonies are detailed in Table 21. No significant variation among the treatments was observed in the unsealed brood area one WAT.

The unsealed brood area of diseased Indian bee colonies in two WAT revealed that the treatment with turmeric powder 0.2 % has recorded significantly higher mean area (214.77 cm^2) which is on par with all other treatments except the treatment with $CaSO_4$ (homeo medicine) and the control treatment with mean area 161.68 cm^2 and 160.74 cm^2 respectively.

In three WAT, maximum unsealed brood area was observed for colonies treated with Crushed garlic 0.25 % with a mean area of 219.49 cm^2. This was on par with crushed leaves of *Ocimum* sp. 0.05 %, turmeric powder 0.2 %, crushed leaves of *C. asiatica* 0.1 % and crushed garlic 0.5 %, the mean unsealed brood area being 214.27 cm^2, 212.44 cm^2, 209.34 cm^2 and 205.07 cm^2 respectively. The mean unsealed brood area of colonies treated with $CaSO_4$ (145.53 cm^2) was on par with the control treatment (142.84 cm^2).

In four WAT, the Indian bee colonies treated with crushed garlic 0.25 % recorded maximum mean unsealed brood area (236.80 cm^2) which was on par with crushed leaves of *Ocimum* sp. 0.05 %, turmeric powder 0.2%, crushed garlic 0.5 %, and crushed leaves of *C. asiatica* 0.1 % with a mean unsealed brood area of 222.22 cm^2, 215.72 cm^2, 212.52 cm^2 and 208.37 cm^2 respectively. The mean unsealed brood area was significantly reduced for the colonies treated with $CaSO_4$ with the mean area of 127.53 cm^2 which was on par with the control treatment with a mean area of 124.71cm^2.

3.3.5 Effect of Botanicals on the Pollen Storage of the Treated Indian Bee Colonies

The results of mean pollen storage in the diseased Indian bee colonies are represented in the Table 22.

In one WAT, the mean pollen storage area of the diseased Indian bee colonies was maximum for crushed garlic 0.25 % with a mean area of 34.17 cm^2. This was on par with the control treatment with a mean area of 31.33 cm^2. It was also found on par with crushed leaves of *Ocimum* sp. 0.05 %, crushed garlic

Table 21. Unsealed brood area (cm^2) of the treated Indian bee colonies

Treatments	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	182.80	199.10	207.00	219.49	236.80
Crushed garlic 0.5%	188.20	193.50	200.00	205.07	212.52
Crushed leaves of *Centella asiatica* 0.05%	187.50	193.00	194.00	201.20	205.80
Crushed leaves of *Centella asiatica* 0.1%	193.60	194.90	193.00	209.34	208.37
Crushed leaves of *Ocimum* sp. 0.05%	180.10	197.50	206.00	214.27	222.22
Crushed leaves of *Ocimum* sp. 0.1%	176.50	185.20	195.00	184.60	198.30
Turmeric powder 0.2%	194.90	185.00	214.77	212.44	215.72
$CaSO_4$ (homeo medicine)	199.40	199.90	161.68	145.53	127.53
Oxytetracycline hydrochloride (40 ppm)	195.10	206.60	210.00	215.00	220.00
Control	188.90	183.90	160.74	142.84	124.71
CD (0.05)	NS	NS	22.515	16.709	29.670

0.5 %, and the check treatment oxytetracycline hydrochloride (40 ppm) with a mean pollen storage area of 29.73 cm^2, 28.14 cm^2 and 29.14 cm^2 respectively.

The mean pollen storage area was significantly different in colonies treated with crushed leaves of *C. asiatica* 0.1 % with a mean area of 18.80 cm^2 which was on par with crushed leaves of *C. asiatica* 0.05 %, crushed leaves of *Ocimum* sp. 0.1 % and $CaSO_4$ (homeo medicine), the mean pollen storage area being 20.21 cm^2, 24.60 cm^2 and 25.23 cm^2 respectively in one WAT.

In two WAT, the colonies treated with crushed garlic 0.25 % recorded highest mean pollen storage area of 36.11 cm^2, which was on par with crushed garlic 0.5 %, crushed leaves of *Ocimum* sp. 0.05 % and the check oxytetracycline hydrochloride (40 ppm) with a mean area of 33.10 cm^2, 32.74 cm^2 and 30.74 cm^2 respectively. Colonies treated with $CaSO_4$ recorded the lowest mean area of 21.93 cm^2. This was on par with crushed leaves of *C. asiatica* 0.05 %, crushed leaves of *C. asiatica* 0.1 % and crushed leaves of *Ocimum* sp. 0.1 %, the mean pollen storage area being 21.93 cm^2, 22.40 cm^2 and 25.76 cm^2 respectively.

In three WAT, the mean pollen storage area was highest for the colonies treated with crushed garlic 0.25 % with the mean area 42.34 cm^2 which was on par with oxytetracycline hydrochloride (40 ppm) having a mean area of 38.85 cm^2. Lower mean pollen storage area was obtained for the control treatment (17.39 cm^2). This was on par with $CaSO_4$, crushed leaves of *C. asiatica* 0.1% and crushed leaves of *C. asiatica* 0.05 % with a mean pollen storage area of 19.70 cm^2, 20.37 cm^2 and 21.15 cm^2 respectively.

The mean pollen storage area of the diseased Indian bee colonies has reached its peak for the colonies treated with crushed garlic 0.25 % (49.19 cm^2) in 4 WAT. This was on par with crushed leaves of *Ocimum* sp. 0.05 % (47.70 cm^2). Lowest mean pollen storage area was recorded from the control treatment (14.88 cm^2) which was on par with crushed leaves of *C. asiatica* 0.1 % and $CaSO_4$, the mean pollen storage being 16.79 cm^2 and 17.86 cm^2 respectively.

Table 22. Pollen storage (cm^2) of the treated Indian bee colonies

Treatment	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	29.41	34.17	36.11	42.34	49.19
Crushed garlic 0.5%	25.85	28.14	33.10	36.82	39.48
Crushed leaves of *Centella asiatica* 0.05%	20.84	20.21	21.93	21.15	22.57
Crushed leaves of *Centella asiatica* 0.1%	17.13	18.80	22.40	20.37	16.79
Crushed leaves of *Ocimum* sp. 0.05%	28.94	29.73	32.74	35.88	47.70
Crushed leaves of *Ocimum* sp. 0.1%	23.70	24.60	25.76	32.12	35.56
Turmeric powder 0.2%	30.71	26.63	28.41	34.15	37.13
$CaSO_4$ (homeo medicine)	26.20	25.23	21.93	19.70	17.86
Oxytetracycline hydrochloride (40 ppm)	26.01	29.14	30.74	38.85	42.77
Control	32.00	31.33	22.05	17.39	14.88
CD (0.05)	NS	6.447	6.573	5.28	4.634

3.3.6 Effect of Botanicals on the Honey Storage of Treated Indian Bee Colonies

The results of mean honey storage area in the treated Indian bee colonies are presented in the Table 23.

Highest mean honey storage area was recorded for the diseased Indian bee colonies treated with crushed leaves of *C. asiatica* 0.05 % (58.69 cm^2) at one WAT. This was on par with crushed garlic 0.25 % and crushed garlic 0.5 % which had the mean area of 56.70 cm^2 and 55.44 cm^2 respectively.

In two WAT, the mean honey storage was recorded highest for the Indian bee colonies treated with crushed garlic 0.25 % with a mean of 66.88 cm^2. This was found significantly different from the other treatments. The mean honey storage area was lowest for the control treatment (48.12 cm^2) which was on par with $CaSO_4$ (53.34 cm^2) and crushed leaves of *C. asiatica* 0.1 % (52.24 cm^2).

In three WAT, the mean honey storage area has reached its peak for the Indian bee colonies treated with crushed garlic 0.25 % (76.49 cm^2). This was found significantly different from the other treatments. The next higher mean was recorded for treatment with crushed leaves of *Ocimum* sp. 0.05 % with the mean area 66.69 cm^2, which was also found significantly different from the other treatments. Lowest mean honey storage area was obtained for $CaSO_4$ (51.70 cm^2), which was on par with crushed leaves of *C. asiatica* 0.1 % and turmeric powder 0.2 %, the mean honey storage area being 53.78 cm^2 and 56.01 cm^2 respectively.

The mean honey storage area has attained the peak for the Indian bee colonies treated with crushed garlic 0.25 % (84.74 cm^2) in four WAT. This was on par with crushed leaves of *Ocimum* sp. 0.05 % with a mean area of 77.13 cm^2. Lowest mean honey storage was obtained for $CaSO_4$ (47.59 cm^2) which was on par with crushed leaves of *C. asiatica* 0.1 % and crushed garlic 0.5 % with a mean area of 53.50 cm^2 and 52.92 cm^2 respectively.

Table 23. Honey storage (cm^2) of the treated Indian bee colonies

Treatments	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	55.96	56.70	66.88	76.49	84.74
Crushed garlic 0.5%	53.77	55.44	57.75	60.98	52.92
Crushed leaves of *Centella asiatica* 0.05%	56.11	58.69	58.92	58.08	57.93
Crushed leaves of *Centella asiatica* 0.1%	54.01	51.81	52.24	53.78	53.5
Crushed leaves of *Ocimum* sp. 0.05%	52.56	54.09	58.61	66.69	77.13
Crushed leaves of *Ocimum* sp. 0.05%	53.47	54.70	59.60	60.39	60.13
Turmeric powder 0.2%	54.17	52.15	55.01	56.01	59.56
$CaSO_4$ (homeo medicine)	54.48	53.44	53.34	51.70	47.59
Oxytetracycline hydrochloride (40 ppm)	52.91	49.20	55.37	57.45	60.79
Control	56.31	49.03	48.12	43.17	39.68
CD (0.05)	NS	3.657	5.333	5.254	9.975

3.3.7 Effect of Botanicals on the Number of Foragers in Treated Indian Bee Colonies

The number of foragers min^{-1} in the Indian bee colonies four weeks after treatment is detailed in Table 24. No significant variation among the treatments was observed in the mean number of foragers min^{-1} at one WAT.

The mean number of foraging bees was found significantly higher for the Indian bee colonies treated with crushed leaves of *Ocimum* sp. 0.05 % (2.20 bees min^{-1}) in two WAT. This was on par with crushed garlic 0.25 % and the check oxytetracycline hydrochloride (40 ppm) with a mean of 2.13 and 1.93 bees min^{-1} respectively. The mean number of foragers min^{-1} was observed lowest for $CaSO_4$ (homeo medicine) (0.80) which was on par with crushed leaves of *C. asiatica* 0.05 %, crushed garlic 0.5 % and crushed leaves of *C. asiatica* 0.1 % with a mean of 0.93, 1.00 and 1.07 bees min^{-1} respectively in two WAT.

In three WAT, the maximum mean number of foragers entering into the Indian bee colony was observed for treatments with crushed garlic 0.25 % (2.80 bees min^{-1}). This was on par with crushed leaves of *Ocimum* sp. 0.05 % and oxytetracycline hydrochloride (40 ppm) with a mean of 2.60 and 2.47 bees min^{-1} respectively. The minimum number of foragers min^{-1} was observed for the treatment, crushed leaves of *C. asiatica* 0.05 % with a mean of 1.00 bee min^{-1} which was on par with $CaSO_4$ and crushed garlic 0.5% , the bees min^{-1} being 1.07 and 1.13 respectively in three WAT.

Maximum number of foraging bees entering into the Indian bee colony was recorded for the colonies treated with crushed garlic 0.25 % (3.13 bees min^{-1}) in four WAT. This was on par with crushed leaves of *Ocimum* sp. 0.05 % and oxytetracycline hydrochloride (40 ppm) with a mean of 3.00 and 2.93 bees respectively. Lowest number of foragers was recorded for the treatment $CaSO_4$ (1.07 bees min^{-1}) which was on par with crushed leaves of *C. asiatica* 0.05 % and crushed leaves of *C. asiatica* 0.1 % with a mean of 1.13 and 1.60 bees min^{-1} respectively.

Table 24. Number of foragers per minute in the treated Indian bee colonies

Treatments	Pre-count	Post count			
		1 WAT	2 WAT	3 WAT	4 WAT
Crushed garlic 0.25%	1.00	1.47	2.13	2.80	3.13
Crushed garlic 0.5%	0.93	1.13	1.00	1.40	1.93
Crushed leaves of *Centella asiatica* 0.05%	0.87	1.00	0.93	1.00	1.13
Crushed leaves of *Centella asiatica* 0.1%	0.93	0.93	1.07	1.67	1.6
Crushed leaves of *Ocimum* sp. 0.05%	1.13	1.60	2.20	2.60	3.00
Crushed leaves of *Ocimum* sp. 0.05%	1.00	1.13	1.67	1.60	2.07
Turmeric powder 0.2%	0.87	0.80	1.27	2.07	2.27
$CaSO_4$ (homeo medicine)	1.07	1.00	0.80	1.07	1.07
Oxytetracycline hydrochloride (40 ppm)	1.20	1.07	1.93	2.47	2.93
Control	1.00	0.93	0.93	1.13	1.13
CD (0.05)	NS	NS	0.408	0.453	0.556

Discussion

5. DISCUSSION

The present study entitled "Etiology of honey bee brood disease of Southern Kerala" was conducted at AICRP on Honey Bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani. The study was carried out with the objective to characterize, identify and confirm the infectivity of the microbial isolate from diseased Indian honey bee brood and also to test the efficacy of botanicals against it. The results obtained in the present study are discussed below.

5.1 ESTIMATION OF DISEASE INTENSITY

The survey was conducted in the apiaries of three southern districts of Kerala *viz.,* Thiruvananthapuram, Kollam and Pathanamthitta during 2016-2018, to estimate the intensity of brood disease affecting Indian honey bee (*Apis cerana indica).*

The symptoms observed during the study included scattered brood cells causing "pepper box symptom"; change in colour of the infected larvae from pearly white to yellow and finally brown; perforated cell cappings with dead pupae; the infected larvae decay in the cell and transform into dark brown scales and adhere to the bottom of the cells. Similar symptoms were observed by Bailey (1961) and Forsgren (2010) in bacterial disease infected *Apis mellifera* colonies in Western countries.

The symptoms observed in the present study was found identical to the symptoms reported by Bahman and Rana (2002) in the *A. mellifera* colonies while, Rana *et al.* (2004) and Singh (2005) reported similar symptoms from the *A. cerana indica* colonies of Himachal Pradesh, India.

Perforated cell cappings observed in the diseased colonies is an indication of the hygienic behaviour of the adult bees. Palacio *et al.* (2000) also

reported about the hygienic behaviour of the adult bees to prevent the spread of the disease within the colony.

Apart from these, reduced foraging activity of the worker bees, loss of their gentle temperament where they become more aggressive and a high tendency for absconding were also observed. There was also reduction in the honey yield from the infected Indian bee colonies. This is in accordance with the investigation of Rao (2009) in the *A. mellifera* colonies at Nauni, Solan.

Amritha *et al.,* (2014) reported that the Indian bee colonies infected with the brood disease in Kerala exhibited the symptoms *viz.*, scattered egg laying; uncapped cells; loss of appetite; colour change in larvae, more aggressive adult bees. These symptoms are similar to that of the present investigation.

In the present study, the disease incidence in the southern districts varied among different locations. Maximum disease incidence was observed during the dearth season (May to August) in Thiruvananthapuram district and the disease subsided after October. This may be due to the timely management measures taken by the bee keepers of the district. In Kollam district, the disease incidence attained its peak during the brood rearing season (September to December), which further receded in the honey flow season (January to April). This is in agreement with the finding of Shimanuki (1978), who has reported a drop in the disease incidence during the honey flow season in the *A. mellifera* colonies of U.S., due to the availability of ample food and active foraging population. While in Pathanamthitta district, the disease incidence was observed only in March and April in the current study.

In the brood rearing season, nurse bees aid in rapid spread of the disease to the early stage of the larvae through the bee bread fed to them. This is in harmony with the study conducted by Bahman and Rana (2002) where they also reported the incidence of brood disease during the colony building stage of honey bees which is due to the increased brood strength. The dearth season also favours the disease incidence which may be due to the stress conditions of the colony. An

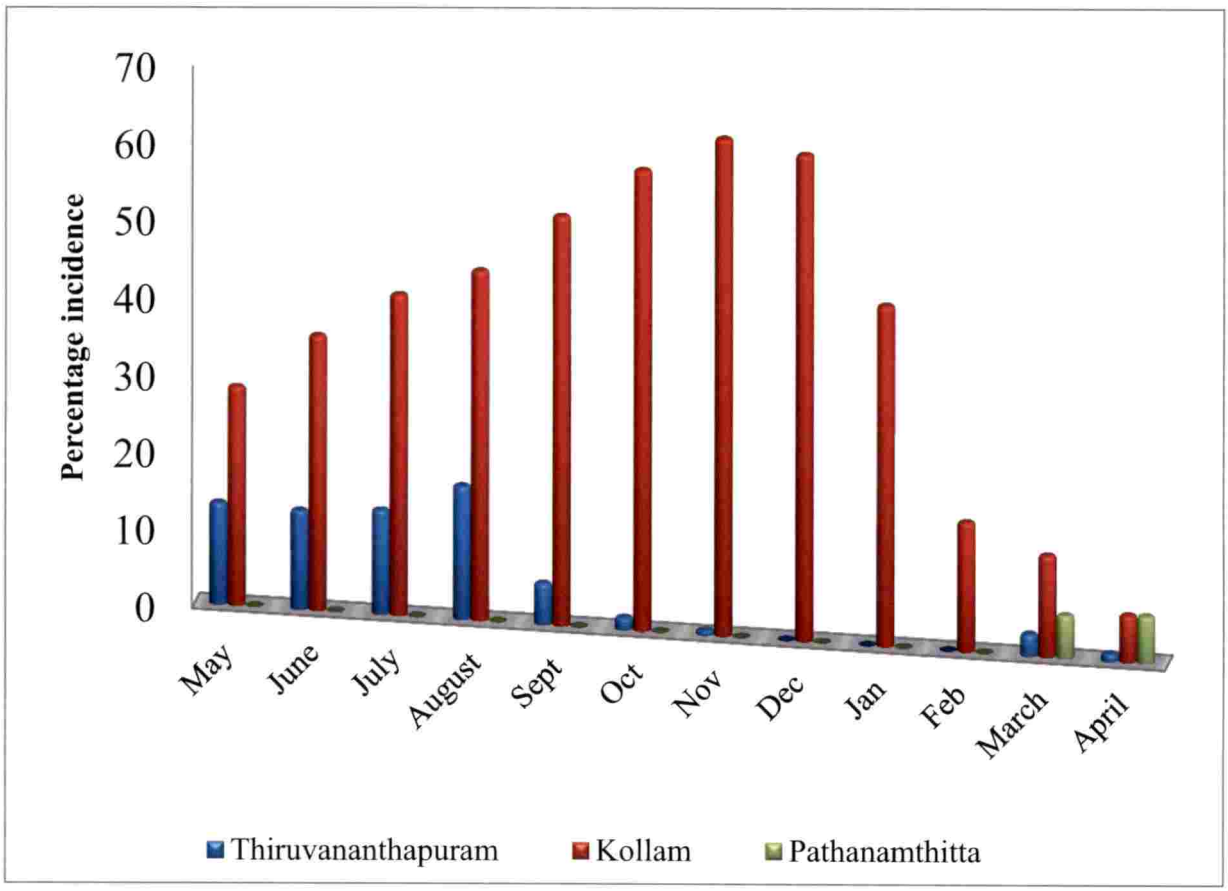

Fig.2 Percentage incidence (month wise) of Indian bee brood disease in Southern Kerala

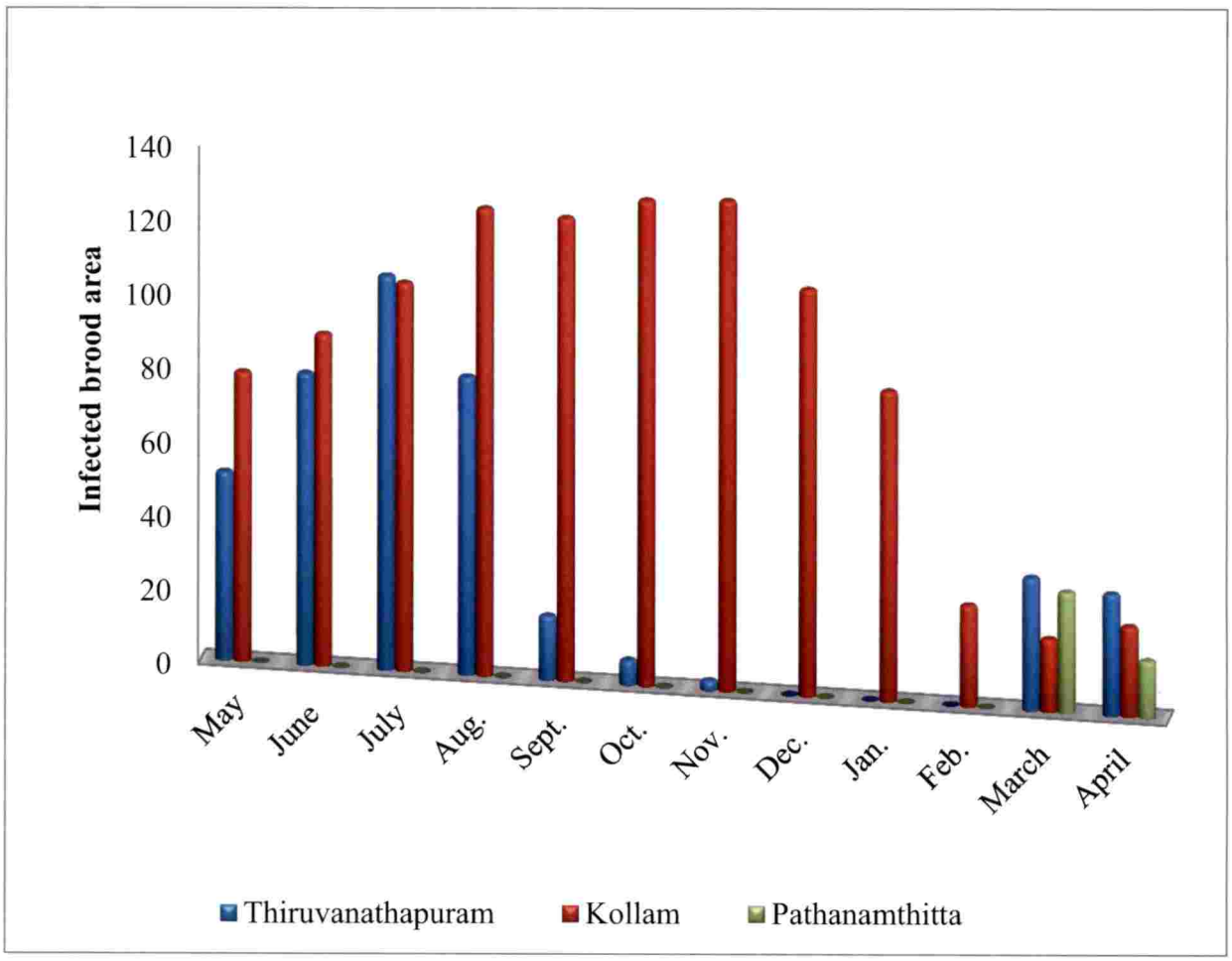

Fig.3 Infected brood area (cm^2) of the Indian bee apiaries in Southern Kerala

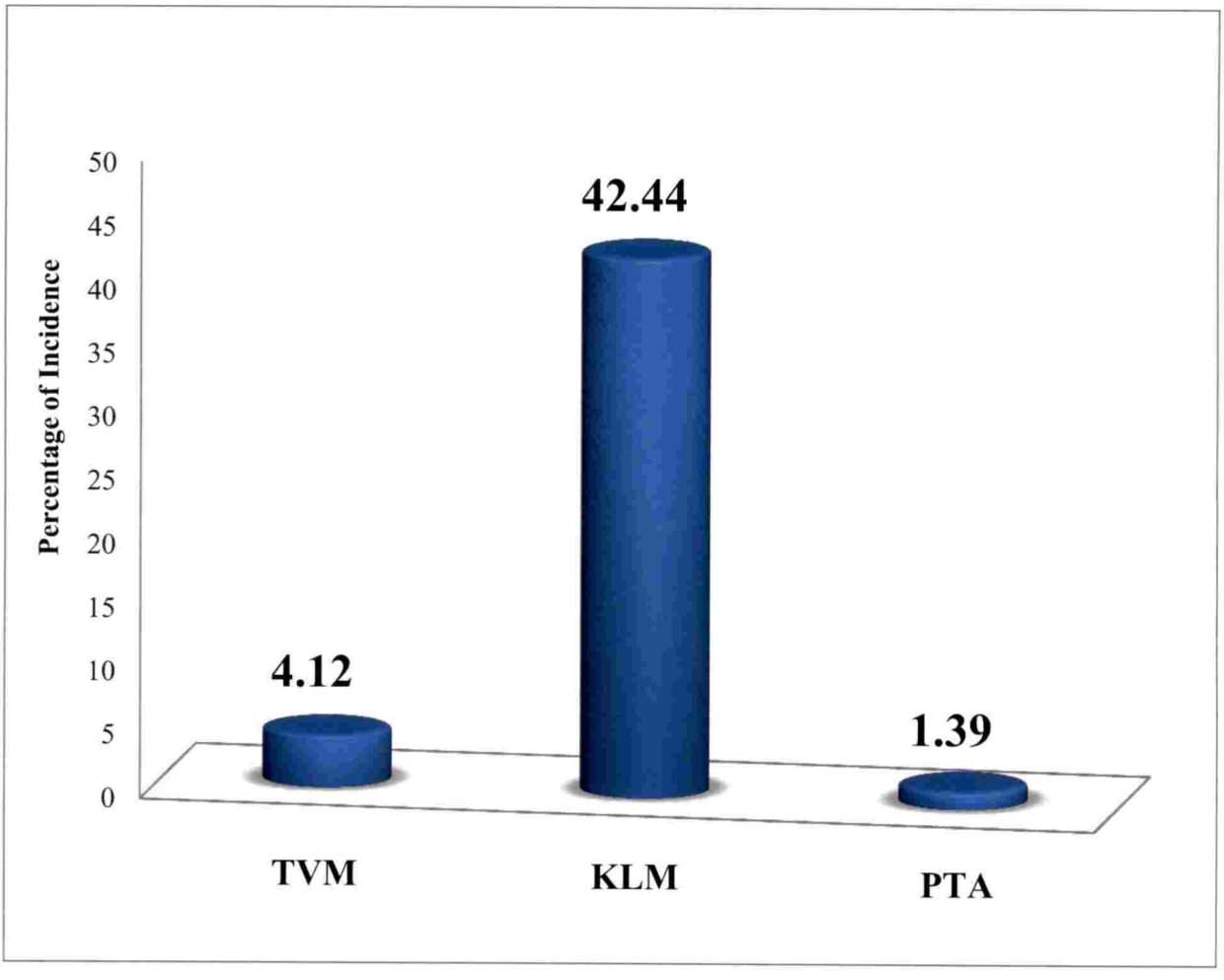

Fig. 4 District wise incidence of Indian bee brood disease in Southern Kerala

increased disease incidence has been reported by Somerville (2004) due to the stress factors affecting the bee colony such as nutritional deficiencies and shifting of bee colonies to newer locations.

Highest mean percentage incidence of the disease was recorded from the apiaries of Thiruvananthapuram district in August (17.26 %), while in Kollam district the peak stage was observed in November (64.18 %) (Fig. 2). Rao (2009) stated that the agroclimatic variations and variation in the strain of the pathogen causing the disease had a great influence on the disease incidence at various locations.

In the present study, highest infected brood area of the diseased Indian bee colonies was recorded in July (106.59 cm^2) from Thiruvananthapuram district. While in Kollam district, the peak brood area infection was observed in the month of November (132.56 cm^2) and that of Pathanamthitta it was more prominent in March (32.52 cm^2) (Fig. 3). Similar results were also reported in the study of Rana *et al.* (2004) in the *A. cerana indica* colonies maintained in Solan. According to the study conducted by Rao (2009), the maximum brood area infection was observed in September (18.52 %) in the *A. mellifera* colonies, while for the Indian bee colonies it was in August (25.33 %).

Thus in the present study, maximum disease incidence was recorded from the Indian bee apiaries of Kollam district with a mean percentage infection of 44.36, followed by Thiruvananthapuram district (4.45) and Pathanamthitta district (1.07) (Fig. 4).

5.2 ISOLATION, CHARACTERISATION AND IDENTIFICATION OF THE PATHOGEN

In the present study out of the three bacterial colonies isolated, two (J_1 and J_3) were proven pathogenic to bees in five days after inoculation. Similar works on the pathogenicity was done by Bailey (1957c) and Forsgren (2010) where they reported the appearance of symptoms 6-8 days after inoculation with

M. plutonius suspension along with the feed in *A. mellifera* colonies. Studies by Rao (2009) also revealed the appearance of symptoms in two days old larvae on 6th day of inoculation of *M. plutonius* in *A. cerana indica.*

The isolates with pathogenicity were identified by morphological, molecular and biochemical characterisation. Morphological characterisation was done by studying the colony characters and gram staining. The colony characters of J_1 isolate were yellow in colour with irregular margins and spreading appearance, 0.311 µm in size and were gram positive. The bacteria were identified as *Bacillus pumilus* which is in accordance with the description by Logan and Vos (2009).

The J_3 isolate colonies were cream in colour, transperent with glassy appearance, 0.212 µm in size and were gram negative in nature. These were found similar to the morphological characters of *Achromobacter* sp. Erler *et al.* (2018) has reported the bacterium, *Achromobacter eurydice* which was isolated along with *Melissococcus plutonius*, the major causative organism of European Foul Brood (EFB) disease, from the infected colonies of *A. mellifera.*

For further identification, the isolates were subjected to molecular characterisation. The J_1 isolate was showing 99 per cent similarity to *Bacillus pumilus* and J_3 isolate was showing 91 per cent similarity to *Achromobacter* sp. Govan *et al.* (1998) has developed the PCR technique in *A. mellifera* for the identification of *M. plutonius* and *P. larvae* in the pure cultures of the bacterium, infected bee larvae and crude cell lysates.

Bailey (1963) reported *Bacillus* sp. as a secondary invader of the European foul brood disease of honey bees. Gilliam and Morton (1978) found *B. pumilus* as the most frequently isolated bacteria from the honey bees. *B. pumilus* was isolated from the worker bees of the bacterial disease infected honey bee colonies (Gilliam, 1997). Gaggia *et al.* (2015) isolated *B. pumilus* from the EFB infected honey bee larvae.

In the present study, J_1 isolate was showing higher similarity to *B. pumilus* and *B. cereus.* Also, it was showing relation to the causative agent of EFB, *M. plutonius.* The Phylogenetic relationship of *M. plutonius* and *Enterococcus* sp. was reported by Cai and Collins (1994). Jeyaprakash *et al.* (2003) also studied the phylogeny of different bacteria in the adult worker bees of *A. mellifera* and identified the presence of *M. plutonius* and *Bacillus* sp.

Thus, in the present investigation the isolated bacteria were identified to be *Bacillus pumilus* and *Achromobacter* sp. respectively, which were reported as the secondary bacteria of the European Foul Brood (EFB) disease of honey bees, caused by *M. plutonius.*

5.3 FIELD EVALUATION OF BOTANICALS

Administration of antibiotics was recommended for the management bacterial brood disease infecting the honey bees. Oxytetracycline is the commonly used antibiotic for the management of the bacterial disease of honey bees. Bahman and Rana (2002) were able to control the EFB disease by feeding oxytetracycline @ 200 mg in *A. mellifera* colonies in eight days after feeding. Feeding ciprofloxacin @ 40 mg along with the sugar solution has resulted in cent per cent reduction of the EFB disease in *A. mellifera* colonies after 9 days of feeding (Rao *et al.,* 2011).

Studies by Solomon *et al.* (2006) reported a reduction in the honey export from India due to the presence of antibiotic residue in honey. Thus, it is necessary to develop an alternate method for the management of brood disease. Therefore, present study focuses on the evaluation of botanicals for the management of brood disease of honey bees.

In the present study, the field evaluation of the botanicals against the brood disease revealed a significant reduction in the disease of the colonies treated with crushed garlic 0.25 % (68.52 %) over the pre-count observation followed by

the treatment with crushed leaves of *Ocimum* sp. 0.05 % with 67.57 per cent reduction in the disease in four WAT (Fig. 5).

The infected brood area of the Indian bee colonies treated with crushed garlic 0.25 % was reduced to 2.78 cm^2 in four WAT. The treatment with crushed leaves of *Ocimum* sp. 0.05 % has shown a reduction in infected brood area 3.76 cm^2 after four weeks of treatment, whereas the control treatment had an infected brood of 18.44 cm^2 in four WAT (Fig. 6).

The role of garlic in the improvement of brood health of *A. mellifera* colonies and also its effect in the management of *Nosema apis* parasite was demonstrated by Bura *et al.* (2004). Chaimanee *et al.* (2017) has found that garlic has an inhibitory effect on *Ascosphaera apis*, causal organism of chalk brood disease of honey bees. They also studied the effect of garlic against the pathogen of American Foul Brood (AFB) disease, *Peanibacillus larvae*.

Ocimum basilicum was found effective in the management of the bacterium *P. larvae*, causative agent of AFB (Albo *et al.,* 2003). The anti-bacterial property of *O. sanctum* was reported against *Bacillus cereus* and *Bacillus subtilis* (Joshi *et al.,* 2009). Marghitas *et al.* (2011) found that the extract of *O. basilicum* has enough anti-microbial property to control the *P. larvae* causing AFB. *O. tenuiflorum* was found effective in the management of the bacterial diseases of honey bees (Ansari *et al.,* 2016). These studies are in account with the results obtained in the present study.

The findings of the present study revealed an increase in the brood in terms of egg and larvae (unsealed), pupae (sealed) of the colonies treated with crushed garlic 0.25 % by 286.07 cm^2 and 236.80 cm^2 in four WAT respectively. The colonies treated with crushed leaves of *Ocimum* sp. 0.05 % also resulted in an increase in the sealed brood area with 261.32 cm^2 in 4 WAT. The control treatment was showing a reduction in the sealed and unsealed brood by 34.62 cm^2 and 124.71 cm^2, in four WAT respectively (Fig.7).

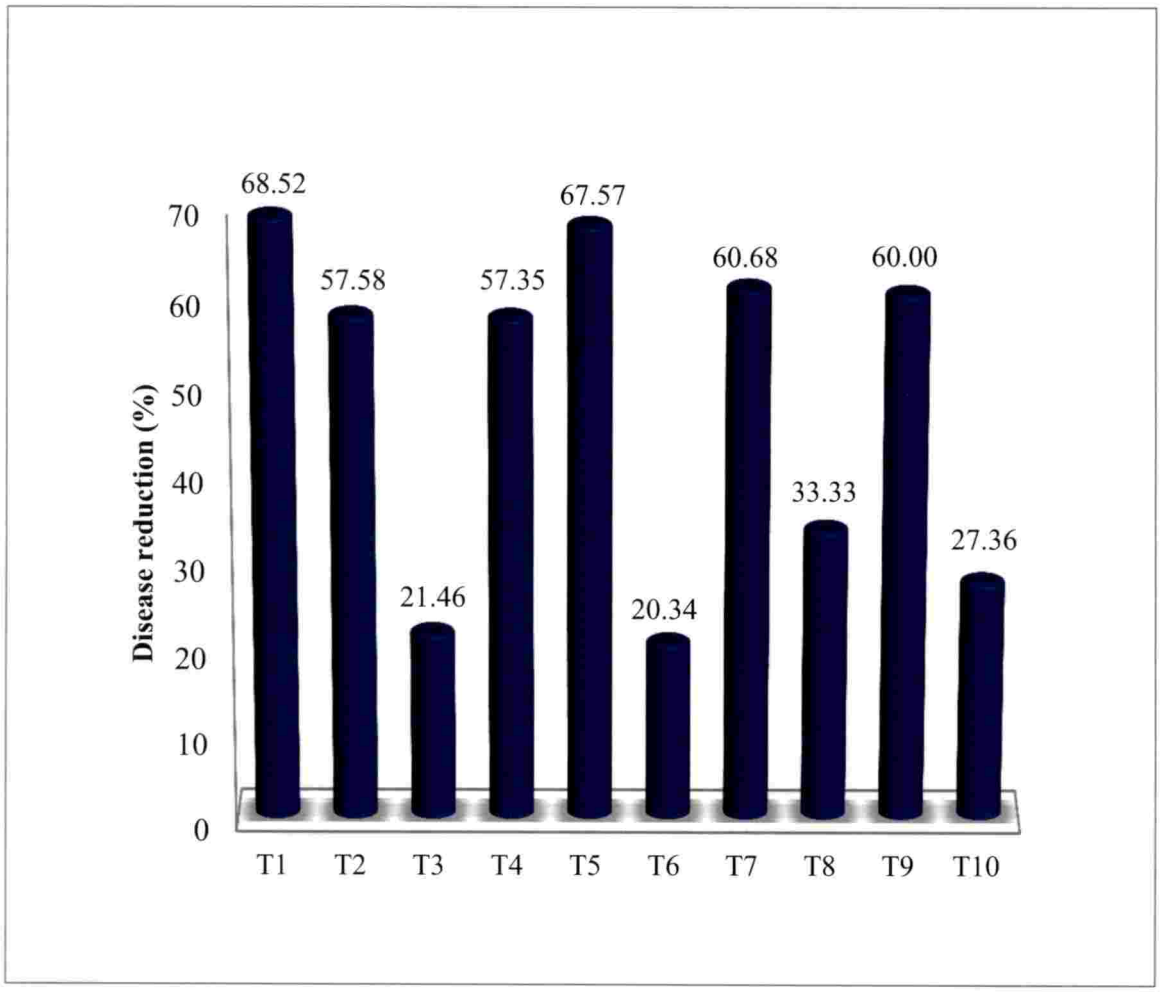

Fig. 5 Percentage reduction in the infected brood cells of Indian bee colonies (4WAT)

T_1 : Crushed garlic 0.25 %

T_2 : Crushed garlic 0.5 %

T_3 : Crushed leaves of *Centella asiatica* 0.05 %

T_4 : Crushed leaves of *Centella asiatica* 0.1 %

T_5 : Crushed leaves of *Ocimum* sp 0.05 %

T_6 : Crushed leaves of *Ocimum* sp 0.1 %

T_7 : Turmeric powder 0.2 %

T_8 : $CaSO_4$ 8 tablets per 250 mL

T_9 : Oxytetracycline hydrochloride 40 ppm

T_{10} : Untreated

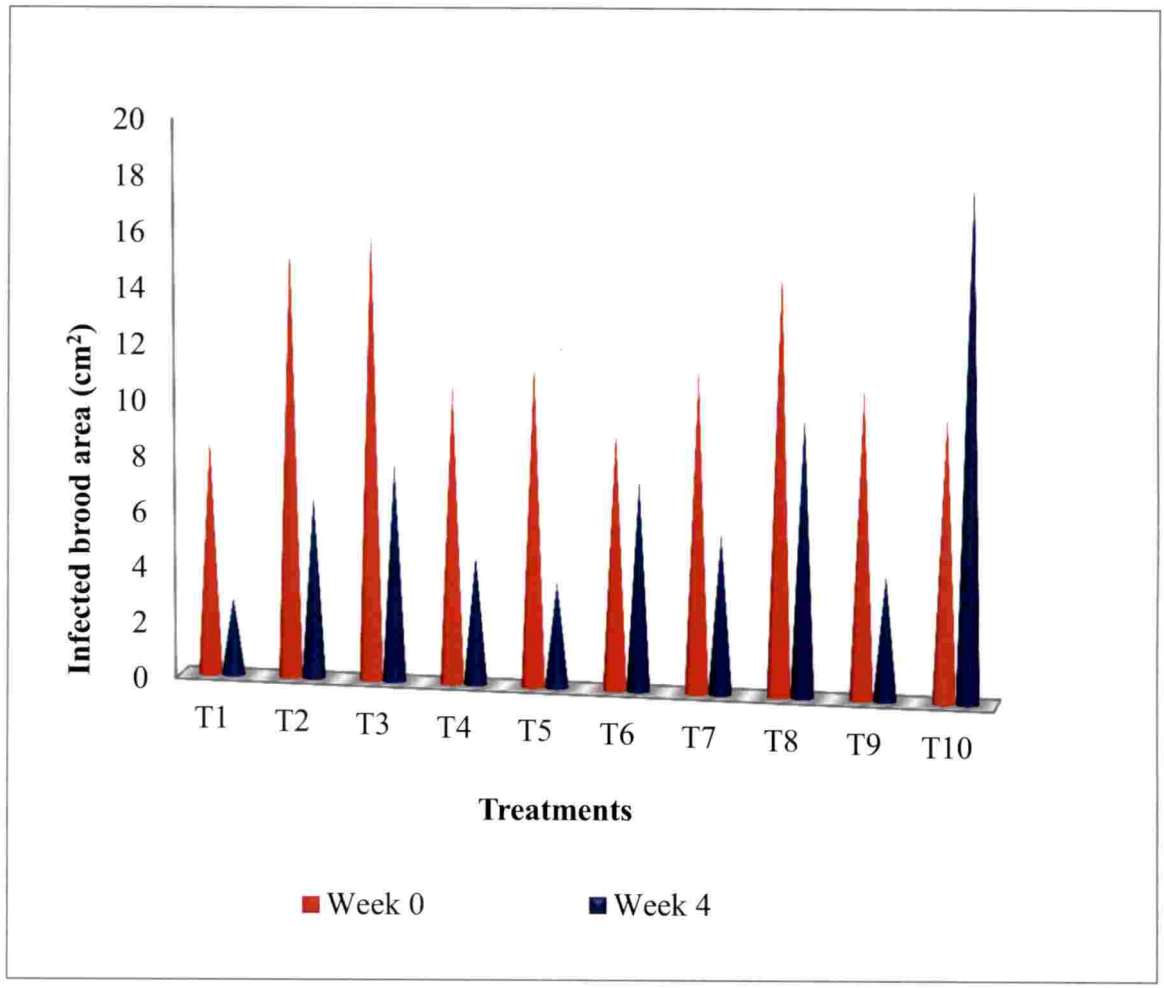

Fig. 6 Infected brood area (cm^2) before and four weeks after treatment

T_1 : Crushed garlic 0.25 %	T_6 : Crushed leaves of *Ocimum* sp 0.1 %
T_2 : Crushed garlic 0.5 %	T_7 : Turmeric powder 0.2 %
T_3 : Crushed leaves of *Centella asiatica* 0.05 %	T_8 : $CaSO_4$ 8 tablets per 250 mL
T_4 : Crushed leaves of *Centella asiatica* 0.1 %	T_9 : Oxytetracycline hydrochloride 40 ppm
T_5 : Crushed leaves of *Ocimum* sp 0.05 %	T_{10} : Untreated

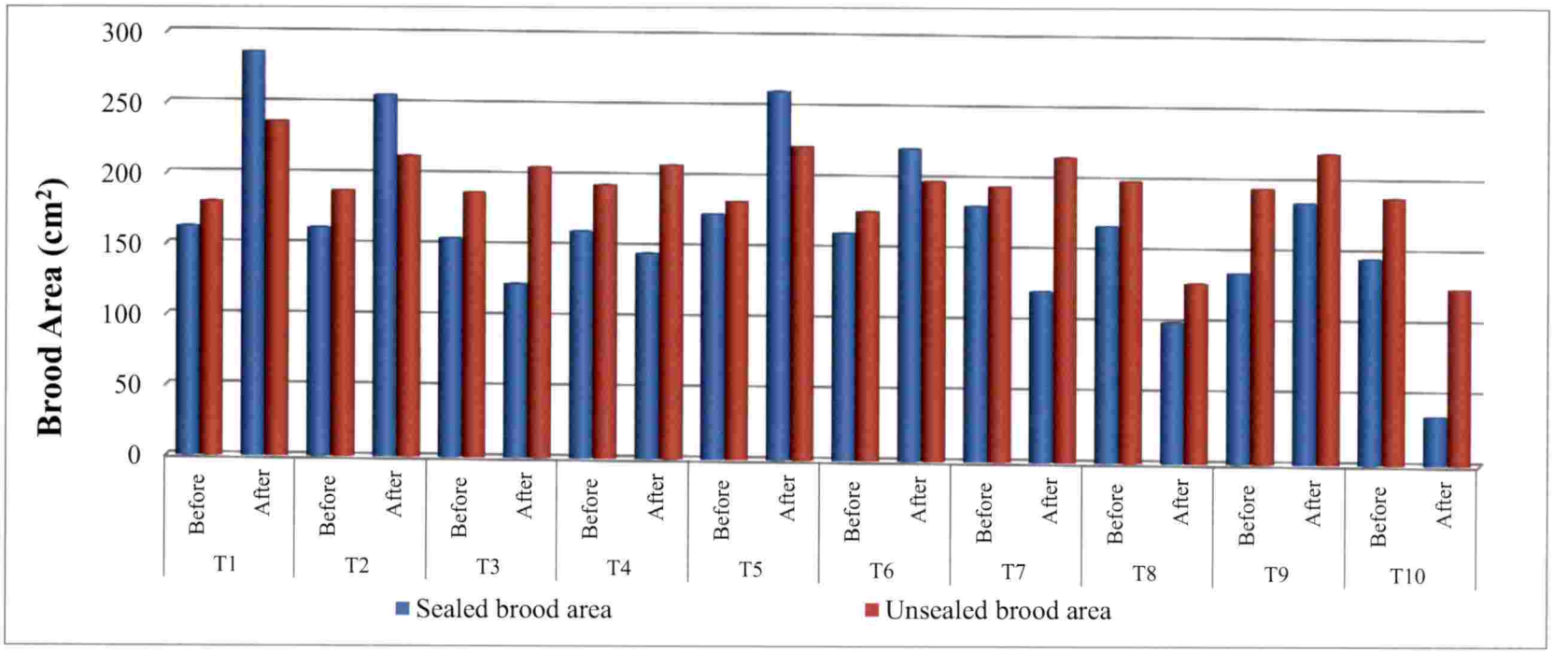

Fig. 7 Brood area (sealed and unsealed) of the Indian bee colonies before and four weeks after treatment

T_1 : Crushed garlic 0.25 %

T_2 : Crushed garlic 0.5 %

T_3 : Crushed leaves of *Centella asiatica* 0.05 %

T_4 : Crushed leaves of *Centella asiatica* 0.1 %

T_5 : Crushed leaves of *Ocimum* sp 0.05 %

T_6 : Crushed leaves of *Ocimum* sp 0.1 %

T_7 : Turmeric powder 0.2 %

T_8 : $CaSO_4$ 8 tablets per 250 mL

T_9 : Oxytetracycline hydrochloride 40 ppm

T_{10} : Untreated

An increase in the pollen storage and honey storage was also observed in the current study. The pollen storage was increased in the colonies treated with crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 % by 49.19 cm^2 and 47.70 cm^2 in four WAT respectively. The honey storage area was increased by 84.74 cm^2 and 77.13 cm^2 in four WAT for the colonies treated with crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 % respectively. While in the colonies maintained as control, a decrease in the pollen storage and honey storage of 14.88 cm^2 and 39.68 cm^2 four WAT respectively was observed (Fig. 8).

The management of honey bee disease using botanicals are practised and studied by the bee keepers and researchers globally. Patruica *et al.* (2017) studied the effect of garlic extract in the health of honey bee colonies. He reported that garlic extract has stimulated the egg laying by the queen and reduced the bacterial load in the gut of the worker bees. In the current study also, the use of garlic has shown an increase in the sealed and unsealed brood area representing more egg laying by the queen bee.

The use of turmeric by the beekeepers for the management of honey bee disease has already been reported by the researchers (Saville, 2000). The antibacterial property of turmeric was reported to be due to the presence of curcumin (Tajbakhsh *et al.*, 2008). Strachecka *et al.* (2015) found that curcumin stimulates biochemical mechanisms of resistance and extends the life of *A. mellifera.* The antibacterial property of different medicinal plants against the honey bee diseases were studied and was found effective in the management of honey bee diseases (Khan *et al.*, 2018).

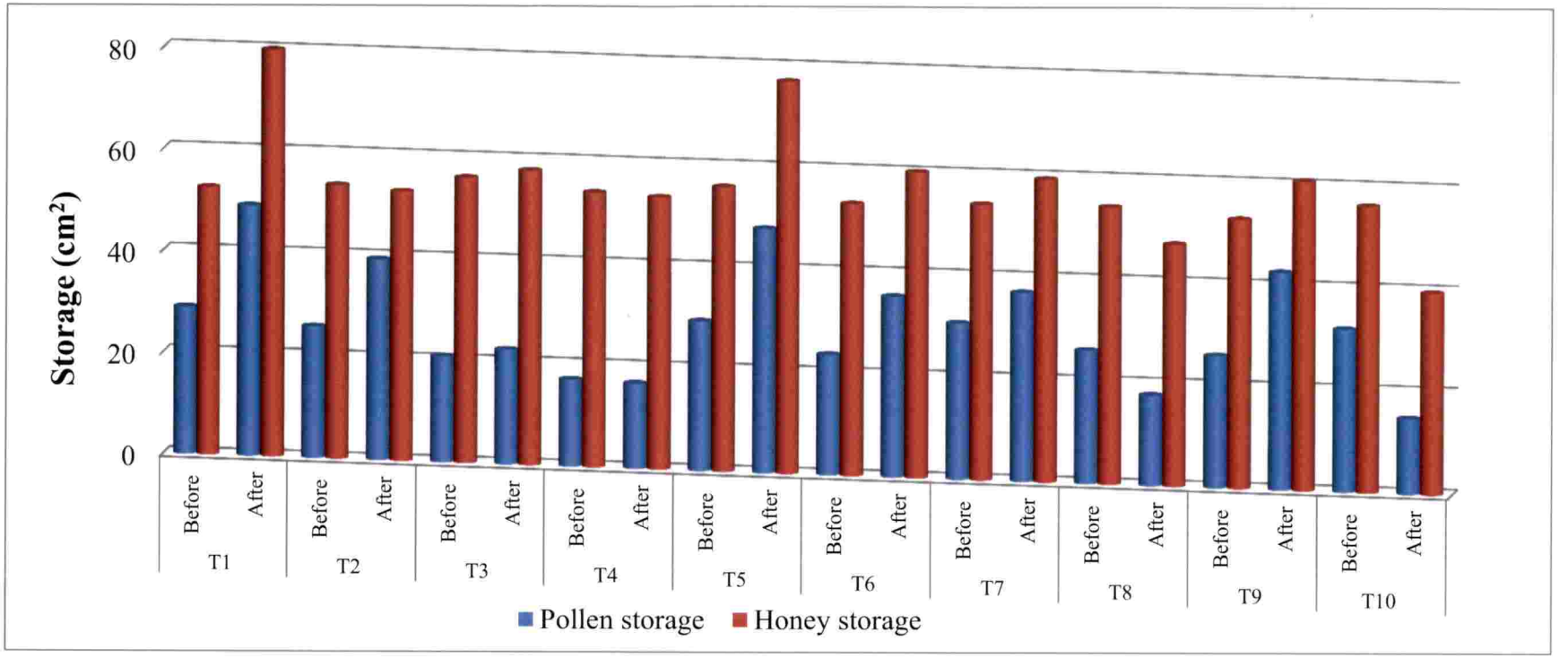

Fig. 8 Honey and pollen storage (cm^2) of the Indian bee colonies before and after four weeks of treatment

T_1 : Crushed garlic 0.25 %	T_6 : Crushed leaves of *Ocimum* sp 0.1 %
T_2 : Crushed garlic 0.5 %	T_7 : Turmeric powder 0.2 %
T_3 : Crushed leaves of *Centella asiatica* 0.05 %	T_8 : $CaSO_4$ 8 tablets per 250 mL
T_4 : Crushed leaves of *Centella asiatica* 0.1 %	T_9 : Oxytetracycline hydrochloride 40 ppm
T_5 : Crushed leaves of *Ocimum* sp 0.05 %	T_{10} : Untreated

Summary

6. SUMMARY

The present investigation on "Etiology of honey bee brood disease in Southern Kerala" was conducted at AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani with the objective to characterize, identify and confirm the infectivity of the microbial isolate from diseased Indian honey bee brood and also to test the efficacy of botanicals against it. The study was carried out as three separate experiments that includes estimation of the Indian bee brood disease in southern Kerala; isolation, characterisation and identification of the pathogen responsible for the disease and the field evaluation of botanicals for the management of the Indian bee brood disease.

Survey was conducted in the apiaries of three districts in southern Kerala *viz.* Thiruvananthapuram, Kollam and Pathanamthitta. Five apiaries with a minimum of ten colonies were selected from each district. The symptoms observed during the survey period includes, the presence of sealed and unsealed brood scattered in the comb giving "pepper-box symptom", change in colour of the larvae, perforated cell cappings with dead pupae inside, the infected larvae decay inside the cell and finally become dark brown scales that adhere to the bottom of the cells. Apart from these symptoms, the adult bees become restless and loss their gentle temperament, high tendency for absconding and reduced honey yield were also recorded.

The monthly incidence of the Indian bee brood disease varied significantly among the three districts. The peak incidence was observed during the dearth season (May to August) from the apiaries of Thiruvananthapuram whereas in the apiaries of Kollam district the incidence was highest during both dearth season (May to Aug.) and brood rearing season (September to December). The disease incidence in Pathanamthitta district was recorded only in March and April.

Highest mean percentage infection of the brood disease in Southern Kerala was recorded from the apiaries of Kollam district (44.36 %) followed by

Thiruvananthapuram (4.45 %) and the least infection was recorded from the apiaries of Pathanamthitta (1.07 %).

The bee strength of the colonies was also severely affected due to the disease incidence. The bee strength was greatly reduced in the apiaries of Kollam district during the brood rearing season (Sept. to Dec.), while in Thiruvananthapuram district; it was severe in the dearth season (May to Aug.).

The infected brood area of the diseased Indian bee colonies was also determined. Highest infected brood area was recorded in July (106.59 cm^2) from the apiaries of Thiruvananthapuram district while in Kollam district the brood area infected attained the peak in November (132.56 cm^2). The brood area infection was very low in Pathanamthitta district compared to other two districts. The infected brood area was showing similar variation as that of percentage incidence of the disease.

The infected brood samples collected from the apiaries were subjected to the laboratory study. Three bacterial colonies were isolated *viz.* J_1, J_2 and J_3 in the nutrient agar (NA) media. Out of the three isolates, J_1 and J_3 isolates which proved the pathogenicity test were taken for morphological, molecular and biochemical characterisation.

The molecular characterisation of the isolates was done by sequencing the 16S rRNA region of the bacterial genome. The BLAST search of amplified DNA in the NCBI data base resulted 99 per cent and 91 per cent homology of J_1 and J_3 isolate with *Bacillus pumilus* and *Achromobacter* sp. respectively. The identity of the bacteria was further confirmed by the biochemical analysis of the isolates.

Field evaluation of botanicals *viz.*, crushed garlic (0.25 % and 0.5 %), crushed leaves of *Centella asiatica* (0.05 % and 0.1 %), crushed leaves of *Ocimum* sp. (0.05 % and 0.1 %), turmeric powder 0.2 %; $CaSO_4$ (homeo medicine) along with oxytetracycline hydrochloride (40 ppm) as check and a control treatment provided through artificial feed (sugar solution 1:1), has revealed a significant reduction in the infected brood area of colonies treated with

crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 %, in four weeks after treatment.

The infected colonies treated with crushed garlic 0.25 % and that with crushed leaves of *Ocimum* sp. 0.05 % were exhibiting a significant increase in the sealed brood area, unsealed brood area, pollen storage area and honey storage area compared to the control treatment in four weeks after treatment. An increase in the number of foragers min^{-1} was also recorded from the infected colonies for the above treatments.

Thus, the present study has revealed that the brood disease of Indian bee has emerged as a serious problem in the apiaries of Southern Kerala with the peak infection recorded from the apiaries of Kollam district (44.36 %) followed by Thiruvananthapuram (4.45 %) and least from Pathanamthitta (1.07 %). The bacterial isolates which proved the pathogenicity were identified as *Bacillus pumilus* and *Achromobacter* sp. Crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 % were found effective against the brood disease with a percentage reduction of 68.52 and 67.57 respectively.

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Abstract

ETIOLOGY OF HONEY BEE BROOD DISEASE IN SOUTHERN KERALA

by

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ABSTRACT

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ABSTRACT

The study entitled "Etiology of honey bee brood disease in Southern Kerala" was conducted with the objective to characterize, identify and confirm the infectivity of the microbial isolate from diseased Indian honey bee brood and also to test the efficacy of botanicals against it. The study was carried out during the period 2016-2018 at AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani.

Survey was conducted in the Indian bee apiaries of three Southern districts of Kerala *viz.* Thiruvananthapuram, Kollam and Pathanamthitta. From each district, five apiaries with a minimum of 10 colonies were selected purposively. The infected brood samples were subjected to laboratory study to understand the etiology of the disease. Evaluation of botanicals against the Indian bee brood disease using different botanicals was also conducted.

Scattered presence of sealed and unsealed brood giving "pepper-box symptom", change in colour of the larvae from pearly white to yellow and finally dark brown, perforated brood cappings with dead pupae and infected larvae transform into brown scales which were seen adhered to the bottom of the cell, were the typical symptoms observed in the infected bee colonies. Apart from these, the adult bees were found restless and more aggressive, reduced foraging activity, tendency for absconding and reduced honey yield were also observed.

The monthly incidence of the disease varied among different locations with the peak infection period during the dearth season (May to August) in Thiruvananthapuram district, while in Kollam district it was observed in both dearth season and brood rearing season (Sept. to Dec.). Among the three districts, highest mean percentage infection was recorded from the apiaries of Kollam district (44.36) followed by Thiruvananthapuram district (4.45) and the least infection percentage from the apiaries of Pathanamthitta district (1.07).

Three bacterial colonies were isolated (*viz.* J_1, J_2 and J_3 isolates) in the nutrient agar (NA) medium from the infected brood samples taken during survey. The bacterial isolates, J_1 and J_3 which were proved pathogenic to the honey bee larvae were subjected to morphological, molecular and biochemical characterisation. The bacteria, identified as *Bacillus pumilus* and *Achromobacter* sp., has already been reported to be associated with *Melissococcus plutonius*, causal agent of European Foul Brood.

Evaluation of botanicals for the management of Indian bee brood disease with crushed garlic (0.25 % and 0.5 %), crushed leaves of *Centella asiatica* (0.05 % and 0.1 %), crushed leaves of *Ocimum* sp. (0.05 % and 0.1 %), turmeric powder (0.2 %), $CaSO_4$ (homeo medicine), along with oxytetracycline hydrochloride (antibiotic) (40 ppm) as check and a control provided with artificial feed, was assessed in terms of infected brood area, sealed brood area, unsealed brood area, pollen storage, honey storage and number of foragers per minute. After four weeks of treatment, bee colonies treated with crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 % recorded highest percentage reduction of the disease with a mean value of 68.52 and 67.57 respectively.

Apart from these, a significant increase in sealed brood area (286.07 cm^2 and 261.32 cm^2), unsealed brood area (236.80 cm^2 and 222.22 cm^2), pollen storage (49.19 cm^2 and 47.70 cm^2), honey storage (84.74 cm^2 and 77.13 cm^2) and number of foragers (3.13 and 3 bees per minute) were also observed in the colonies treated with crushed garlic 0.25 % and crushed leaves of *Ocimum* sp. 0.05 % respectively, when compared to their respective control (34.62 cm^2; 124.71 cm^2; 14.88 cm^2; 39.68 $cm^2$and 1.13 bees per minute).

Thus the present investigation revealed that the Indian bee apiaries of Kollam district recorded the highest incidence of brood disease in southern Kerala during the dearth and brood rearing season, with a mean percentage infection of 44.36 followed by Thiruvananthapuram district (4.45) and Pathanamthitta district (1.07) Two bacterial isolates which proved the pathogenicity were identified as

Bacillus pumilus and *Achromobacter* sp. Crushed garlic 0.25 % and crushed leaves of *Ocimum* sp 0.05 % were found effective in the field evaluation of botanicals, against brood disease, with a mean percentage reduction of 68.52 and 67.57 respectively.

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