# HISTOMORPHOLOGY OF INDIAN BEE (*Apis cerana indica* Fab.) SUPPLEMENTED WITH PROBIOTICS

*by*

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**THESIS**

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## DECLARATION

I, hereby declare that this thesis entitled **"HISTOMORPHOLOGY OF INDIAN BEE (*Apis cerana indica* Fab.) SUPPLEMENTED WITH PROBIOTICS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani,
09/08/2019

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# CERTIFICATE

Certified that this thesis entitled **"HISTOMORPHOLOGY OF INDIAN BEE (*Apis cerana indica* Fab.) SUPPLEMENTED WITH PROBIOTICS"** is a record of research work done independently by Ms. Akhila Pahee under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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| | |
|---|---|
| % | Per cent |
| @ | At the rate of |
| < | Less than |
| > | Greater than |
| °C | Degree celsius |
| E | East |
| N | North |
| µg | microgram |
| AICRP | All India Co- ordinated Research Project |
| cfu | Colony- forming units |
| *et al* | And other co workers |
| g | Gram |
| h | Hour |
| kg | Kilogram |
| L | Litre |
| min. | minute |
| mL | Millilitre |
| ng | nanogram |
| No. | Number |
| ppm | Parts per million |
| Sl. | Serial |
| sp. or spp. | Species (singular and plural) |
| *viz.*, | Namely |

# *Introduction*

# 1. INTRODUCTION

Bees popularly called as "Angels of Agriculture" are essentially recognised as the most important insects in the world. Honey bees are commercially important due to the economic value of its apicultural products. In addition, they significantly increase agricultural production by means of crop pollination process (Morse and Calderone, 2000).

*Apis cerana indica* Fab., the Indian honey bee is the popular honey bee species used for commercial beekeeping in Kerala. Maintaining these bee colonies in a healthy state throughout the year is one of the main concerns of beekeepers. In the dearth season, sugar syrup is used as the artificial feed for honey bees. Sugar can harm the honey bees by means of the impurities present in the commercial sugar (Roy, 1977). According to Doung (2014), raw sugar has a sucrose level of 99.50 per cent with an ash content of 0.2 per cent which can cause digestive issues to the honey bees.

Honey bees, the eusocial beneficial insects are reported to have many diseases. According to Gilliam (1997), there is a large diversity of disease causing microorganisms associated with honey bees. Recently, beekeeping in Kerala faced a serious threat when brood diseases resulted in great economic losses throughout the state. The disease incidence was reported as 5.4 to 63.63 per cent (Amritha *et al.*, 2014). Joseph (2018) also reported the occurrence of similar bacterial brood disease from southern districts of Kerala.

Honey bees are exposed to pesticides while collecting pollen and nectar from flowers, resins from various flora, drinking water from rivers or lakes or ponds etc., breathing and during flight (if the pesticides are airborne) (Mullin *et al.*, 2010). These pesticides are brought back unintentionally into the colony where its level sets concentrated further in the waxy comb (Gregorc and Ellis, 2011).

Studies conducted by Thiboldeaux *et al.* (1998) revealed that midgut is the most important site for terminal digestion and absorption of water and nutrients and even the pesticide molecules present in food can also be absorbed. Thus, the intestine can reveal the morphological alterations induced by environmental contaminants absorbed by the insect.

Probiotics are microorganisms that when consumed provide health benefits to the host. Being the dietary supplement, live bacteria such as *Lactobacillus*, maintain or restore beneficial bacteria in the digestive tract there by stimulating the intestinal defense mechanism in the host body (Corcionivoschi and Drinceanu, 2010). Probiotics have the ability to shape the immune system through their physiological action in the intestine. Once in the intestine, they interact with intestinal cells, triggering an immune response due to which the intestinal cells produce a series of immune- simulator molecules. Thus, the beneficial bacterial population of the bee digestive tract has very important implications for the improvement of bee health (Koch and Schmid-Hempel, 2011). Histomorphological tools have been widely employed for the diagnosis of numerous diseases of animals and have proved to be the most sensitive and specific method for the detection and the identification of numerous pathogens (Maiolino *et al.*, 2013).

The study on histological variations in Indian bee or the impact of probiotics in bee health has not been conducted in Kerala hitherto. The present study focusses to assess the histomorphology of midgut of Indian bee's (*Apis cerana indica* Fab.) suffering from various stressors (pathogens and insecticides) and after being fed with probiotics.

# *Review of Literature*

## 2. REVIEW OF LITERATURE

Productive insects are those important species which, in the normal course of their life activities, produce certain substance that are adopted by man for his own use. Honey bees are one of the most important beneficial insects. Honey bees in India are known mainly for the production of honey and to some extent as wax producers. Other valuable beehive products are pollen, royal jelly, bee venom and propolis. Apart from these rewards, they play an important role in pollination. Bees pollinate fruits, nuts, and vegetables. About ninety percentage of world's food supply is ensured by the bees (Deodikar and Suryanarayana, 1997).

Nowadays, bees are affected by a number of biotic and abiotic factors which affect honey bee health and productivity. Farmer practices, climate change, pests, pathogens, pesticides and its residue on hive environment, whose implications in insect health have been deeply studied.

In order to improve bee health, scientists are researching on the role of bee gut microbiota in health status. Generally lactic acid bacteria (LAB) has been proved to be important inhabitants of animal and human intestinal tracts as they have a multifaceted, antimicrobial potential (Audisio and Benitez- Ahrendts, 2015). The literature pertaining to the beekeeping scenario, major threats and probiotic concept in bees are reviewed here under.

### 2. 1 BEEKEEPING IN KERALA

Apiculture or beekeeping is the art and science of procuring or collecting honey bee species, hiving them in specified standard boxes, installing them in appropriate sites, managing optimum number of honey bees throughout the year and utilizing the benefits of their activities (Thakur, 2014).

The tropical climate and floral diversity of Kerala encourages successful beekeeping. *A. cerana indica* the Indian honey bee is the popular honey bee species used for commercial beekeeping in Kerala. Honey bees play a vital role in pollination of crops. Insect pollination has enhanced seed quality parameters and thus upgraded the quality as well as market value of the produce. By means of insect pollination, 75 per cent of crop species are benefited that contributed a global service worth $ 215 billion to food production. Premila *et al.* (2014) reported that pollination by *A. cerana indica* resulted 25 per cent yield increase in cucumber and it also enhanced the quality parameters of the crop. The total honey production in India during 2015- 2016 was 89 MT. The state has been contributing 70 per cent of this honey production (Kumar and Joy, 2017). As per the National Bee Board data (2019), the honey production in India is 1,50,000MT and Kerala about 2,000MT.

In Kerala, there is a green cover of wild and cultivated forest trees, food crops, ornamental and medicinal plants and plantation crops which supports the commercial beekeeping. Identification of rubber (*Hevea brasiliensis* Muell. Arg.) as a potent source of nectar was an important breakthrough in beekeeping. The extra floral nectaries of rubber produced after the commencement of new flushes serves as rich source of honey during January to April (Padmanabhan, 2003).

### 2. 1. 1 Diversity of Honey Bee in India

Honey bees belong to the order Hymenoptera, superfamily Apidae, family Apoidae and genus *Apis*. There are six species of *Apis viz., Apis cerana indica, A. florea, A. dorsata, A. andreniformis* and *A. laboriosa* which are indigenous to India while *A. mellifera* is an introduced species. Among these species, *A.cerana indica* and *A. mellifera* are widely used for commercial beekeeping in India (Thomas *et al.*, 2002)

### *2. 1. 1. 1 Rock Bee (Apis dorsata* Fab.)

It is the largest of honey bees. It builds often a single comb of huge size which is about one meter in diameter. The comb is fully exposed and hung from inaccessible branches of trees, along the sides of steep rocks in the forest and even from walls, rafters and other parts of buildings. It produces plenty of honey and the annual yield from the colony is about 60-80 kg. It is impossible to domesticate because of its irritable and ferocious nature, peculiar hives and its habit of deserting the hives often. But professional honey gatherers collect honey and wax from wild colonies, often killing the entire colony (Oldroyd and Wongsiri, 2006).

### *2. 1. 1. 2 Indian Bee* (*Apis cerana indica* Fab.)

It is the common Indian honey bee found in the forest as well as in plains throughout the country. It is smaller than rock bee but larger than little bee. It builds many parallel combs in the cavities and hole of trees, caves and such other hidden sites, the combs being parallel to the direction of the entrance in the plains and at right angles to the entrance in cold regions. It is mild and is capable of being domesticated and is commonly reared in South India. The annual yield of honey is 2 to 5 kg per colony. A queen can lay 350- 1000 eggs per day. A race of this is found in hilly tracts of South India. The hill bee is larger in size and darker in colour than the plain race and has the habit of moving away from the broods even at slight disturbances. The Indian honey bees are domesticated in all parts of the country like Himachal Pradesh, Jammu and Kashmir, Assam, Andhra Pradesh, Karnataka, Tamil Nadu and Kerala (Yadav *et al.*, 2017).

### *2. 1. 1. 3 Little Bee (A. florea* Fab.*)*

It is known as the little bee since it is smallest among four species of *Apis*. It is seen only in plants and corners of roofs. It yields only a little amount of honey, about 0.5 - 1 kg per year from a colony and it is not domesticated and reared. A queen lays about 323-365 eggs per day (Abrol, 2010).

### *2. 1. 1. 4 Italian Bee (A. mellifera L.)*

In Europe and America, *Apis mellifera* is extensively reared. It is similar to *A. cerana indica* in structure and habits but has prolific queen and has good honey gathering qualities. They yield about 45 - 180 kg per year. Swarming tendency is less in Italian bees. Now it has well established in the Northern parts of the country thus slowly replacing the Indian bees (Dhaliwal *et al.,* 2015).

## 2. 2 MAJOR THREATS TO HONEY BEES

Maintaining the honey bee colonies in a healthy state throughout the year is one of the main intention of beekeepers. Nowadays, bees are affected by a number of biotic and abiotic factors which affect honey bee health as well as productivity. Farmer practices such as monocropping and declining field size resulted in less variety and quantity of bee food supply. Climate change, habitat loss and invasive species are becoming equally crucial for beehive integrity (Oldroyd, 2007).

In recent years, researchers of United State of America has identified a phenomenon called Colony Collapse Disorder (CCD) which caused 80 to 100 per cent honey bee mortality. The possible explanations included radiations emitted by

mobile towers, genetically modified crops, pest and diseases, environmental factors and the excessive use of pesticides (Ratnieks and Carreck, 2010).

### *2. 2. 1 Diseases of Honey Bees*

The diseases of honey bees are caused by a wide range of pathogens like bacteria, fungi, protozoa and viruses. They infect both brood and adult bees. Major brood diseases include bacterial diseases (American foul brood (AFB) and European foul brood (EFB)), fungal diseases (chalk brood and stone brood) and viral disease (Thai sac brood). Nosema disease caused by a protozoan infect the adult bees (Arbia and Babbay, 2011). Among the brood diseases, AFB and EFB are considered as universal threats to beekeeping (Smith *et al.,* 2014; Goulson *et al.,* 2015).

#### *2. 2. 1. 1 American Foul brood Disease*

American foul brood disease is more widespread in tropical and subtropical areas and was first reported by White in 1907. From India, there is only a single report of American foul brood disease in *A. cerana indica* from Nainital of Uttarakhand during 1960s (Singh, 1961). It is an infectious, highly contagious, cosmopolitan disease affecting the early larval (1 day old larvae) and pre- pupal stages. It is considered as the most contagious disease of honey bees, which can even destroy an entire colony (Alippi *et al.,* 2004).

American foul brood kills the larvae after the cell has been capped. The caps of infected cells are shrunken and dark. It may be punctured by the investigating adult bees. Infected larvae are brown in color (White, 1907). Most of the larvae die in the pre-pupal stage, they are usually stretched along the side of the cell. The resulting cadaver dries down to a black-brown scale, which sticks to the cell wall. A stinking odor will also be produced and hence this disease is also known as "Stinking

disease". In severe condition, the brood exhibits pepper box symptom (Shimanuki, 1990). The causal agent is *Paenibacillus larvae.*

***2. 2. 1. 2 European Foul brood Disease***

The European foul brood disease was first reported from United Kingdom and later from United States of America (White, 1912). The 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).

It is a deadly disease found world-wide. European foul brood affects mainly unsealed brood, killing honey bee larvae when they are 4 to 5 days old (Bailey and Ball, 1991). The colour of the infected larvae changes from bright white to yellowish brown. Finally it turns blackish or dark brown colour. The cell capping of the dead brood were seen as perforated, sunken and convex. The brood pattern becomes irregular and larvae become twisted with creamy white guts visible through the transparent body wall. The disease is usually noticed during dearth period. The larvae died while still in coiled state. Infected larvae are first soft and watery, afterwards they became pasty, but non- ropey. Dead and dried scales were tough, rubbery rather than brittle and can be easily removed. The adult bees become abnormal, sluggish, and are unable to fly. At last they died within a few hours of emergence. Sudden weakening of the colonies was observed as a general symptom. *Melissococcus plutonis* is the causal organism of the disease (Abrol and Ball, 2006).

***2. 2. 1. 3 Bacterial Brood Disease***

Recently, beekeeping in Kerala is facing a threat where brood diseases result in excessive economic losses throughout the state. 5.4 to 63.63 per cent disease incidence is reported (Amritha *et al.*, 2014). The symptoms observed comprise

scattered egg laying, uncapped cells and the infected colonies exhibited loss of appetite and yellow to yellowish brown larvae which are similar to that of bacterial diseases. Colony strength gets reduced and adult bees become more aggressive. This eventually results in decline of honey yield and enormous economic loss to the beekeepers. The bacteria responsible for the brood disease was isolated and identified as *Bacillus pumilus and Achromobacter* sp. (Joseph, 2018).

### *2. 2. 2 Pesticide Toxicity to Honey Bees*

The application of pesticides on crops had made a rapid development in agriculture. Though honey bees are non- target organisms for most of the pesticides, they are often exposed to pesticides while collecting pollen and nectar from flowers, collecting resins from various plants, during flight, drinking water from pesticide contaminated water bodies (Mullin *et al.,* 2010). These pesticides are brought back into the colony where its level sets concentrated further in the waxy comb (Gregorc and Ellis, 2011).

Honey bees are exposed to lethal and sub lethal doses of pesticides during foraging leading to mortality of the bees. Also, their indirect effect has led to weakening of the colonies and its large scale decline (Fairbrother *et al.,* 2014). Neonicotinoid insecticides are neurotoxicants that have specific concern for sub lethal effects in honey bees. This class of insecticides was considered as a major innovatory for integrated pest management programs. They possess broad-spectrum activity, low application rates, low mammalian toxicity, target specificity and upward systemic movement in plants. The neonicotinoid insecticides include imidacloprid, acetamiprid, clothianidin, thiamethoxam, thiacloprid and dinotefuran which are marketed under a variety of trade names (Blacquiere *et al.*, 2012).

Thiamethoxam is an insecticide widely recommended in vegetable ecosystem for the sucking pest's management. Among the neonicotinoids, this insecticide has highest toxicity towards bees. Laboratory experiment on acute toxicity of thiamethoxam against *A. mellifera* was conducted and its $LD_{50}$ was found to be 0.03 µg bee $^{-1}$ (Iwasa *et al.,* 2004). Contact toxicity of thiamethoxam against different strains of *A. mellifera* was evaluated by Laurino *et al.* (2013) and the $LC_{50}$ was found between 3.53 to 3.75 ppm. Later, Stanley *et al.* (2015) conducted a laboratory bioassay of the insecticide on *A. mellifera* and *A. cerana indica.* They detected cent per cent mortality of the bees within 48 hours of treatment. Acute consumption of thiamethoxam along with sugar syrup by *A. mellifera* resulted in diminished motor activity which impaired their locomotion and flight (Tosi *et al.,* 2017).

### *2. 2. 3 Impact of Artificial Feed on Honey Bees*

Honey bees rely on plant based diet like nectar and pollen. In the dearth season, sugar syrup is used as the artificial feed for honey bees. Sugar can poison honey bees and the impurities in the commercial sugar are detrimental for bee health (Roy, 1977).

Sucrose solution, inverted sugar syrup, or other syrups such as starch syrup or high fructose corn syrup (HFCS) are used as artificial feed to honey bees (Barker, 1977). It was found that some sugars in these feeds, such as galactose, mannose and lactose are toxic to honey bees in certain concentrations (Brodschneider *et al.* 2010). In addition to toxic sugars, another toxic substance that can be found in syrups during artificial feed preparation is 5-hydroxymethylfurfural (HMF).

Krainer *et al.* (2015) investigated the toxicity of HMF towards larvae. Artificially reared larvae were exposed to a chronic HMF intoxication for six days using six different concentrations (5, 50, 750, 5000, 7500 and 10,000 ppm) along

with control and their mortality was assessed. Concentrations ranging from 5 to 750 ppm HMF did not show any influence on larval mortality compared to control whereas concentrations of 7500 ppm or higher caused a larval mortality of cent per cent.

The most significant bee mortality was observed when fed with acid hydrolysed invert sugar syrup. Bee mortality can be caused by stomach poisoning or splitting of alimentary canal (Rogers and Illsley, 1992). According to Mirjanic *et al.* (2013) dry brewer's yeast significantly improved the longevity of bees. Bees fed with dry brewer's yeast lived 38 days whereas those bees fed with sugar syrup lived only 22.5 days. According to Doung (2014), raw sugar has a sucrose level of 99.50 per cent with an ash content of 0.2 per cent which can cause digestive problems to honey bees.

## 2. 3 HISTOMORPHOLOGY OF HONEY BEE MIDGUT

The midgut of honey bee is susceptible to poisonous substances or malnutrition and pathogens. The chemicals present in the feed, disease causing microorganisms and the pesticide particles will be adsorbed into the midgut region. The external clinical manifestation of these stressors become evident only in the advanced stages leading to large scale loss of colonies before the adoption of management strategies. In order to discriminate the sublethal effects of these stressors on bees, histomorphological analysis is important as an additional tool for diagnosis (Gregorc and Bowen, 2000). Histomorphological tools have been widely employed for the diagnosis of numerous diseases of animals. It is proved to be sensitive and precise method for the detection and the identification of numerous pathogens (Maiolino *et al.*, 2013).

Nelson (1924) observed that midgut of normal honey bee larvae comprises columnar epithelial cells and its brush bordered microvilli, thick mucus like peritrophic membrane and minute triangular regenerative cells at the base of the epithelial cells. The walls of larval midgut is made up of simple epithelium of cubical to columnar cells, on the inner surface there is a distinct striated border. Snodgrass (1956) described the presence of triangular group of minute regenerative cells next to the basement membrane as well as a homogenous layer of peritrophic membrane apparently having gelatinous consistency.

Ayaad *et al.* (2017) inoculated *A. mellifera* with *Paenibacillus larvae,* the causal organism of American foul brood disease, and studied the gut histology at 48 and 72 h post infection. After 48 h of infection, the midgut showed significant histological alterations such as presence of vacuoles, separation of epithelial layer, lacerations in the basement membrane and damage of circular muscles. At 72 h post infection, the infected brood showed separation and elongation of the midgut epithelial cells and severe deterioration of muscular epithelial layers. Moreover, *P. larvae* bacterial cell aggregations were detected in the gut epithelium. Through electron microscopy, the subcellular level histological alterations such as mitochondrial and nuclei degradation was confirmed.

Histopathology is used a specific method for the detection and identification of *Ascospahera apis,* the causative agent of chalkbrood disease (Maiolino *et al.*, 2013). The stereomicroscopic observations of naturally infected larvae revealed the presence of hyphal filament of the pathogen in fat body. This resulted in the slow degradation of trophocytes and oenocytes, distortion of their shape and disintegration. The hyphae extended from visceral cavity to the cuticle. The fungus destroyed and replaced all the larval tissues except for the trachea and tracheoles.

Maiolino *et al.* (2013) studied the histopathology of adult *A. mellifera* naturally infected by nosemosis caused by *Nosema* spp. The histological examination revealed the presence of infected cells along the midgut epithelial cells. The apical cytoplasm was extremely enlarged and entirely filled with parasitic spores. No spores were found in any other tissue. The infected epithelial cells exhibited evidence of degeneration and lysis only after being completely filled with spores.

The effect of different feed on digestive tract of bees (*A. mellifera*) was studied by Mirjanic *et al.* (2013). Acacia honey, sugar syrup and acid invert syrup were selected and fed to bees. Based on the research, it was found that feeding with different food has significant effect on midgut epithelial cells. Natural source of food for bees such as honey had no harmful effect on the midgut histology and have positive effect on the life span of bees. When fed with sugar syrup, there was pronounced peak damage of epithelial cells with non- homogenous intestinal content. In midgut of bees fed with acid invert syrup, almost totally damaged columnar epithelial cells with loose intestinal content was observed.

The midguts of the *A. mellifera* exposed to 0.0428 ng of thiamethoxam 25 % WG $L^{-1}$ of diet for a period of eight days showed typical characteristics *viz.*, digestive cells with nuclei of spherical shape, organelles with no alterations, especially mitochondria with intact double membranes and cristae and rough endoplasmic reticules. Vacuoles were also observed in the cytoplasm (Catae *et al.*, 2014).

## 2. 4. HONEY BEE GUT MICROBIOTA

Since the last decade, investigations are going on the microbial gut symbionts with a particular focus on the functional aspect of host- symbiont interaction in honey bees. There is a distinctive gut bacterial community, which consists of nine dominant groups, comprising over 95 per cent of the whole microbial community. They belong

to mainly three phylum Proteobacterium, Actinobacterium and Firmicutes. The Gram-negative *Gilliamella apicola* and *Frischella perrara*, belonging to the Gammaproteobacteria class, and the Betaproteobacterium *Snodgrassella alvi* are predominant in the midgut. The rectum is preferentially colonized by phylum Actinobacterium with the Firmicutes clades Firm- 4 and Firm- 5, including different *Lactobacillus* species (e.g. *Lactobacillus mellis, L. mellifer, L. helsingborgensis, L. kullabergensis, L. melliventris and L. kimbladii*) and two species belonging to the genus *Bifidobacterium* (*Bifidobacterium asteroides* and *B. coryneforme*). *Parasaccharibacter, Glucanobacter* and *Bartonella* have been described but they are less abundant (Moran, 2015; Kwong and Moran, 2016).

**2. 4. 1 Spatial Organization of Gut Microbiota**

A few bacteria are found in the proventriculus of alimentary canal, which is used for the storage and transport of nectar for feeding larvae and the production of honey. Correspondingly, the limited numbers of bacteria in the crop mostly consist of *Lactobacillus kunkeei* and *Parasaccharibacter apium,* that inhabit nectar and hive materials. The insect midgut, which functions in the digestion and absorption of food, does not provide a stable substrate for bacterial colonization, as it is lined by a continuously shed chitinous material known as the peritrophic matrix. So there are only a few bacteria in the midgut.

The hindgut is lined with a stable layer of cuticle and harbors a large microbial community of bacterial cells between $10^8$ and $10^9$ that accounts for more than ninety nine percentage of the bacteria in adult worker bees. The hindgut is divided into two discrete regions, the ileum and the rectum, each of which has a distinct microbial community composition. The ileum, a narrow tube with six longitudinal folds, is dominated by the main Gram-negative bacterial species. *S. alvi* forms a layer directly on the gut wall, on top of which forms a layer of *G. apicola.*

This bacterial community extends into the pylorus, a small region at the junction of the midgut and hindgut. In the pylorus, *F. perrara* is abundant and localizes adjacent to the gut epithelium. The microbial community in the rectum is dominated by the fermentative Gram-positive bacteria like *Lactobacillus* Firm- 4, *Lactobacillus* Firm- 5 and *B. asteroides* cluster (Kwong and Moran, 2016).

### 2. 4. 2 Transmission of Gut Microbes

Occasionally, in individual adult worker bees, there is considerable deviation from the normal composition of the gut microbial community. Sociality, which includes the sharing of the hive environment by a social group, is vital to the host transmission of the bee gut microbiota. After pupating in capped cells inside the colony, adult honey bees emerge from the cells that are free of germ- guts. The establishment of a stable microbial community occurs before worker bees leave the hive, which indicates that transmission occurs through nest mates or hive components, such as wax surfaces. The transmission is mainly of three ways - oral trophallaxis, from hive material and faecal- oral route. Oral trophallaxis, a common behaviour for communication and food transfer, is not a major route for transmission, which is in line with the observations that foregut harbors only a few bacteria. A faecal route seems important, mostly for *S. alvi*, *G. apicola* and *F. perrara*, even though members of the gut microbial community may also be attained through contact with hive components that have been in recent contact with live bees. The most important one is feacal- oral route since, almost all the microbiota are harboring in the distal part of the gut (Kapheim *et al.,* 2015).

### 2. 4. 3 Variation with Age and Caste

Young honey bee larvae are devoid of bacteria, but they are fed by worker bees throughout their development and these interactions may lead to the buildup of bacterial species from hive materials in their microbe less guts, as well as

of some species that are usually found in the guts of adult bees. However, both the composition and abundance of this larval gut microbiota seems erratic. The gut lining is shed during the metamorphosis of larvae to pupae and adult bees. The newly emerged adult bees have very few or no gut bacteria and get colonized by the normal gut microbial community in the first few days of adult life, before leaving the hive (Kapheim *et al.,* 2015).

The microbiome of a queen bee varies in size and composition and often lacks certain characteristic species of bacteria that are present in the guts of workers. The guts of queen bees are frequently dominated by *P. apium*. The differences in the composition of the gut microbiomes are perhaps due to the unique physiology and diet of queens, which feed solely on highly nutritious secretions that are produced by attendant workers. Drones have a microbiome composition more similar to that of workers. The reason for this is unclear (Kapheim *et al.,* 2015).

Adult workers have a relatively even set of bacterial species in their gut compared with that of the male or queen bees. Older foraging workers may have a lower abundance of the core species of bacteria than younger adult bees (Kapheim *et al.,* 2015).

### 2. 4. 4 Benefits of Microorganisms in their Host

The microbes provide nutritional and immunity support to the host.

#### *2. 4. 4. 1 Nutritional Support*

Social insects develop a partnership with the microbial gut symbionts as they possess genes encoding for enzymatic activities (i.e. cellulases, hemicellulases and lignase) crucial for the energy uptake from a plant- based diet (Newton *et al.* 2013).

Moreover, the microbial consortium produces fatty acids, amino acids and other essential nutrients and metabolites. The gut symbionts utilize the plant complex molecule lignin, which is a component of pollen, thus beginning the breakdown of this important high- protein plant-derived food (Rokop *et al.* 2015).

Interestingly, the energy uptake of the *S. alvi* exclusively relies on the aerobic oxidation of the products of the fermentation process (citrate, malate, acetate and lactic acid), thus avoiding the competition for nutrients with other species (Kwong *et al.* 2014). A further interesting finding (Engel and Moran, 2013) is the pectin degradation activity of *G. apicola* that is strain specific and leads to pollen cell wall degradation, thus providing the protein content for the host. Among *Bifidobacteria*, some isolates from social insects are known to possess a complete trehalose degradation pathway, which is lacking in majority of the other Bifidobacterial taxa. Trehalose is indeed used as carbohydrate storage and haemolymph sugar by several insects including honey bee (Milani *et al.,* 2015).

Alberoni *et al.* (2016) identified that the bacterial groups are the major contributors of the protein- coding transcripts, involving in the breakdown of plant-derived macromolecules and in the fermentation of the monomeric subunits.

#### *2. 4. 4. 2 Immunity Support*

Host protection is another important aspect that is often associated with a balanced gut microbiota. It is a fact that different stressors, such as parasites or pathogens, deficient nutrition and pesticides, can result in immunosuppression. The microorganisms could play a role in host protection by directly stimulating the bee's immune system or by directly inhibiting pathogens through antimicrobial compound production (Klaudiny *et al.*, 2005).

Honey bee genome has significantly fewer immune genes than that expected. Thus, the gut endosymbionts contribute to immunity to bees (Evans and Pettis, 2005). One of the main effectors of the innate immunity in honey bee is denoted by antimicrobial peptides (AMPs). Honey bees possess six AMPs, mostly activated at epithelial surfaces: abaecin, hymenoptaecin, apidaecin, defensin-1, defensin-2 and apisimin (Klaudiny *et al.* 2005).

## 2. 5 PROBIOTIC CONCEPT IN BEES

All members of the kingdom Animalia, including humans, have helpful symbiotic microbiota which are extremely important for the proper functioning of the gastrointestinal tract. These symbiotic microorganisms are responsible for the fermentation of carbohydrates as well as the production of some vitamins and amino acids that are needed by the host. Furthermore, gut microbiota prevent pathogenic microorganisms from colonizing the gastrointestinal tract. In order to improve bee health, scientists are researching on the role of bee gut microbiota in health status. Generally lactic acid bacteria (LAB) are proved to be the important inhabitants of animal and human intestinal tracts as they have a multidimensional, antimicrobial potential (Audisio and Benitez- Ahrendts, 2015).

The original modern hypothesis of the positive role played by certain bacteria was first introduced by Russian scientist Elie Metchnikoff where he suggested that it would be possible to modify the gut microbiota and to replace harmful microbes with beneficial microbes. The word probiotics is a composite of the Latin preposition '*pro*', meaning 'for', and the Greek adjective '*biotikos*', meaning 'fit for life, lively'. Thus the word can be translated as 'beneficial for life'. The World Health Organization (WHO) (2002) defined probiotics as live microorganisms that when administered in adequate amounts confer a health benefit to the host.

There is a mutualistic dependence between the LAB and honey bees. The LAB flora acquire a niche in which nutrients are abundant. Honey bees in turn got protected by the bacteria from harmful pathogens (Olofsson and Vasquez, 2008). In particular, beyond the health aspect, probiotic microorganisms should fulfil a list of biological requirements and safety criteria. They should be non-toxic and non-pathogenic. They should have an accurate taxonomic identification and should be normal inhabitants of the targeted host- species. Apart from these, the LAB should adhere to the gut epithelium (Gaggia *et al.*, 2010).

### 2. 5. 1 Applications of Probiotics

Studies on probiotics are mainly concentrated on the improvement of nutrients in the food, management of diseases and establishment of intestinal microflora.

#### *2. 5. 1. 1 Improvement of Feed*

Honey bees rely on plant based diet like nectar and pollen. During the dearth period, the beekeepers supplement sugar syrup and pollen substitute instead of nectar and pollen respectively.

Audisio and Benitez- Ahrendts (2015) delivered the bees with 125 g $L^{-1}$ cane syrup with a final concentration of $10^5$ cfu $ml^{-1}$ of *Lactobacilli* sp. The bees accepted the new nourishment, which was consumed within 24- 48 hours and was administered in two independent trials. The results revealed significant differences in the open and the operculated (closed) brood areas in the treated group compared with the control. Also a higher number of bees were measured in the treated group (54%

increase with respect to initial bee population) than the control. Furthermore, honey storage was higher, 40 per cent for the treated group than the control groups.

In an experiment by Kazimierczak- Baryczko and Szymas (2006), two probiotic preparations were added to the substitute in three doses immediately before administration to bees, i.e. "Biogen-N" (a biological stimulant of immunity and growth containing 4 strains of the genus *B. bifidum*, *Enterococcus faecium*, *L. acidophilus* and *Pediococcus acidlactiti*) and "Trilac" (a preparation restoring functions of gastrointestinal microflora containing *L. acidophilus, L. delbrueckii* and *B. bifidum*). Total bacteria count per 1 mg of Biogen-N accounted for $11x10^9$ which was similar to that of 1.267 g of Trilac. The administration of probiotics decreased the number of fatal cases among bees. However, the decrease seemed to be statistically significant with pollen substitute supplementation using Trilac in a dose of 1.267 g per 100 g of substitute and with the Biogen-N in a dose of 1mg.

### *2. 5. 1. 2 Disease Management*

The suppression of bacterial numbers could be produced by production of antibacterial substances. Primary metabolites, such as organic acids and hydrogen peroxide, are known to be effective *in vitro*. Another mechanism for preventing colonization by pathogens is competition for adhesion sites on the gut epithelial surface. The probiotic bacteria use most of the nutrients by hindering the growth and multiplication of pathogen (Fuller, 1989).

Inhibition assays to investigate the effects of honey bee LAB on *Paenibacillus larvae* growth were done by Forsgren *et al.* (2010). Adding the LAB mixture to the larval food significantly reduced the number of AFB infected larvae in exposure bioassays. The results demonstrated that honey bee specific LAB possess beneficial properties for honey bee health where 10 per cent decrease in the larval death was documented than that of the control.

The LAB strains isolated from honey crop were orally administered to honey bee larvae infected with *Mellisococcus plutonius* at three concentrations ($10^7$, $10^6$ and $10^5$ bacteria ml $^{-1}$). Irrespective of the infectious dose, mortality was significantly reduced to 20 percentage in groups treated with the LAB mixture (Vasquez *et al.*, 2012).

With respect to adult honey bees, beneficial microorganisms are targeted against the pathogen, *Nosema* spp. which multiplies within gut epithelial cells. The nosema affected worker bees become unable to fly. So they crawl about at the hive entrance. Body hairs are lost and bees become shiny. The disease is caused by *Nosema apis* and *N. ceranae*. In the experiment done by Andrearczyk *et al.* (2014), the bees infected with *Nosema* spp. on ingesting the probiotics with 0.5 µl and 1.5 µl ml $^{-1}$ of sugar syrup exhibited low mortality rate.

***2. 4. 3. 3 Effect of Probiotics on Intestinal Microflora***

Two probiotics, Biogen-N and Trilac, were used as supplements to pollen substitute in feeding honey bees, *A. mellifera*. The probiotics were given either throughout the entire 14-day experiment or only for 2 days, just after bee emergence. The midgut of worker bees was colonized by bacteria present in probiotics, including *Lactobacillus* spp., *P. acidilactici, B. bifidum* and *E. faecium*. Advantages of probiotic supplementation include enhanced bee survival and higher dry matter and crude fat level in comparison with bees fed with pollen substitute alone (Kaznowski *et al.*, 2005).

The practice of stimulating the bee colonies using sugar syrup fed with probiotics Enterobiotics (1.25 and 2.5g $L^{-1}$ of sugar syrup) and Enterolactis Plus (1.2 and 2.4g $L^{-}$

[1] of sugar syrup) for a period of three weeks, resulted in a significant reduction in the total number of harmful bacteria in the digestive tracts of the bees compared with that of the control group. Intestinal colonization of beneficial bacteria in probiotics products was detected. This resulted in an improved health status and productivity of the bee colonies (Patruica and Mot, 2012).

### 2. 5. 2 Histomorphology of Honey Bee Midgut Supplemented with Probiotics

Szymas *et al.* (2012) assessed the morphological changes in the midgut epithelium of bees fed with pollen substitute or pollen substitute fortified with probiotic preparation. One day old worker bees were kept in cages placed in a temperature controlled environment. Workers were fed with bee bread (control), pure pollen substitute or pollen substitute fortified with three different doses of probiotic preparations: Biogen or Trilac for a period of two weeks. The evaluation of histological changes of the bee midgut was carried out in bees feed for 8 and 14 days. Slight changes in the epithelium as well as strong merocrine type secretion were noticed in bees nourished with pollen substitute supplemented with probiotic preparations. Significant differences were observed, primarily, in quantities of the developed peritrophic membranes. Their quantities were particularly high after 14 days of feeding with the pollen substitute enriched with probiotic preparations. The development of numerous peritrophic membranes could have contributed to better utilization of nutrients in the feed and better condition of bees.

# *Materials and Methods*

## 3. MATERIALS AND METHODS

The study entitled "Histomorphology of Indian bee (*Apis cerana indica* Fab.) supplemented with probiotics" was conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani during 2017-19. The study was conducted with the objective to assess the histomorphology of midgut of Indian bee's (*A. cerana indica*) suffering from various stressors (pathogens and insecticides) and after being fed with probiotics.

### 3.1 SELECTION OF APIARIES AND SAMPLE COLLECTION

The larvae of Indian bee (*A. cerana indica*) were collected from different parts of Kerala by purposive sampling. The samples of the larvae were taken from naturally occurring feral colony, domesticated colonies which were fed with and without artificial feed (artificial feed), naturally infected ones with bacterial brood disease as well as larvae fed with insecticide (Thiamethoxam 25% WG) treated royal jelly (Plate1). Twelve larvae (3 to 4 days old) each from these colonies were collected from the brood.

Table 1. Geographical details of the selected apiaries

| Sl. No. | Type of sample | Locations | District | Latitude | Longitude |
|---|---|---|---|---|---|
| 1. | Feral colony | Edamon, | Kollam | 9° 0' 22. 392"N | 76° 58' 53. 715"E |
| 2. | Colony fed without artificial feed | Vellimala, | Idukki | 9° 70' 75.563"N | 77° 16' 09. 188"E |
| 3. | Colony fed with sugar syrup | Vellayani, | Thiruvananthapuram | 8° 26' 57.09"N | 77° 1' 0. 030"E |
| 4. | Colonies naturally infected with disease | Prakkanam, | Pathanamthitta | 9° 27' 14. 291"N | 76° 73' 59. 48"E |

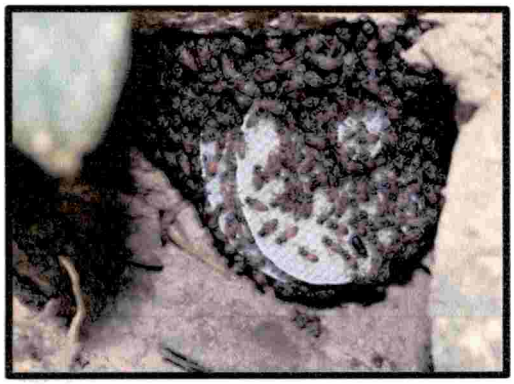

Plate 1a. Feral colony

Plate 1b. Colonies fed with sugar syrup

Plate 1c. Colony fed without artificial feed

Plate 1d. Diseased colonies

Plate 1. Apiaries selected for larval sample collection

### 3. 1. 1 Feral Colonies

Naturally occurring non-domesticated bees dwelling in wooden logs, caves and such undisturbed areas are called feral colonies. The feral colony was obtained from an earthen bund at Edamon, Kollam district from which the larval samples were collected. The height of the feral colony from the ground was also measured.

### 3.1. 2 Colonies Fed with Sugar Syrup

From the apiary maintained by AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani, larval samples that are fed with sugar syrup were collected. The colonies were provided with 1:1 sugar and water as feed.

### 3. 1. 3 Colonies Fed without artificial feed

Domesticated colonies which were not fed with sugar syrup were selected from Vellimala of Idukki district.

### 3. 1. 4 Colonies Naturally Infected with Disease

The diseased colonies were identified from Prakkanam of Pathanamthitta district. The symptoms were similar to that of the existing bacterial disease like reduced colony strength, change in color of larvae from pearly white to yellow or brown, perforated brood capping, and "pepper- box symptom" which was reported in 2018.

### 3. 1. 5 Colonies Treated with Insecticide

Domesticated colonies from the apiary of AICRP on Honey bees and Pollinators, College of Agriculture, Vellayani were selected to standardize the sub lethal dose of the insecticide thiamethoxam 25% WG. Two doses which were above

and below to that of the field dose were selected for the study. 0.05, 0.1, 0.2, 0.3 and 0.4 g $L^{-1}$ of insecticide solution was prepared. The cells with larvae of the brood chamber were marked and 0.5 mL solution from each dose of insecticide was provided to the larvae of selected cells using a 2 mL syringe (Plate 2). Cells provided with sterile water were maintained as control. Mortality of larvae was recorded in every five minutes interval by observing its movement while touching with a camel hair brush. The dose of insecticide that took more time for the death of the larvae was selected as the sub lethal dose. The standardized sub lethal dose of insecticide was selected for the experiment and was given to twelve marked cells with larvae. Such treated larvae were taken from the cells before its death for histological evaluation.

### 3. 1. 6 Shade Conditions in the Apiary

Based on visual canopy coverage, shade conditions in the apiary were categorized as low (<30 % canopy coverage), moderate (30- 80 % canopy coverage) and high (>80 % canopy coverage) to understand if the shade has any influence on the disease incidence.

Apart from these, observations on the total number of colonies and foraging sources in and around the apiary were also recorded.

## 3. 2 HISTOMORPHOLOGY OF HONEY BEE MIDGUT

### 3. 2. 1 Procedure for Histological Evaluation

Twelve representative larvae (3 to 4 days old) from each group (treatment) were taken from the comb carefully using forceps for histomorphology study. The histomorphology study was conducted at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Biomedical Technology Wing, Poojapura, Thiruvananthapuram.

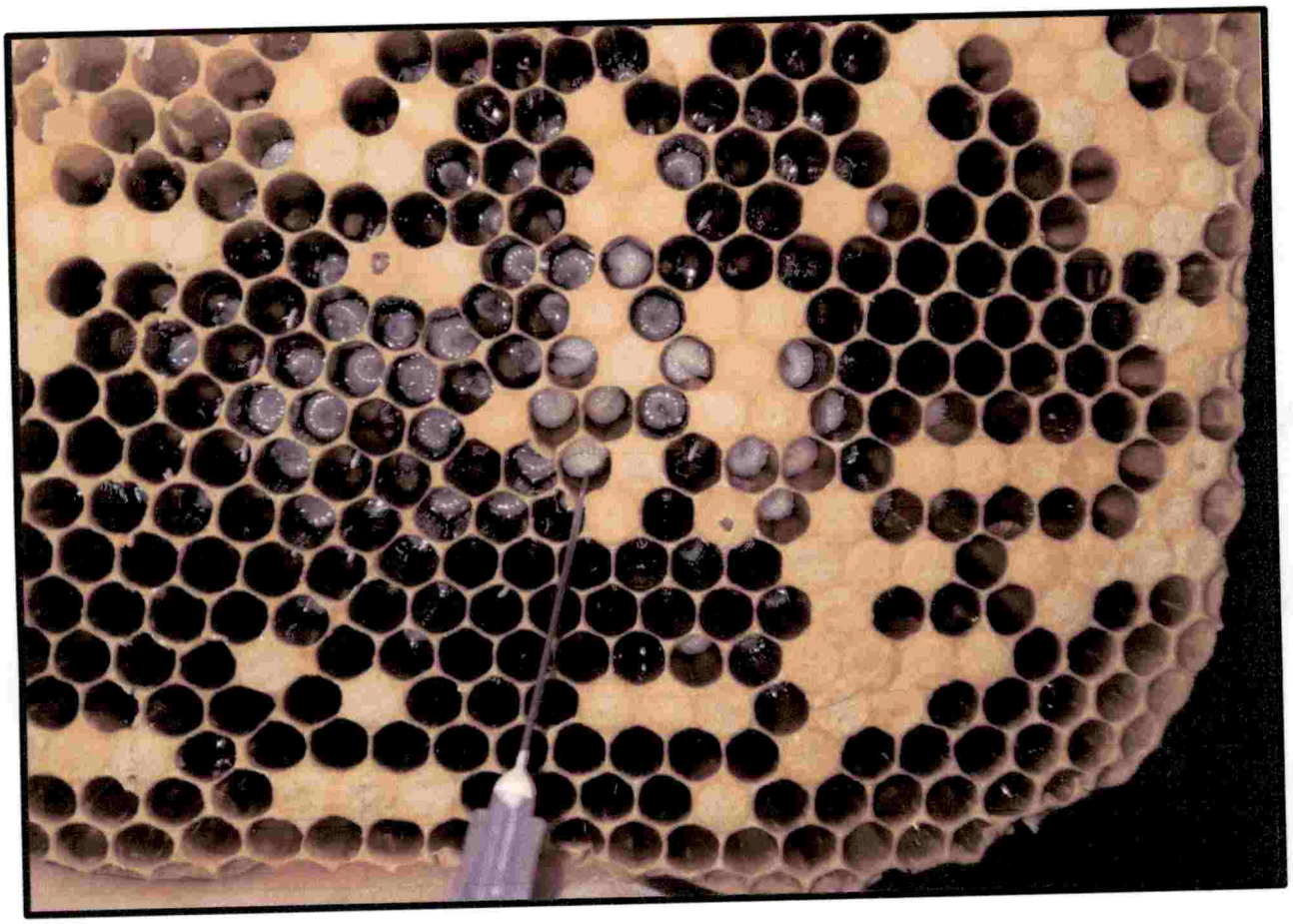

Plate 2. Provision of insecticide solution into the brood cells

The histology procedure of larval honey bee samples by Silva- Zacarin *et al.* (2012) was followed with slight modifications. The steps involved in the histology procedure are detailed below:

***3. 2. 1. 1 Fixation of Larvae***

The larvae were taken in vials filled with 10 % NBF (Neutral Buffered Formalin) to preserve natural tissue structure and maintain the cell structure from degradation (Plate 3a). The quantity of formalin taken in the vial was ten times than that of the collected sample. The larvae were handled with utmost care to avoid the tissue damage. Following were the reagents used for the preparation of 10 % NBF.

Sodium phosphate, monobasic - 4.0 g

Sodium phosphate, dibasic - 6.5 g

Formaldehyde (37- 40%) - 100 ml

Distilled water - 900 ml

The reagents were mixed well and the pH was maintained at 6.8. The collected honey bee larvae were retained in NBF for two to three days.

***3. 2. 1. 2 Dehydration of Fixed larvae***

In a laminar air flow chamber, the larval samples from each group were transferred from the vials to respective labeled plastic cassettes with perforations (Plate 3b). These cassettes were then kept in NBF in a glass bottle in order to prevent the drying of larval samples (Plate 3c). These cassettes were taken from the bottle with NBF using a forceps and then immersed into a glass bottle containing 50 % alcohol (diluted in distilled water) for 3 h. After 3 h, these cassettes were taken out and kept in 70 % alcohol for 2 h (Plate 3d). After 2 h, the cassettes were introduced into the inlet portal of the tissue processor where the larval samples were

automatically subjected to infusion with a series of alcohol (70%, 80%, 85%, 90%, 95% & 100%) in the descending grade and was replaced with xylene for eight and a half hours (Plate 3e). These processed larvae were then subjected to embedding.

***3. 2. 1. 3 Embedding***

The larvae from the cassettes were transferred to embedding molds and these molds were then placed on a hot plate at 60°C as it is the congealing point of wax. Molten paraffin wax was poured on the respective molds and the larvae were oriented in molten wax with the help of warmed tweezers (Plate 3f). These molds were then placed over a cold plate for hardening of the wax blocks. The wax blocks removed from the mold were stored at room temperature until further process (Plate 3g).

***3. 2. 1. 4 Microtomy***

Before microtomy, the edges of paraffin embedded wax blocks were trimmed and the wax was sliced into ribbons of 4 µm thickness with the help of a rotary microtome (Plate 3h). Such sections were put over hot water bath to remove the wax layer surrounding the sections. When the wax from the sections were about to melt, the sections were taken from the hot water and was carefully placed over a clean glass slide. The slides were then dried at 37°C overnight using a hot air oven. Such slides were then subjected to staining process.

***3. 2. 1. 5 Staining***

Hematoxylin and eosin (H&E) staining, the most common staining technique in histopathology, was used as this stain clearly demonstrates the nucleus and cytoplasm of tissues. The following were the steps for staining.

All the respective chemicals were filled in the staining dishes before the process starts. The glass slides containing the sections were inserted into staining racks (Plate 3i).

1. In order to deparaffinize, the staining rack with slides were placed in xylene for 30 min.
2. Hydrated the tissue section by passing through decreasing concentration of alcohol (100%, 80%, 70%) for 5 min. each.
3. Washed in running tap water for 5 min.
4. Stained in hematoxylin for 30 min. Again washed in running tap water for 5 min.
5. Dipped three times in 1% acid alcohol (1% HCl in 70% alcohol).
6. Washed in running tap water and were dipped in an alkaline solution (ammonia water) for 2 min. followed by tap water wash.
7. Stained in 1 % eosin for 3 min.
8. Dehydrated in increasing concentration of alcohols (95% and 100%) for 10 min. and cleared in xylene.
9. Mounted in mounting media DPX (a mixture of distyrene, a plasticizer and xylene).

### 3. 2. 2 Observations taken during Histology Study

The DPX mounted slides were then observed under light microscope to study the histology (Plate 3j). The midgut histology of the treatments were compared with respect to the epithelial integrity, presence of microvilli, peritrophic membrane and regenerative cells. Then the representative photographs revealing the histological variations were taken at magnification power 4X, 10X and 40X.

## 3.3 EFFECT OF PROBIOTICS ON THE MIDGUT OF HONEY BEE LARVAE

For the experiment, the probiotic formulation Darolac available in the local pharmacies was used (Plate 4). Each 1g of Darolac contained 1.25 x $10^9$ live cells of *Lactobacillus acidophilus, L. rhamnosus, Bifidobacterium longum* and

Plate 3a. Collecting larvae in glass vials

Plate 3b. Plastic cassettes

Plate 3c. Plastic cassettes with larvae kept in 10 per cent NBF

Plate 3d. Dehydration of samples in 50% and 70% ethanol


Plate 3e. Automatic tissue processor

Plate 3. Steps involved in histology study

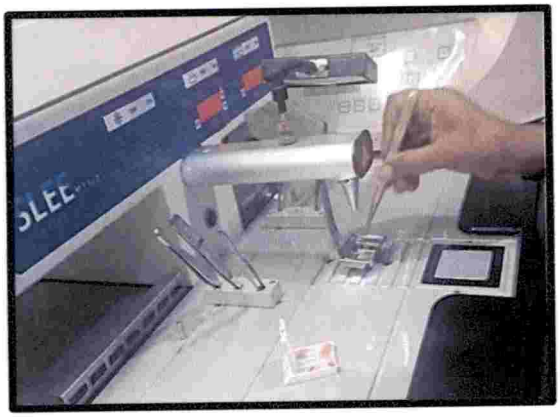

Plate 3f. Orienting the larvae in molten paraffin wax

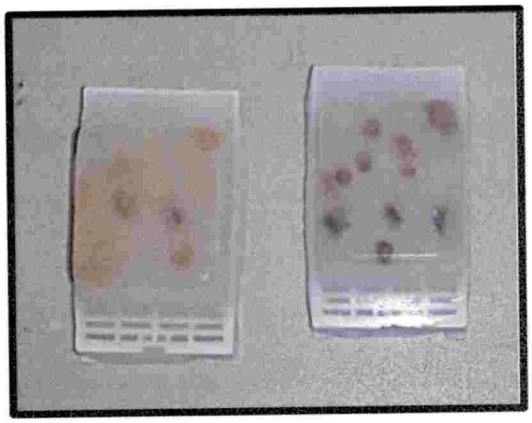

Plate 3g. Hardened paraffin wax blocks


Plate 3h. Rotary microtome

Plate 3i. Staining rack with slides

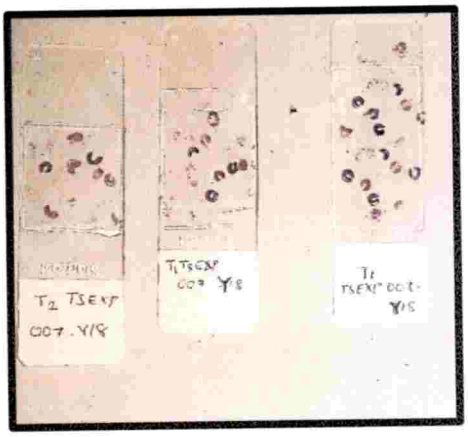

Plate 3j. Hematoxylin-Eosin stained glass slides

Plate 3. Steps involved in histology study (continued)

*Saccharomyces boulardii.* The dosage was fixed as 1. 2 g $L^{-1}$of water or sugar syrup (Patruica and Mot, 2012).

### 3. 3. 1 Probiotics alone in Domesticated Colonies

Domesticated colonies were selected from the apiary of AICRP on honey bees and pollinators. To one litre of boiled and cooled water, 1.2 g of probiotic was added from which 250 mL of the solution was given to the hive in a coconut shell (Plate 5). Freshly prepared probiotic solution was provided weekly for four weeks.

### 3. 3. 2 Probiotics along with Sugar Syrup in Domesticated Colonies

Sugar syrup was prepared by dissolving 125g of sugar in 1L of water and boiled. The syrup was cooled to which 1.2 g of probiotic was added. This solution was fed to the colonies maintained at the apiary of AICRP on honey bees and pollinators. Freshly prepared probiotic solution was provided weekly for four weeks.

### 3. 3. 3 Probiotics along with Sugar Syrup in Domesticated Diseased Colonies

Diseased colonies were identified from Prakkanam of Pathanamthitta district. Sugar syrup with probiotics as mentioned in 3. 3. 2 was prepared. This solution was provided to the diseased colony in a coconut shell. Freshly prepared probiotic solution was provided weekly for four weeks.

All the treated larval samples were collected and processed as mentioned in 3. 2. 1 and 3. 2. 2.


Plate 4. Probiotic used in the experiment (Darolac)

Plate 5. Providing prepared probiotic solution to the colony

# Results

# RESULTS

The results of the investigation on histomorphology of Indian bee (*A. cerana indica*) supplemented with probiotics was conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani during 2017 - 2019 are presented in this chapter.

## 4.1 SELECTION OF APIARIES AND SAMPLE COLLECTION

### 4. 1. 1 Apiaries Selected for Sample Collection

Purposive sampling was conducted at Thiruvananthapuram, Kollam, Pathanamthitta and Idukki districts for the present study. Twelve larvae (3 to 4 days old) each from naturally occurring feral colony, domesticated colonies which were fed with and without artificial feed, naturally infected ones with bacterial brood disease as well as larvae fed with insecticide treated royal jelly (Thiamethoxam 25% WG) were collected and subjected to histomorphological evaluation (Table 2).

The feral colony was attained from an undisturbed earthen bund which was 0.6 m above the ground level. The shade condition was moderate (30 – 80 % canopy coverage) for the apiaries selected for feral colony, colonies fed with and without artificial feed. The diseased colonies were found to be in highly shaded condition (>80% canopy coverage).

### 4. 1. 2 Diseased Colony

Out of the 17 colonies in the apiary, 13 were diagnosed with the symptoms of bacterial brood disease. In the healthy hive, the brood was observed as uniformly capped and fully covered with bees (Plate 6a and 6b). The larvae were pearly white in colour and with characteristic 'C' shape (Plate 6c). The symptoms observed in the diseased bee colonies were presence of scattered sealed and unsealed cells called "pepper box symptom". Perforated cell cappings were also noticed with dead pupae

Plate 6a. Comb uniformly covered with Indian bee

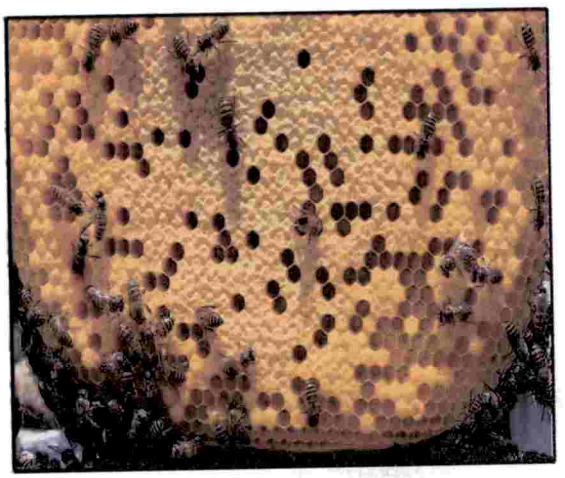

Plate 6b. Uniformly capped cells of Indian bee

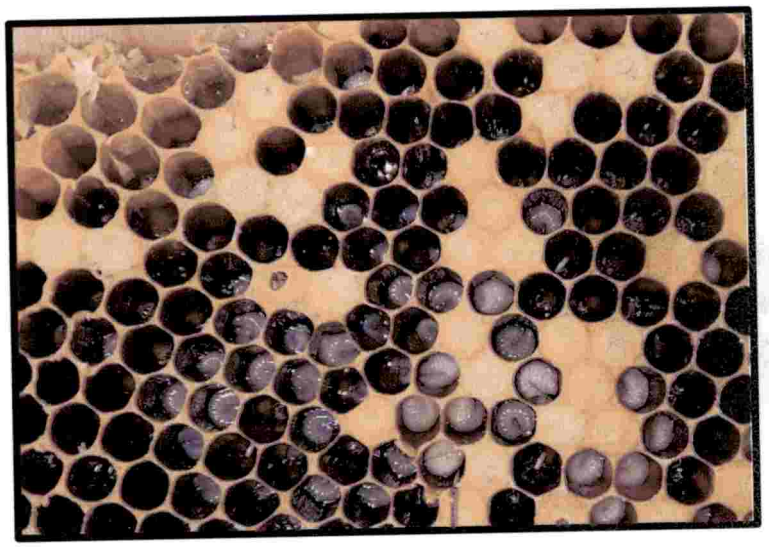

Plate 6c. Pearly white larvae of Indian bee

Plate 6. Healthy brood of Indian bee

Table 2. Details of the apiaries selected for sample collection

| Sl. No. | Type of apiary | Location | Total no.of colonies in apiary | No. of diseased colony | Shade condition | Floral sources | |
|---|---|---|---|---|---|---|---|
| | | | | | | Common name | Scientific name |
| 1 | Feral colony | Edamon, Kollam | 1 | 0 | Moderate | Rubber | *Hevea brasiliensis* |
| | | | | | | Coconut | *Cocos nucifera* |
| | | | | | | Rose | *Rosa* sp. |
| 2 | Colonies fed without sugar syrup | Vellimala, Idukki | 7 | 0 | Moderate | Terminalia | *Terminalia arjuna* |
| | | | | | | Tamarind | *Tamarindus indica* |
| | | | | | | Black plum | *Syzygium cumini* |
| | | | | | | Asoka tree | *Saraca indica* |
| | | | | | | Elanji | *Mimusops elanji* |
| | | | | | | Banana | *Musa* sp. |
| 3 | Colonies fed with sugar syrup | AICRP on HB& P apiary, COA, Vellayani | 10 | 0 | Moderate | Coconut | *Cocos nucifera* |
| | | | | | | Banana | *Musa* spp. |
| | | | | | | Rangoon creeper | *Quisqualis indica* |
| | | | | | | Mexican creeper | *Antigonon leptopus* |
| | | | | | | Coatbuttons | *Tridax procumbens* |
| 4 | Diseased colonies | Prakkanam, Pathanamthitta | 17 | 13 | High | Rubber | *Hevea brasiliensis* |
| | | | | | | Coconut | *Cocos nucifera* |
| | | | | | | Rambutan | *Nephelium lappacium* |
| | | | | | | Papaya | *Carica papaya* |

(Plate 7a). The pearly white larval colour changed to yellow and then to yellowish brown (Plate 7b). The larvae and pupae were found dead in the infected cells. Apart from these, the colony strength was also significantly reduced and the combs possessed only scanty bee population (Plate 7c).

**4. 1. 3 Standardization of Sublethal Concentration of Thiamethoxam 25 % WG**

The larvae were marked and different concentrations of thiamethoxam 25 % WG (0.05, 0.1, 0.2, 0.3 and 0.4 g $L^{-1}$) and water (control) were provided to the cells and observations were recorded at every five minutes interval. The experiment revealed that the field dose (0.2 g $L^{-1}$) as well as the higher concentrations above the field dose (0.3 and 0.4 g $L^{-1}$) resulted cent per cent mortality of larvae within 30 min. of treatment. When the larvae were fed with thiamethoxam 25 % WG @ 0.05 g $L^{-1}$, mortality observed within 65 min. (Table 3).

4. 2 HISTOMORPHOLOGY OF HONEY BEE MIDGUT

**4. 2. 1 Histoanatomy of Normal Honey Bee Larvae**

The anatomy of honey bee larvae (Plate 8) was studied from the larvae taken from feral colony which was chosen as the control group for the entire experiment.

***4. 2. 1. 1 Cuticle***

The cuticle (Ct) was observed as an external covering of the larval body. It was very soft and thin layer. The cuticle formed an exoskeleton, running throught the body except at the natural openings like mouth, anus and spiracle. The grooves on the cuticles marked the segmentation of bee larvae (Plate 9).

***4. 2. 1. 2 Brain***

The brain appeared to be a flattened lobed structure. It was located in the head above the stomodaeum or foregut. The cell body of the neurons with prominent

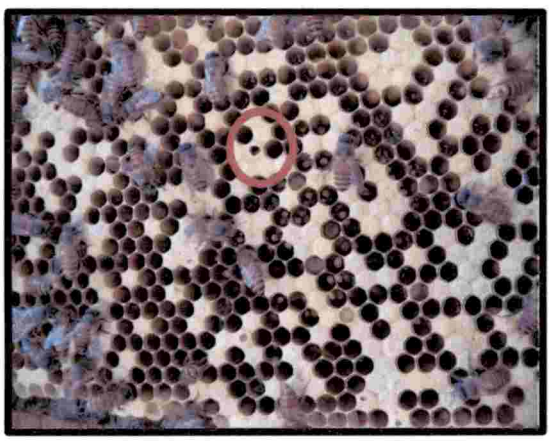

Plate 7a. "Pepper box symptom" in Indian bee brood

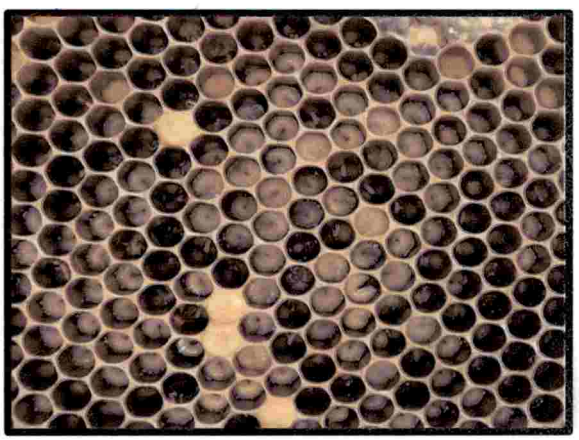

Plate 7b. Yellow and yellowish brown larvae

Plate 7c. Reduced population of Indian honey bee

Plate 7. Indian bee colony infected with bacterial brood disease

Table 3. Mortality of honey bee larvae orally treated with different concentrations of thiamethoxam 25% WG

| Dosage | No. of dead larvae after each 5 min ( Cumulative )* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (g $L^{-1}$) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 |
| 0.05 | 0 | 1 | 1 | 1 | 1.5 | 2 | 2.5 | 3.5 | 3.5 | 4 | 4 | 4.5 | 5 |
| 0.1 | 0 | 0 | 1 | 1 | 1.5 | 2 | 2 | 3 | 3.5 | 4 | 5 | - | - |
| 0.2 | 0 | 1 | 1.5 | 2 | 3 | 3.5 | 4 | 5 | - | - | - | - | - |
| 0.3 | 0 | 1 | 1 | 3 | 3.5 | 5 | - | - | - | - | - | - | - |
| 0.4 | 0 | 1.5 | 2.5 | 3.5 | 5 | - | - | - | - | - | - | - | - |
| control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

n = 5,*mean of two replication

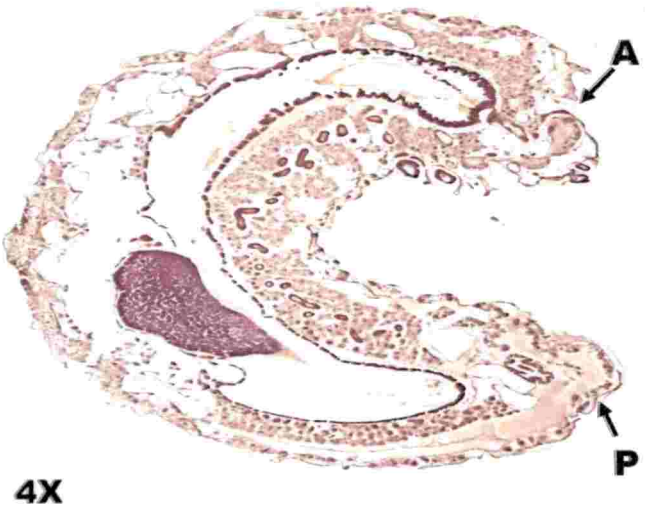


Plate 8. Transverse section of honey bee larvae
A: Anterior region, P: posterior region

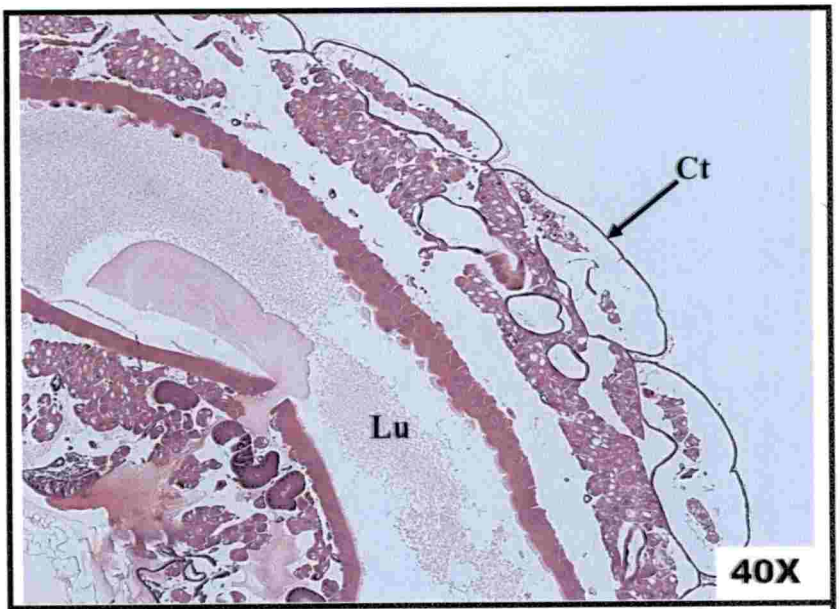


Plate 9. Cuticle
Ct- cuticle, Lu- lumen

nucleus was visible in the transverse section of the brain. The brain cells (BC) were closely packed towards the outer region of brain. The connection between lobes of brain was distinguished with the median cell less area. The brain was externally covered by a thin membrane (M) (Plate 10).

#### *4. 2. 1. 3 Ventral nerve cord*

The ventral nerve cord (V) was a long chain of ganglions ventrally present in the thorax and abdomen of larvae. There were three ganglions (G) in the thorax and eight in the abdomen. These ganglions were interconnected by means of interganglionic connectives. The thoracic ganglions lie in their respective segments between the leg rudiments (Plate 11).

#### *4. 2. 1. 4 Salivary gland*

The salivary gland (SG) also known as labial gland was observed in the ventral part of the head region. It was a pair of gland having a common duct opening at the base of the labium. It was composed of a single layer of closely packed cells with a central lumen into which the saliva is secreted (Plate 12).

#### *4. 2. 1. 5 Fat body*

The larval fat body was made up of many small polygonal shaped cells and was closely pressed together. This mass of cells occupied most of the space within the body cavity surrounding the alimentary canal. The cytoplasm of the fat cells appeared granular and contained numerous small fat globules (FG) filled with oil droplets. The nuclei were irregularly oval with relatively little oily globules. The whiteness of the fat tissue pressed against the transparent skin made the larvae appear white.

In the larvae, there were irregularly oval to polygonal cells embedding in the fat body called oenocytes (O). The size of these cells were larger than the fat cells (FC). The cytoplasmic granulation was not present in the oenocytes like that of fat

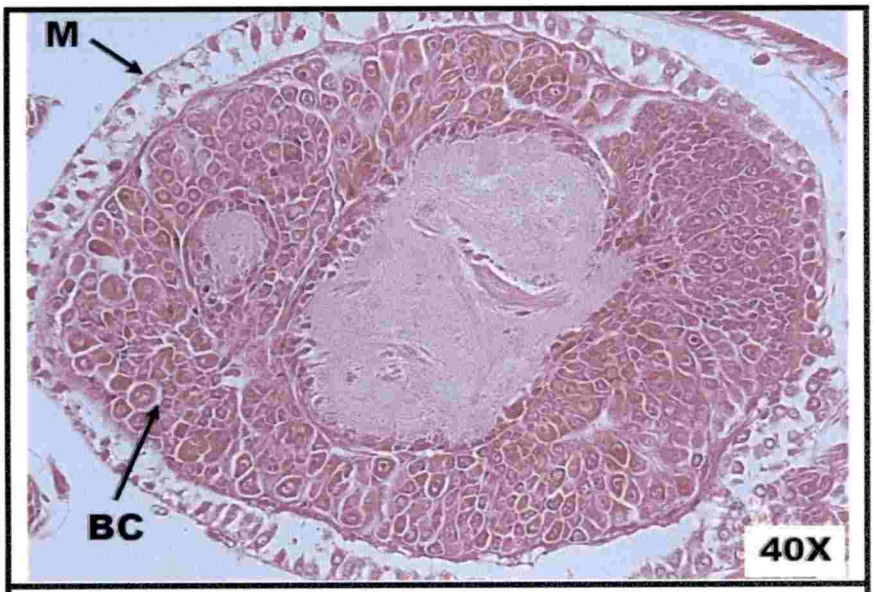


Plate 10. Brain
BC: brain cells, M:membrane

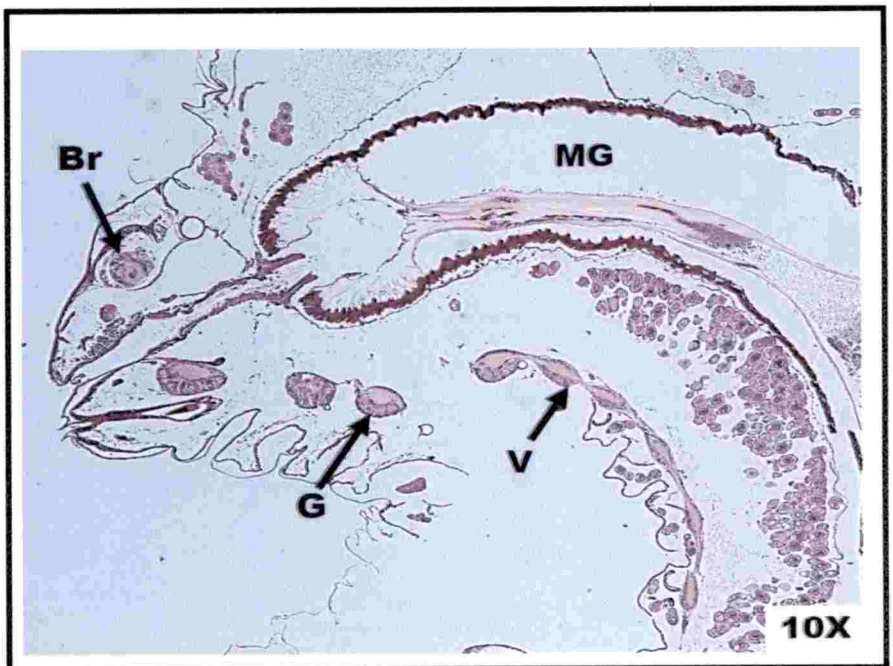


Plate 11. Ventral nerve cord
Br: brain, G: ganglion, MG: midgut,
V: ventral nerve cord

cells. It was filled with perfectly clear substance without any oil globules. The nuclei were round with little granulation than that of fat cells (Plate 13).

### *4. 2. 1. 6 Muscle*

The muscles (M) were arranged in definite patterns in all the body segments. They were arranged as bundles of fibre and had no retaining sheath. The muscles appeared as striated type with light and dark alternate bands. Each muscle fiber was multinucleated (Plate 14).

### *4. 2. 1.7 Malpighian tubule*

The Malpighian tubules (MT) were long, thread like tubes opening into the anterior region of the hindgut. The wall of the tubule was made of a single layer of cells with striations towards the lumen (Lu). This cells were resting on an outer basement membrane. The excretory products were discharged into its lumen (Plate 15).

### *4. 2. 1. 7 Trachea*

The tracheal system of the bee larvae was observed to be simple with main tracheal trunks and numerous ramifying branches reaching all parts of the body. The spiracles (S) appeared in the lateral side of the body segments. The spiracle was a simple opening without having any kind of closing apparatus. It opened into a spacious spherical atrial chamber (A). From this chamber, a narrow trachea (Tr) started that lead to the main lateral trunks (Plate 16 a). The trachea again branched to numerous tracheoles (Trl) and ended in the tissues. The trachea was ribbed by closely set spiral thickenings called taenidia (Ta). The taenidial thickening gave the characteristic appearance and rigidity to the tracheal walls that kept the tubes open. Also this structure maintained a free space for the passage of air (Plate 16 b).

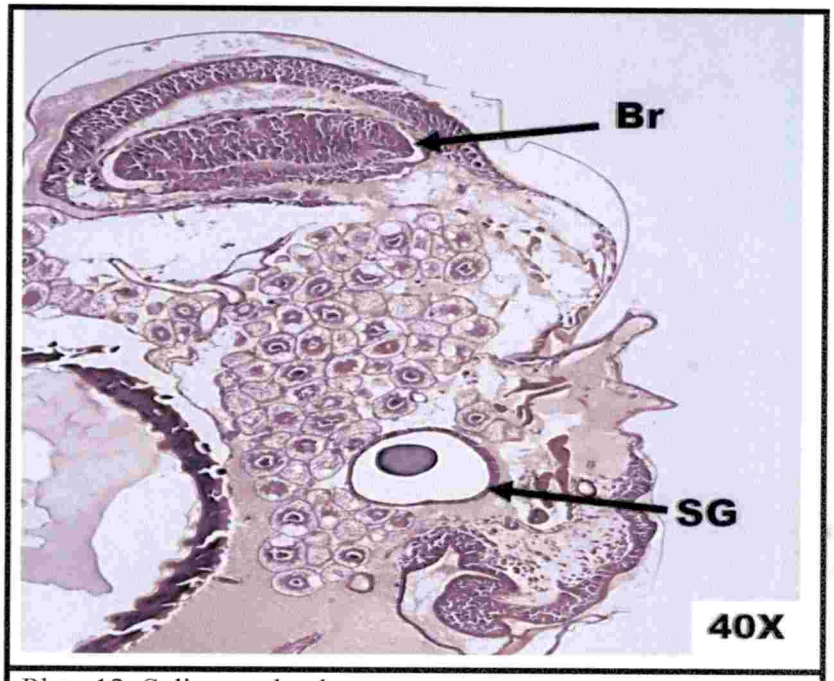


Plate 12. Salivary gland
Br: brain, SG: salivary gland

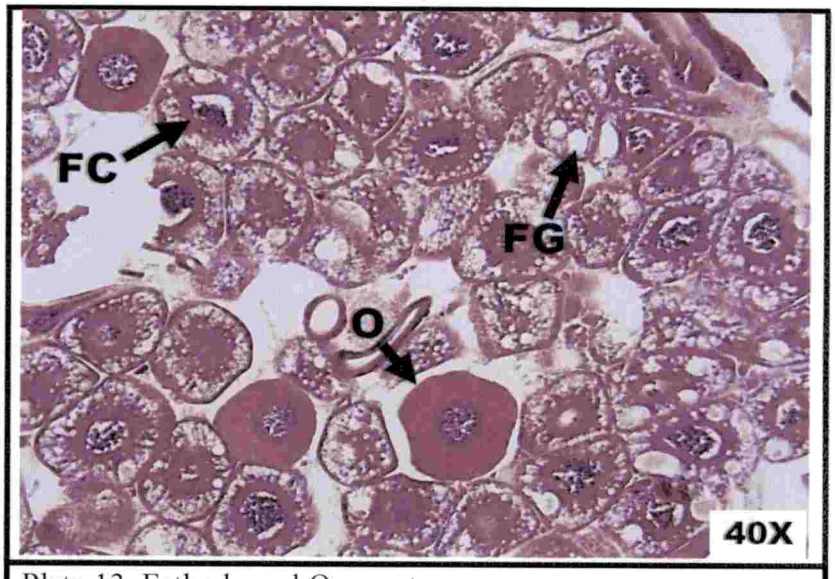


Plate 13. Fatbody and Oenocyte
FC: fat cells, FG: fat globule, O: oenocyte

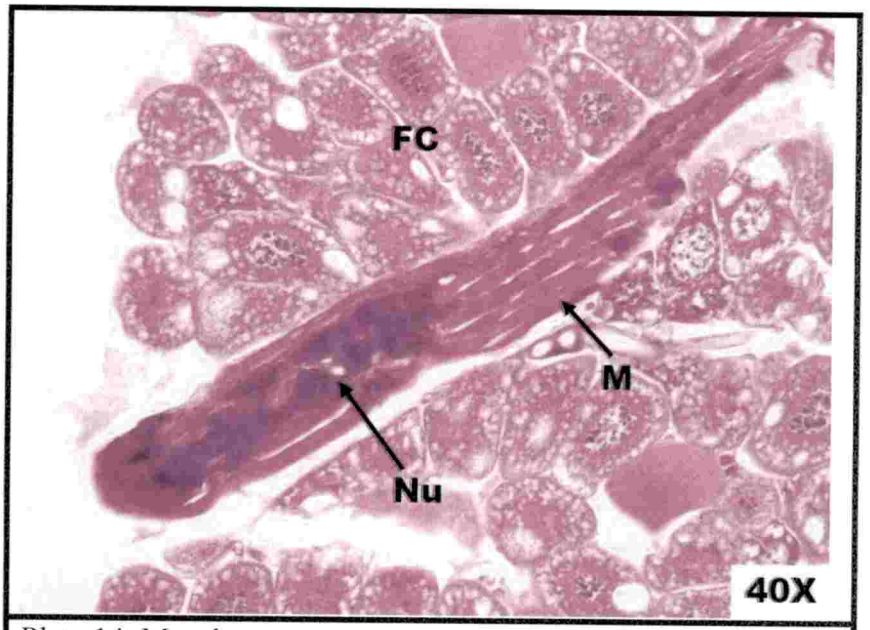


Plate 14. Muscle
FC: fat cells, M: muscle, Nu: nucleus

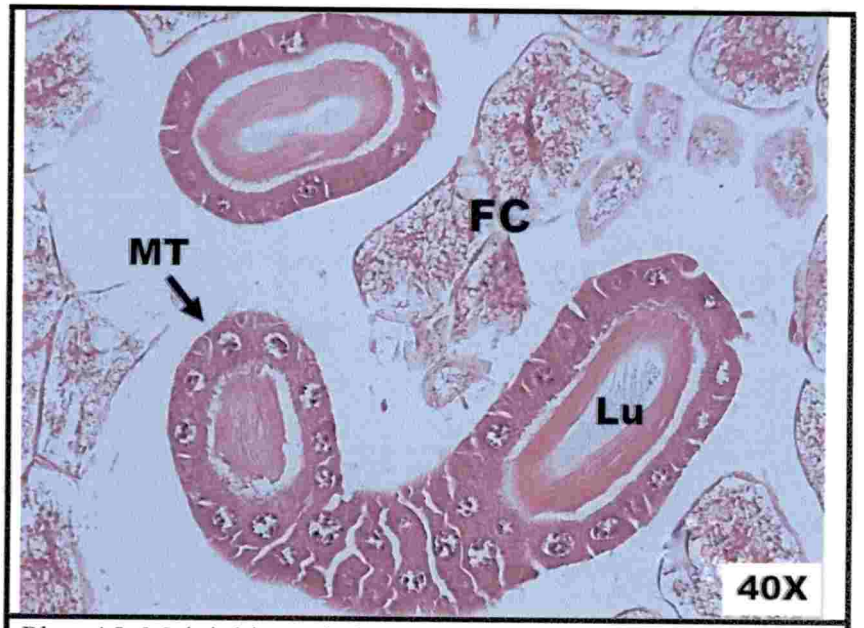


Plate 15. Malpighian tubules
FC: fat cells, Lu: lumen, MT: Malpighian tubule

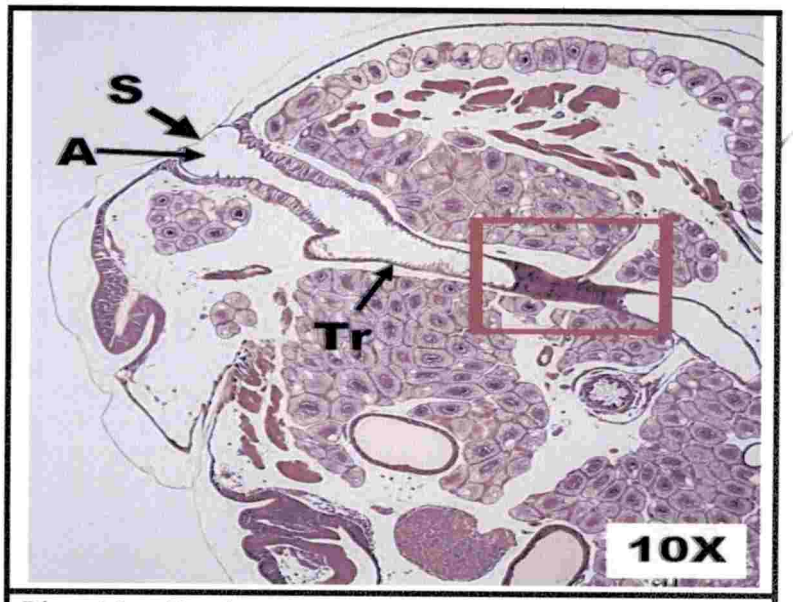


Plate 16 a. Trachea
A: atrial chamber, S: spiracle, Tr: trachea
(The highlighted portion is plate 16 b)

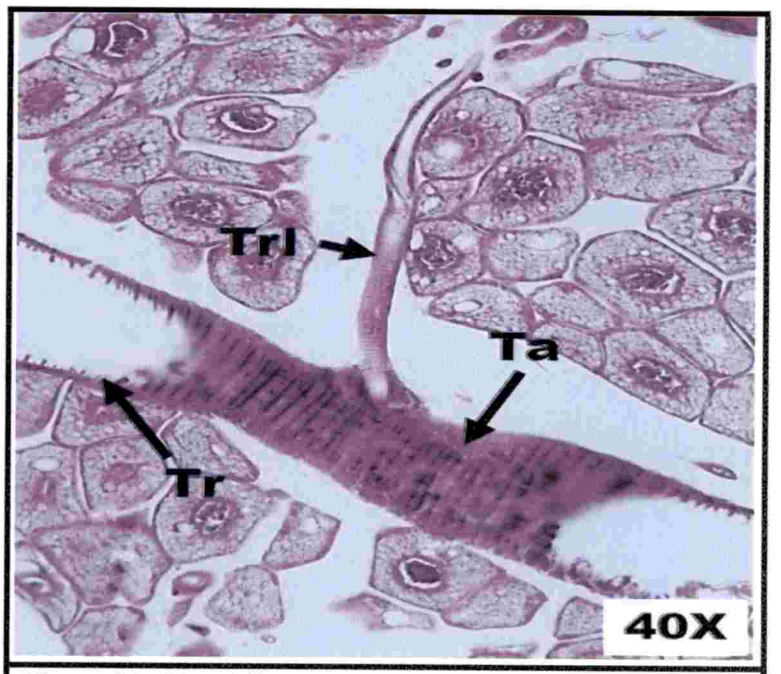


Plate 16 b. Taenidia
Ta: taenidia, Tr: trachea, Trl: tracheole

Plate 16. Tracheal system

*4. 2. 1. 8 Alimentary canal*

The alimentary canal of the larvae was composed of anterior foregut or stomodaeum, middle midgut or mesentron and posterior hindgut or proctodaeum. The alimentary canal started from the mouth and ended in the anus.

The foregut (FG) appeared as a slender tube starting from the oral cavity (Plate 17 a). It was separated from the midgut by a stomodaeal valve (SV) (Plate 17 b). The valve appeared as a tubular fold extending into the anterior portion of the midgut.

The midgut (MG) of the larvae was lined internally with columnar epithelial cells, which lies on a thin basement membrane. On the inner surface of the epithelium, there was brush border like microvilli. Triangular shaped minute regenerative cells were observed at the base of the epithelial cells. The midgut also possesses peritrophic membrane. This membrane appeared as a lining to the epithelium and was present throughout its length. It also covered the food that reaches the midgut (Plate 17 c).

The hindgut (HG) continued as an open tubular structure from the midgut. But wall of the hindgut appeared as multilayered and was more thickened than midgut wall. The hindgut opened externally through the anus (A) (Plate 17 d).

**4. 2. 2 Histomorphology of Midgut of Honey Bee Larvae under Different Stress**

The histomorphological evaluation of midgut of honey bee larvae from domesticated colonies which were fed without artificial feed, fed with sugar syrup, suffering from bacterial brood disease and orally treated with thiamethoxam 25 % WG were done and are compared with that of feral colony larvae.

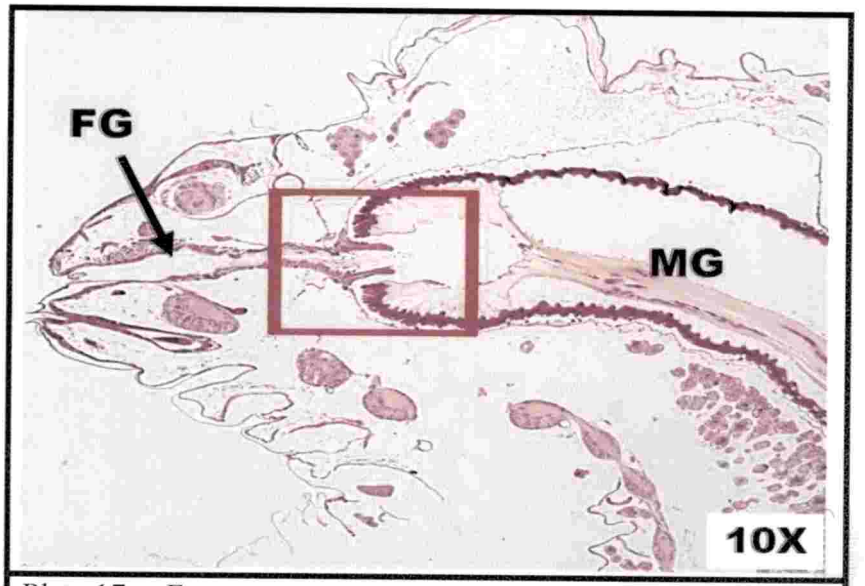


Plate 17 a: Foregut
FG: foregut, MG: midgut
(The highlighted portion is plate 17 b)

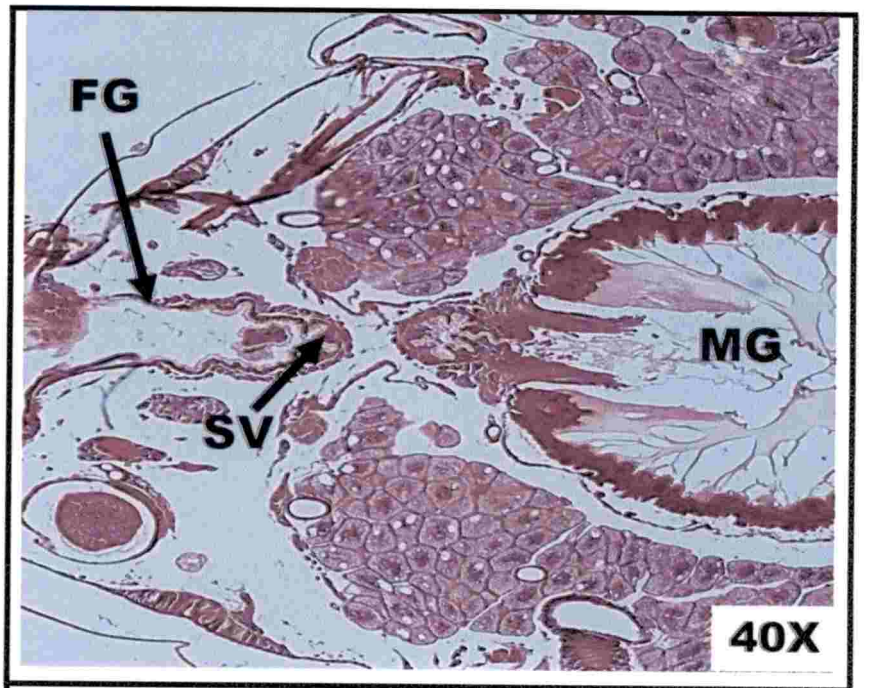


Plate 17 b: Stomodeal valve
FG: foregut, MG: midgut, Sv: stomodeal valve

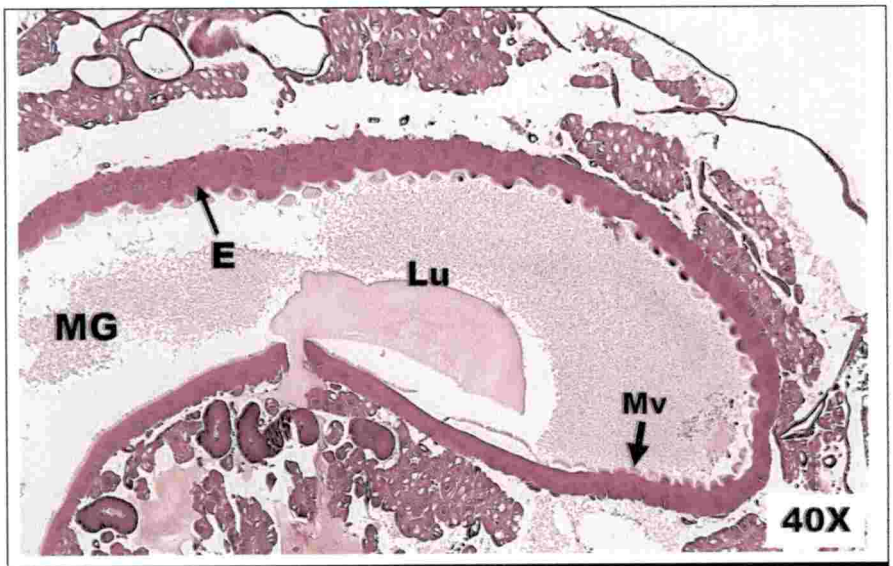


Plate 17 c. Midgut
E: epithelial cells, Lu: lumen, MG: midgut, Mv: microvilli

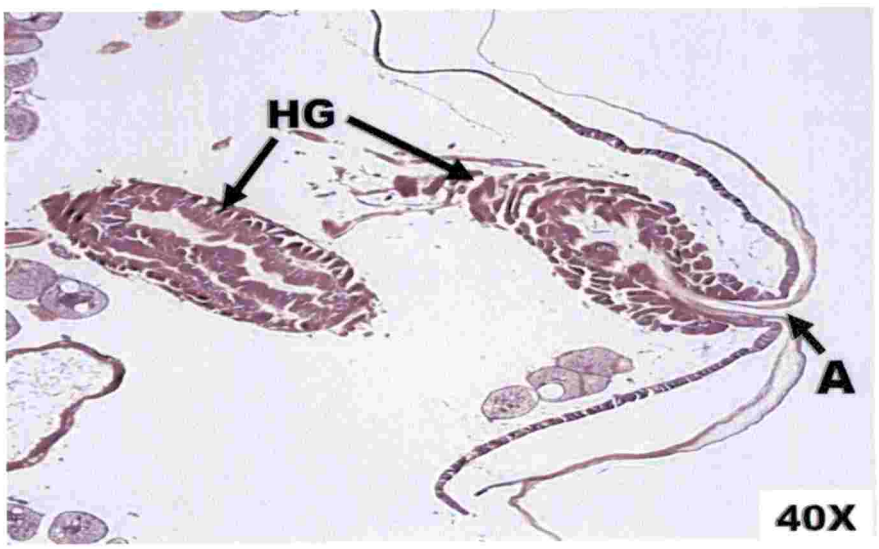


Plate 17 d. Hindgut
A: anus, HG: Hindgut

Plate 17. Alimentary canal

*4. 2. 2. 1 Feral Colonies (Control)*

When the midgut of honey bee larvae taken from feral colony was examined, the structure of midgut cells was found to be normal. The columnar epithelial cells (E) were tightly packed with higher integrity. These cells had predominant nucleus (Nu) which were uniform and oval shaped. Also, there were well defined brush bordered microvilli (Mv) on the surface of the epithelial cells. Copious secretion of peritrophic membrane (PM) was visible over the microvilli towards the lumen (Lu). Prominent regenerative cells (R) could be appreciated at the base of epithelial cells (Plate 18).

*4. 2. 2. 2 Colonies Fed without artificial feed*

The midgut of honey bee larvae fed without artificial feed was found to be similar to that of feral colony. The epithelial cells (E) were homogenously arranged with characteristic columnar shaped cells. The nuclei (Nu) were of uniformly oval shaped and the cytoplasm was less granulated. The brush bordered microvilli (Mv) were of similar length and uniformly arranged over the epithelial cells towards the lumen (Lu). Regenerative cells (R) were well distributed near the base of the epithelial cells. No necrotic cells were present in the midgut of these larvae. The peritrophic membrane was not thick but it was in the initiation stage. (Plate 19).

*4. 2. 2. 3 Colonies Fed with Sugar Syrup*

When the larvae were fed with sugar, it was found that the histomorphology of midgut has deviated from that of the normal one. The typical columnar shape of the epithelial cells (E) was altered and appeared wrinkled towards the apical region of the cells. Slightly degenerating cells were perceivable in the midgut. Also, the number of regenerative cells (R) were reduced when compared to that of the normal

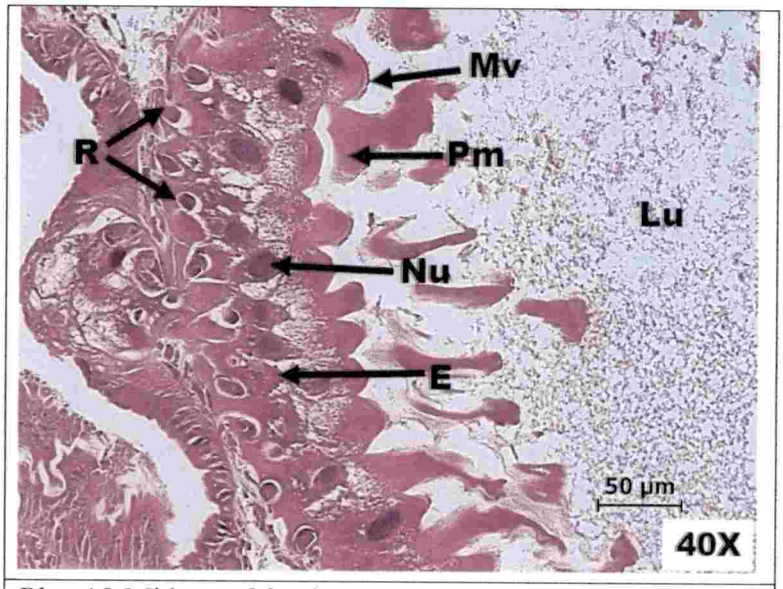


Plate18.Midgut of feral colony
E: epithelium, Lu: lumen, Mv: microvilli,
Nu: nucleus, , Pm: peritrophic membrane,
R:regenerative cell

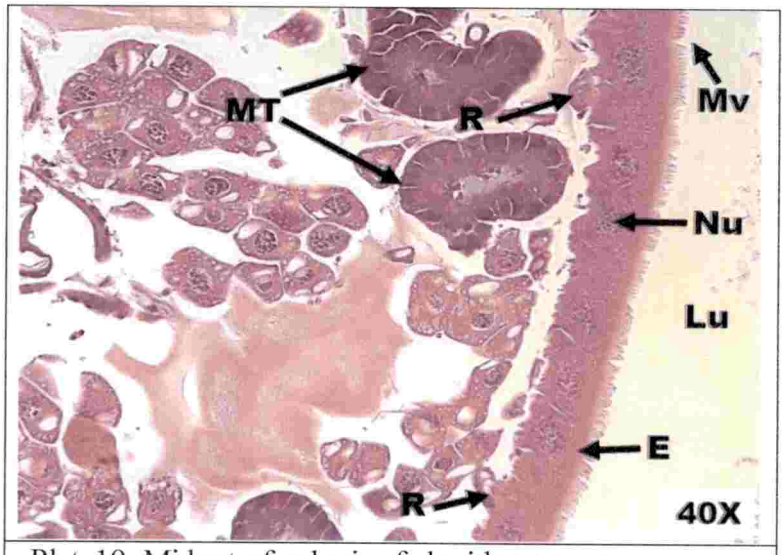


Plate19. Midgut of colonies fed without sugar syrup
E: epithelium, Lu: lumen, MT: Malpighian tubule
Mv: microvilli, Nu: nucleus, R: regenerative cell

healthy feral colony larvae. The nucleus (Nu) of the epithelial digestive cells appeared swollen. Some of the cells were found flat. The nucleus was completely disappeared (Karyolysis) from such cells and was represented as necrotic cells. The cytoplasmic granulation was comparably more than the normal cells. The brush bordered microvilli (Mv) was inconspicuous from the epithelial cells. Though peritrophic membrane (PM) was present, its secretion and distribution over the epithelial cells was uneven (Plate 20).

#### *4. 2. 2. 4 Colonies Naturally Infected with Disease (Symptoms Similar to that of Existing Bacterial Brood Disease)*

The histopathological analysis of diseased honey bee larvae revealed significant morphological variations in the midgut. The epithelial cells (E) were found deviated from the basic columnar cell shape and were flattened. In severely infected cells, disintegration of cell content occurred towards the lumen (Lu) and lead to cell lysis. The epithelial cell integrity was completely distorted and they were loosely arranged to one another. Cell necrosis was prominent in this case which was indicated by the presence of karyorrhectic or picnotic nucleus (PN). These are the cells with fragmented, dense and dark nuclear fragments. The microvilli (Mv) was inconspicuous which are unclear and broken towards the lumen end. Moderately vacuolated cytoplasm was evident. Only occasional regenerative cells were apparent and the peritrophic membrane was found to be distorted (Plate 21).

#### *4. 2. 2. 5 Colonies Treated with Insecticide*

The midgut of honey bee larvae treated with Thiamethoxam 25% WG at 0.05 g $L^{-1}$ was analysed. The characteristic columnar shape of the epithelial cells (E) were visible. The nuclei (N) were dense, dark and granulated. Thick uniform microvilli (Mv) was distributed throughout the length of the epithelium. The significant observation was the presence of vacuoles (V) of variable size (Plate 22).

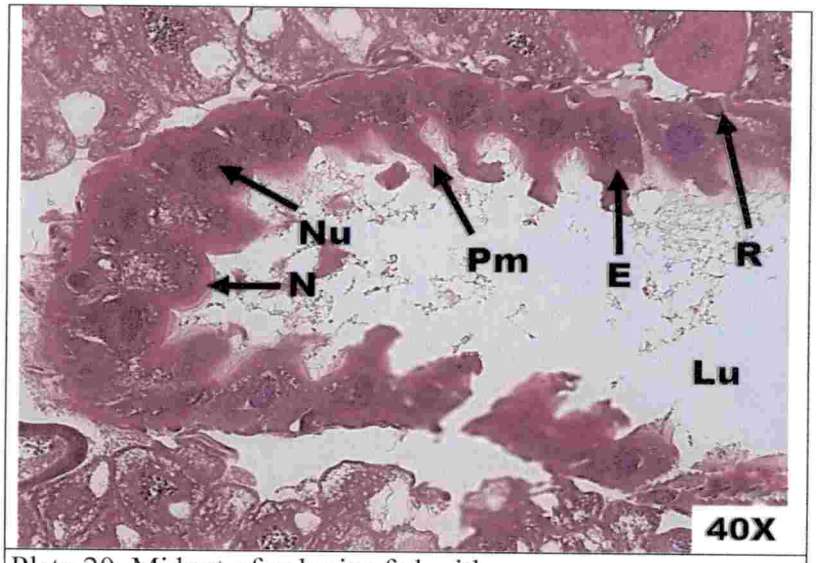


Plate 20. Midgut of colonies fed with sugar syrup
E: epithelium, Lu: lumen, N: non nucleated cell,
Nu: nucleus, Pm: peritrophic membrane,
R: regenerative cell

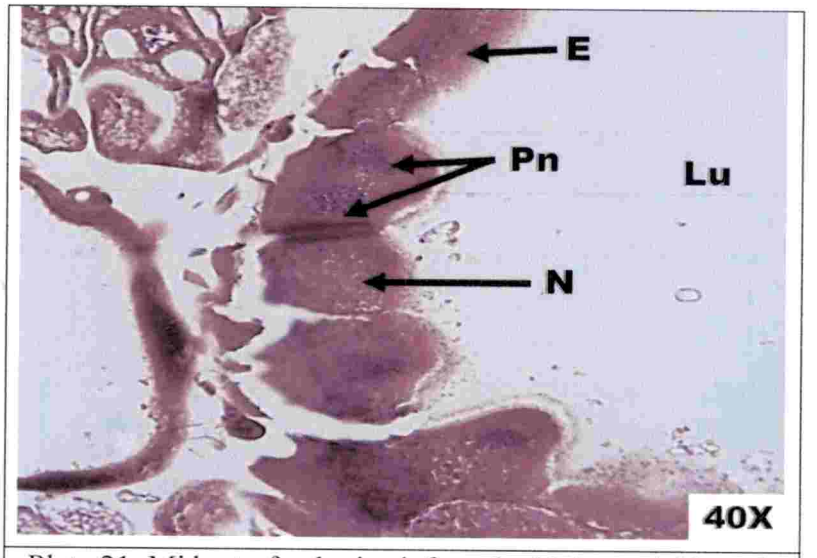


Plate 21. Midgut of colonies infected with bacterial brood disease
E: epithelium, Lu: lumen, N: non nucleated cell,
Pn: picnotic nucleus

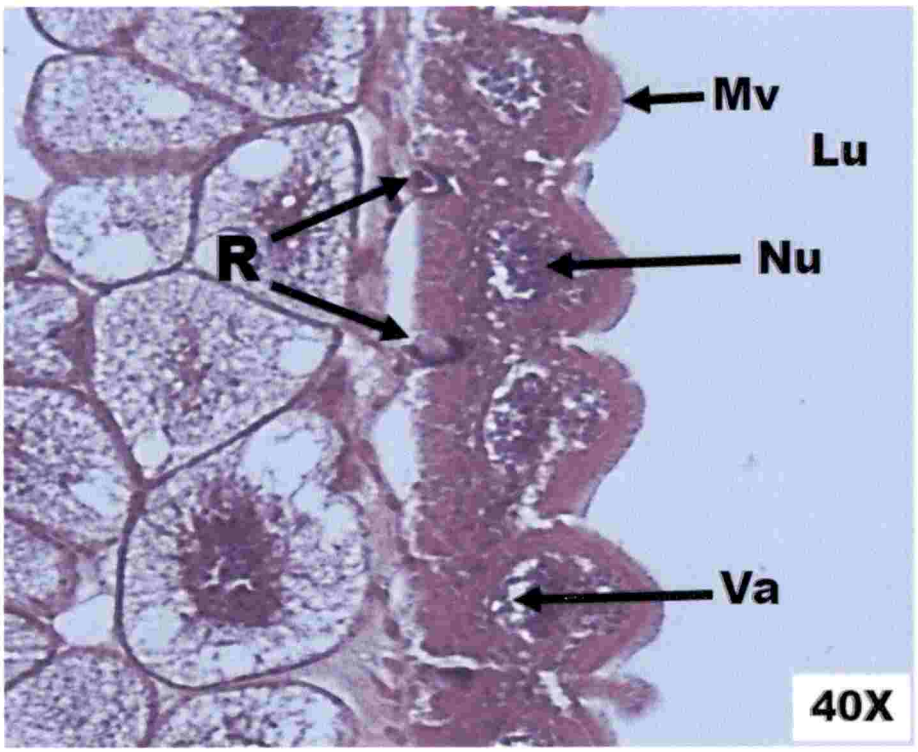


Plate 22. Midgut of colonies orally treated with thiamethoxam 25% WG @ 0.05 g $L^{-1}$
Lu: lumen, Nu: nucleus, Mv: microvilli, R: regenerative cell, Va: vacuoles

4.3 EFFECT OF PROBIOTICS ON THE MIDGUT OF HONEY BEE LARVAE

The probiotic Darolac was provided at 1.2 g $L^{-1}$ of water for normal domesticated colonies and 1.2 g $L^{-1}$ of sugar syrup for normal as well as the diseased colonies.

**4. 3. 1 Probiotics Alone in Normal Colonies**

When the larvae was fed with probiotic, Darolac at 1.2 g $L^{-1}$ of water for a period of four weeks, it was found that in some places, the midgut epithelium cells (E) were projecting towards the lumen (Lu) side. The nuclei of epithelial cells were darker and prominent. The microvilli (Mv) was present all over the epithelial cells. Triangular shaped minute regenerative cells (R) were found at the base of the epithelial cells. When it was compared with that of feral colony, significant difference was seen in the case of strong aposematic secretions (AS) towards the lumen (Plate 23).

**4. 3. 2 Probiotics along with Sugar Syrup in Normal Colonies**

When probiotic Darolac 1. 2g $L^{-1}$ was dissolved per litre of sugar syrup and fed to the bee larvae for four weeks, it was observed that the structure of the epithelial columnar cells (E) started to regain its integrity. But the base of these cells were still under recovering stage. It was conceivable to observe the normal elimination of apocrine secretion (AS) from the digestive epithelial cells. Microvilli (Mv) as well as peritrophic membrane (PM) were also visible over the epithelial cells. A few regenerative cells (R) were also present at the base of the epithelial cells (Plate 24).

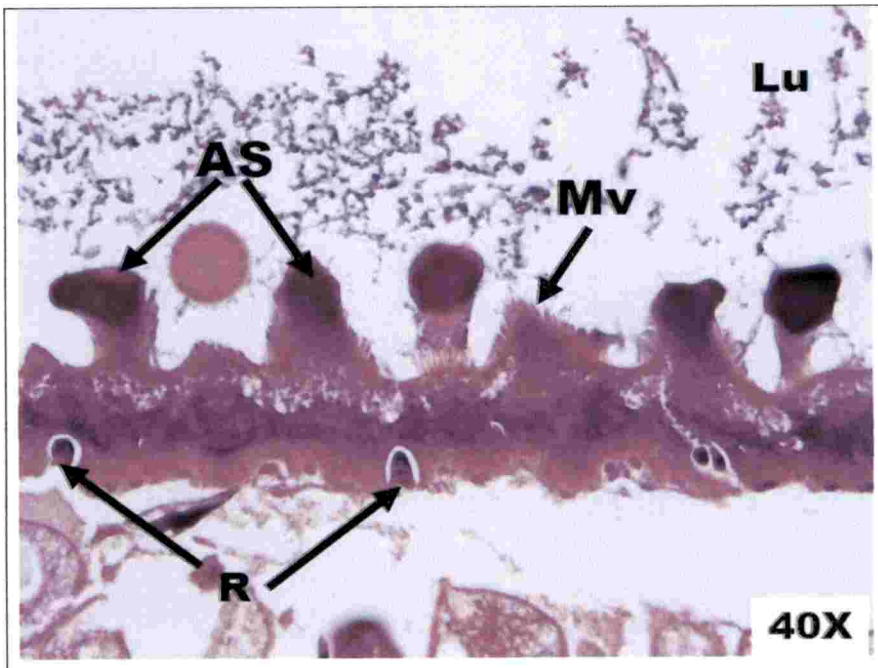


Plate 23. Midgut of colonies fed with probiotics alone
AS: aposematic secreation, Lu: lumen, Mv: microvilli, R: regenerative cell

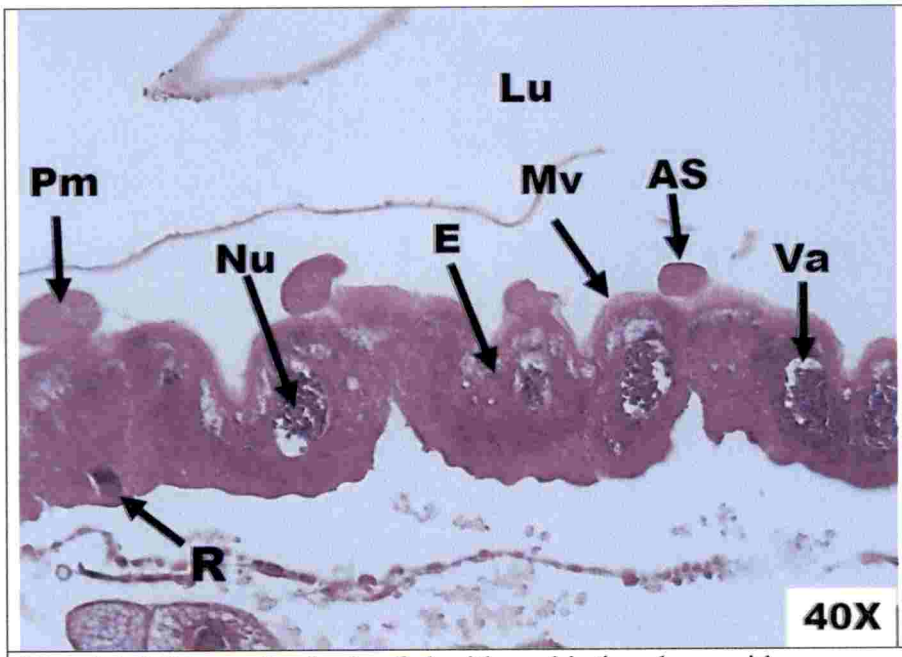


Plate 24. Midgut of colonies fed with probiotics along with sugar syrup
AS: aposematic secreation, E: epithelium, Lu: lumen, Nu: nucleus, Mv: microvilli, Pm: peritrophic membrane, R: regenerative cell, Va: vacuole

### 4. 3. 3 Probiotics along with Sugar Syrup in Diseased Colonies

When the diseased larvae were treated with probiotic for a period of five weeks, a significant difference in the histomorphology was observed from that of the diseased colonies. The epithelial integrity which was totally lost in the diseased larvae tend to became normal when treated with probiotics. The epithelial cells (E) were columnar and the nuclei was dense and darker. The presence of prominent brush bordered microvilli (Mv) was visible throughout the length of midgut. The most important character that can be appreciated was the presence of numerous regenerative cells (R) at the base of the cells (Plate 25).

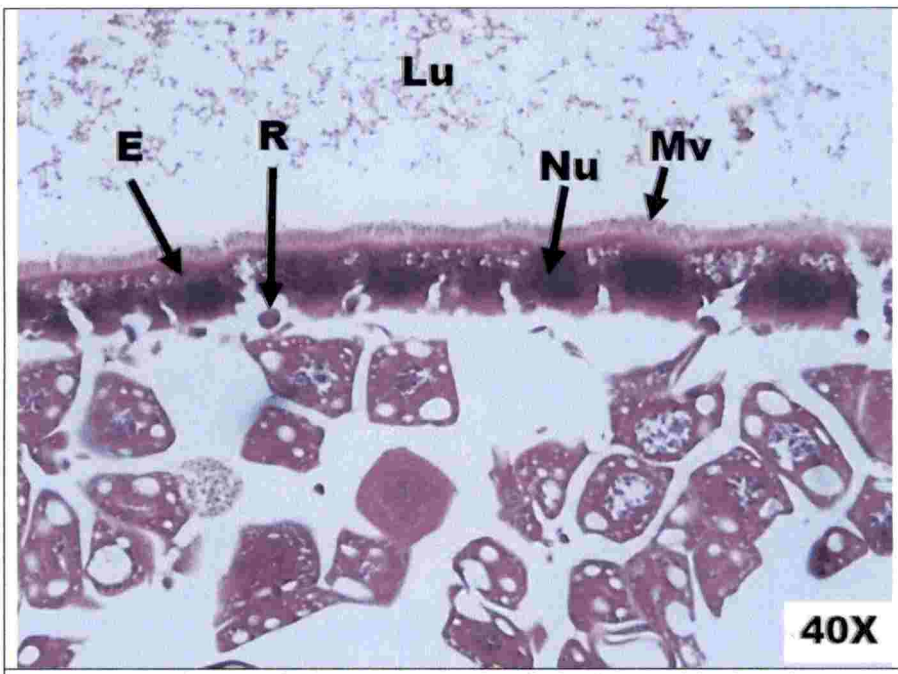


Plate 25. Midgut of diseased colonies fed with probiotics along with sugar syrup
E: epithelium, Lu: lumen, Mv: microvilli, Nu: nucleus, R: regenerative cell,

# *Discussion*

## 5. DISCUSSION

The present investigation was conducted under AICRP on Honey Bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani to study the histomorphology of Indian bee (*A. cerana indica*) supplemented with probiotics. The study was conducted with the objective to assess the histomorphology of midgut of Indian bee suffering from various stressors (pathogens and insecticides) and after being fed with probiotics. The results of the study are discussed below:

### 5. 1 SELECTION OF APIARIES AND SAMPLE COLLECTION

The honey bees rely on plant nectar as energy source. During the dearth season, due to the lack of adequate nectar, beekeepers of Kerala usually supplement artificial feed, sugar syrup (Barker, 1977). In the high range districts of Kerala (Idukki, Wayanad, etc.), the practice of providing artificial feed is very less since adequate foraging plants which provide ample amount of nectar and pollen are available throughout the year. There is continuous availability of nectar and pollen from the natural fauna like terminalia, tamarind, black plum, asoka tree, elanji, and banana.

Studies on the shade conditions revealed that the colonies of the apiaries with >80 % canopy coverage are more susceptible to bacterial brood disease. Shade condition plays an important role in the case of pest incidence. According to Bose (2017), 50.70% incidence of small hive beetle (*Epuraea latissima)*, an important pest of stingless bee hives, was recorded from the apiaries with high shade condition (> 80 % canopy coverage).

In western countries bacterial brood disease was reported by Bailey and Ball (1991) and Forsgren (2010) from *A. mellifera* colonies. In Kerala, it was reported by Amritha *et al.* (2014) and Joseph (2018). The symptoms reported included scattered presence of sealed and unsealed cells ("pepper box symptom"), yellow to yellowish

brown larvae, dead larvae and pupae inside infected cells, perforated cell cappings with dead pupae and reduced colony strength. The diseased colony selected from Prakkanam of Pathanamthitta district also shown similar symptoms.

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

## 5. 2 HISTOMORPHOLOGY OF HONEY BEE MIDGUT

The anatomy of normal honey bee larvae was studied from the feral colony larvae which was taken as the control. Different organ and organ systems of larval honey bee were examined from the sample. All these findings were in accordance with anatomy and physiology studies in honey bee larvae of *A. mellifera* by Snodgrass (1956) and Chapman (1969).

In the present study, the cuticle of honey bee larvae was found as an external covering of the larval body which was running throught the body except from the natural openings like mouth, anus and spiracle. The brain appeared to be a flattened lobed structure with closely packed brain cells. The ventral nerve cord was a long chain of ganglions ventrally present in the thorax and abdomen of larvae.The salivary gland was composed of a single layer of closely packed cells with a central lumen. The fat body cells and oenocytes are found together throughout the larval body. The muscles were of striated type and arranged in definite patterns in all the body segments. The tracheal system of the bee larvae was observed to be simple with main tracheal trunks and numerous ramifying branches which reaches all parts of the body. Malpighian tubules were long, threadlike tubes with single layer of cells with striations towards the lumen.

Histomorphological tools have been employed for diagnosis of numerous infectious and parasitic diseases of animals. Hence it have proved to be the most sensitive and specific method for the detection and identification of numerous pathogens (Maiolino *et al.*, 2013).

The midgut of the honey bee larvae was found to be composed of columnar epithelial cells and its brush bordered microvilli, thick mucus like peritrophic membrane and minute triangular regenerative cells at the base of the epithelial cells (Plate. 18). Studies by Nelson (1924) also revealed that the walls of larval midgut is made with simple epithelium of cubical to columnar cells, on the inner surface of which is a distinct striated border. He also described the presence of triangular group of minute regenerative cells on the basement membrane as well as a homogenous layer of peritrophic membrane over the epithelium apparently having gelatinous consistency. These observations are in line with the findings of Snodgrass (1956). Natural source of food for bees (nectar) had no harmful effect on the midgut histology and have positive effect on the life span of bees (Mirjanic *et al.*, 2013). So the midgut of the larvae that were not fed with sugar syrup had similar histomorphology of feral colony larvae (Plate 19).

Thus the midgut with characteristic columnar to cuboidal epithelial cells and oval nucleus, intact brush bordered microvilli, thick layer of peritrophic membrane in lumen and numerous triangular regenerative cells at the base of the epithelial cells were considered as normal healthier honey bee larva.

According to Doung (2014), sugar syrup is used as a supplement to prevent starvation of bee colony. It may also be useful to increase the number of field bees foraging for pollen from the hive which automatically enhance their role as pollinators. Among different feed, cane sugar have a sucrose level of 99.85 per cent with 0.03 per cent ash content and organic sugar have 99.5 per cent of sucrose with

0.20 per cent of ash. When the ash content levels in the standard cane sugar product was less, there will have only less digestive issues. It is unadvisable to feed waste sugar to bees unless aware of the constituents included in it. White cane sugar provide least risk to bees in digestive complaints which usually manifested as dysentery in bees. White sugar is economically feasible as a supplement for bees when compared to other sugars. 1:1 concentration of sugar and water by volume is preferable for feeding the colonies in every few days.

The present study on histomorphology of honey bee larvae fed with sugar syrup revealed the adverse effect of sugar on midgut cells *viz*., presence of vacuoles in the epithelium, absence of microvilli, reduced number of regenerative cells and uneven peritrophic membrane secretion (Plate 20). This findings were in accordance with the study on impact of sugar on midgut of *A. mellifera* larvae by Mirjanic *et al.* (2013) where they reported pronounced peak damage of epithelial cells with non-homogenous intestinal content in the larvae fed with sugar syrup. In midgut of bees fed with acid invert syrup, almost totally damaged columnar epithelial cells and uneven distribution of microvilli was observed.

According to Maiolino *et al.* (2013), the extensive necrosis of ventricular epithelial cells would lead to ulceration and severe malabsorption of water and nutrients. This will ultimately leads to effusion of tissue fluid and fast death of larvae as a result of dehydration.

In the present study on the histomorphology of honey bee larvae infected with bacterial brood disease, necrosis of epithelial cells with picnotic or karyorrhectic nuclei, moderate to severely vacuolated cytoplasm, inconspicuous microvilli and distorted peritrophic membrane was recorded (Plate 21). Ayaad *et al.* (2017) inoculated *A. mellifera* with *Paenibacillus larvae,* the causal organism of American foul brood disease, and studied the gut histology at 48 and 72 h of post infection. After 48 h of infection, the midgut showed significant histological alterations such as

presence of vacuoles, separation of epithelial layer, lacerations in the basement membrane and damage of circular muscles. At 72 h post infection, the infected brood showed separation and elongation of the midgut epithelial cells. This report is in corroboration with the present study.

In the midgut cells of the larvae infected with bacterial disease, the picnotic nuclei observed are often described in organs undergoing degeneration in Hymenoptera (Silva- Zacarin *et al.,* 2007). The presence of highly picnotic nuclei indicates low transcriptional activity due to chromatin compacting. These features suggested that the cells are in advanced cell death process (Silva- Zacarin *et al.,* 2008).

It has been established that the bacterial brood diseases are contagious disease, in which the infection starts in the initial instars, which ultimately results in an entire colony collapse. After passing through the gut, the ingested spores germinate in the midgut around 12 h of post- ingestion. These spores adheres on the peritrophic membrane and starts bacterial proliferation. These bacteria then penetrate and destroy this protective layer. Breaching of the epithelial layer paves the way to spreading of bacteria all over the body. Finally, rupturing of the cells cause the death of the larvae (Genersch, 2010). This may be the possible reason for the disintegration and lysis of epithelial cells which was evident in the present investigation.

Thiamethoxam 25 % WG was chosen for the present study since it was reported as the most toxic insecticide among the neonicotinoids causing cent per cent mortality to bees within one hour of treatment (Raeesa, 2018). Although the action of thiamethoxam occurs in the nervous system, secondary targets like alimentary canal may also be affected by this compound. Therefore, it is important to analyze the cytotoxicity of thiamethoxam in tissues reached via the metabolism of contaminated food containing insecticides. Thus, one important organ for toxicity analysis is the midgut, since it is responsible for digestion and absorption of ingested food.

Moreover, it is one of the primary sources of contact when the insect comes in contact with the insecticide orally.

Kakamand *et al.* (2008) carried out research on the effect of minimal concentration of deltamethrin, malathion and thiamethoxam on the midgut epithelial tissue of bees (*A. mellifera*). The effect of thiamethoxam on the midgut epithelium was in line with the observations of present study. The abnormality of epithelial cells was clearly observed where the cytoplasm was granulated and vacuolated. Among these observations, the most prominent observation was the presence of various sizes of cytoplasmic vacuolization for minimum concentration of malathion and thiamethoxam.

According to Catae *et al.* (2014), the electron microscopy of midguts of the *A. mellifera* exposed to 0.0428 ng of thiamethoxam 25 % WG $L^{-1}$ of diet for a period of eight days showed typical characteristics *viz.*, digestive cells usually with nuclei of spherical shape, organelles with no alterations, especially mitochondria with preserved double membranes and evident cristae, and rough endoplasmic reticules. Vacuoles were also observed in the cytoplasm. In the present study, similar presence of vacuoles of variable size was observed (Plate 22).

Apart from the effect on honey bee midgut, ultrastructural results showed that cytotoxicity in Malpighian tubules begins in bees which were exposed to thiamethoxam for one day. The most important alteration was the loss of basal labyrinth which is essential for promoting contact with haemolymph and the further uptake of metabolized products (Cruz- Landim, 1998).

Cruz *et al.* (2010) conducted an experiment to study the morphological alterations induced by the insecticides, boric acid and fipronil, in the midgut of worker honey bee (*A. mellifera*) larvae. The larvae were fed with diets containing different concentrations of boric acid (1.0, 2.5 and 7.5 mg $g^{-1}$) and fipronil (0.1 and 1 µg $g^{-1}$) and compared with control. After 4 days of treatment, when the larval midgut was

subjected for morphological analysis, it was observed with cytoplasmic vacuolization, with the absence of autophagic vacuoles and chromatin condensation of the treated groups. In the midgut of the larvae treated with fipronil, the picnotic nuclei was observed. The morphological alterations in the larval midgut cells caused by boric acid was far greater than that by the fipronil. This study significantly pointed out the effect of these insecticides from the ecotoxicological perspective.

## 5. 3 EFFECT OF PROBIOTICS ON THE MIDGUT OF HONEY BEE LARVAE

The bee digestive tract with beneficial bacterial species has very important implications for the improvement of bee health. Bee health is seriously affected when a disturbance arises in the normal balance of beneficial microflora (Koch and Schimid- Hempel, 2011). The nine dominant gut bacterial community included *Gilliamella apicola, Frischella perrara*, *Snodgrassella alvi*, *Lactobacillus mellis, L. acidophilus L. mellifer, L. helsingborgensis, L. kullabergensis, L. melliventris, L. rhamnosus, L. kimbladii, Bifidobacterium asteroids*, *B. longum* and, *B.coryneforme* (Moran, 2015; Kwong and Moran, 2016).

According to World Health Organization (WHO) (2002), probiotics are live microorganisms that when administered in sufficient amounts, offer a health benefit on the host. Probiotics have the ability to shape the immune system through their physiological action on the intestine level. Once in the intestine, they interact with intestinal cells, triggering an immune response due to the fact that intestinal cells produce a series of immune-simulator molecules when stimulated by bacteria (Corcionivoschi and Drinceanu, 2010).

The honey bee gut microbiota displays high affinity with that of mammals. Both microbial communities grow best under oxygen concentrations lower than that of air. The microbial communities are dominated by host-adapted species that are not typically found outside the gut. All major bacterial species are cultivable in the laboratory. But the microbial community of honey bee is simple, with nine bacterial

species clusters comprising over 95 per cent of the community (Martinson *et al.*, 2012). Because of these similarities human grade probiotic Darolac was chosen for the experiment. Also, Darolac contains *Lactobacillus acidophilus, L. rhamnosus and B. longum* which are present among the enlisted natural gut endosymbionts of honey bee.

The probiotic *Enterolactis* (*L. acidophilus*, *L. lactis* and *Bifidiobacterium* sp.) consumed by the bees together with sugar syrup @ 1.2 g $L^{-1}$ increased the population beneficial microbes in the larval intestines and lead to the significant decrease in the number of potential pathogenic bacteria in the gut (Patruica and Mot, 2012). With this reference, the dose of Darolac is taken as 1.2 g $L^{-1}$ of sugar syrup.

Szymas *et al.* (2012) assessed the morphological changes in the midgut epithelium of bees nourished with pollen substitute or pollen substitute enriched with a probiotic preparation, Biogen and Trilac. The assessment of histological changes of the bee midgut was carried out in bees feed for 8 and 14 days. Slight changes in the epithelium as well as strong merocrine type secretion were recorded in bees nourished with pollen substitute supplemented with probiotic preparations. Differences were observed, primarily, in quantities of the developed peritrophic membranes. Their quantities were particularly high after 14 days of feeding with the pollen substitute fortified with probiotic preparations.

The result of the present study on effect of probiotics on midgut histomorphology is in line with the findings of Szymas *et al.* (2012). An increased cell activity which was indicated by the prominent aposematic secretion towards the gut lumen was observed in those larvae supplemented with probiotics along with water or sugar syrup for four weeks (Plate 23 and 24). Apart from these, greater integrity of the epithelial columnar cell, even distribution of microvilli and augmentation in the secretion of peritrophic membrane was also recorded when probiotics was

supplemented to bacterial brood disease infected colony along with sugar syrup (Plate 25).

Kaznowski *et al.* (2005) reported that application of probiotic (Trilac and Biogen-N) supplemented feed can favour bee survival. The average value of bee mortality was about 30 to 50 per cent for groups supplemented with probiotic along with feed. They also compared the results of feeding same probiotic formulation for 2 and 14 days. It showed no significant difference in total viable count of *Lactobacillus* sp. or *Bifidobacterium* sp, dry body mass, total protein and fat content. These results suggests that short- time feeding of bees with probiotic for even two days is enough for bacteria to colonize the bee intestine. But Kazimierczak- Baryczko and Szymas (2006) revealed that the application of probiotic preparation failed to significantly contribute to increase the feed intake.

The present investigation revealed that the larvae fed with sugar syrup as well as those exposed to various stressors (disease and insecticide) had contrary effect on the midgut. The histomorphology of such larvae exhibited necrosis of epithelial cells, broken and unclear microvilli and distorted peritrophic membrane. Thus, the disruption of epithelial cells hindered the absorption of nutrients and water which led to the death of honey bee larvae. When probiotic (Darolac @ 1.2 g $L^{-1}$) was supplemented to honey bee larvae under different stressors, it helped in alleviating the intensity of cell necrosis by restoration of regenerative cells and the larval health was found to be recovered which was indicated by the even distribution of microvilli.

# *Summary*

## 6. SUMMARY

The present investigation on "Histomorphology of Indian bee (*Apis cerana indica* Fab.) supplemented with probiotics" was conducted in AICRP on Honey bees and Pollinators, Department of Agricultural Entomology, College of Agriculture, Vellayani during 2017–19. The study was conducted with the objective to assess the histomorphology of midgut of Indian bee's (*A. cerana indica*) suffering from various stressors (pathogens and insecticides) and after being fed with probiotics.

Purposive sampling was conducted at Thiruvananthapuram, Kollam, Pathanamthitta and Idukki districts for the present study. Twelve larvae each from naturally occurring feral colony, domesticated colonies which were fed with and without sugar syrup (artificial feed), naturally infected ones with bacterial brood disease as well as larvae fed with insecticide amended royal jelly (Thiamethoxam 25% WG) were collected and subjected to histomorphological evaluation. Apart from these, the effect of probiotics on larvae under different stress conditions were also evaluated.

Twelve representative larvae (3 to 4 days old) from each group were taken from the comb carefully using forceps for histomorphology evaluation. The histology procedure of larval honey bee samples by Silva-Zacarin *et al.* (2012) were modified for the histomorphology evaluation at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Biomedical Technology Wing, Poojapura, Thiruvananthapuram.

The procedure comprised of fixation of larvae, its dehydration, embedding in paraffin wax, sectioning of wax blocks, staining of tissue and observation under light microscope. The Neutral Buffered Formalin (NBF) was used as fixative as it preserves natural tissue structure and maintains the cell structure from degradation. In the dehydration process, the larval samples are passed through a series of ethanol (70%, 80%, 85%, 90%, 95% & 100%). Such processed samples are made into paraffin wax blocks and then subjected to sectioning with the help of a rotary microtome. Thus prepared sections of 4 µm thickness were stained by using hematoxylin and eosin

(H&E) staining technique. The DPX mounted slides were then observed under light microscope to study the midgut histology with respect to the epithelial integrity, presence of microvilli, peritrophic membrane and regenerative cells.

As the studies on histological variations in Indian bee larvae has not been conducted in Kerala hitherto, the normal anatomy of honey bee larvae was recorded from the feral colony larvae which was taken as the control treatment in the entire experiment. From the study, the cuticle of honey bee larvae was found as an external covering of the larval bodywhich was running throught the body except from the natural openings like mouth, anus and spiracle. The brain appeared to be a flattened lobed structure with closely packed brain cells and the ventral nerve cord was a long chain of ganglions ventrally present in the thorax and abdomen of larvae.The salivary gland was composed of a single layer of closely packed cells with a central lumen. The fat body cells and oenocytes are found together throughout the larval body which provides the whiteness to the larvae. The muscles were of striated type and arranged in definite patterns in all the body segments. The tracheal system of the bee larvae was observed to be simple with main tracheal trunks and numerous ramifying branches which reaches all parts of the body. Malpighian tubules were long, thread like tubes with single layer of cells with striations towards the lumen.

The study mainly focused on the variations in the midgut histology of the larvae. The midgut of the honey bee larvae from feral colony had high epithelial integrity, well defined brush bordered microvilli which probably helps in the easy absorption of nutrients, prominent regenerative cells that is responsible for epithelial cell replacement and copious secretion of peritrophic membrane that are intended for the protection of the midgut from abrasive food particles, pathogen and toxins. The larvae of the colonies which were not fed with artificial feed also had similar histology. The midgut of such larvae had homogenously arranged characteristic columnar shaped epithelial cells, uniformly arranged brush bordered microvilli and triangular shaped minute regenerative cells.

Histomorphological studies of honey bee larvae fed with sugar syrup, revealed an adverse effect of sugar on midgut cells *viz*., absence of microvilli, reduced number of regenerative cells and uneven peritrophic membrane secretion. Apart from these, the cytoplasmic granulation was comparably more than that of the normal cells. Though peritrophic membrane was present, its secretion and distribution over the epithelial cells was uneven.

Studies on the midgut histomorphology of bacterial brood disease infected larvae indicated necrosis with picnotic or karyorrhectic nuclei, moderately vacuolated cytoplasm, inconspicuous microvilli and distorted peritrophic membrane. Only occasional regenerative cells were apparent. The midgut epithelial cells of the larvae fed with insecticide, Thiamethoxam 25% WG @ 0.05g $L^{-1}$ exhibited only the presence of vacuoles of variable sizes.

The midgut histomorphology of honey bee larvae treated with the probiotic, Darolac @ 1.2 g $L^{-1}$ at weekly intervals for a period of four weeks was also evaluated. An increased cell activity which was indicated by the prominent aposematic secretion towards the gut lumen in those larvae supplemented with probiotics when compared with that of the normal larvae from feral colony. Apart from these, enhanced integrity of the epithelial columnar cell, even distribution of microvilli and augmentation in the secretion of peritrophic membrane was also recorded when the probiotic was fed to bacterial brood disease infected larvae.

The present investigation revealed that the larvae fed with sugar syrup or those exposed to various stressors (disease and insecticide) had adverse effect on the midgut where they exhibited necrosis of epithelial cells, broken and unclear microvilli and distorted peritrophic membrane. When probiotic, Darolac @1.2 g $L^{-1}$ was supplemented to honey bee larvae under different stress, it helped in mitigating the intensity of cell necrosis by restoration of number of regenerative cells. The larval health was found to be recovered which was indicated by the even distribution of microvilli.

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# *Abstract*

# HISTOMORPHOLOGY OF INDIAN BEE (*Apis cerana indica* Fab.) SUPPLEMENTED WITH PROBIOTICS

*by*

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**ABSTRACT**

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## ABSTRACT

The study entitled "Histomorphology of Indian bee (*Apis cerana indica* Fab.) supplemented with probiotics" was undertaken at College of Agriculture, Vellayani, Thiruvananthapuram during 2017- 19. The main objective was to assess the histomorphology of midgut of Indian bee's (*Apis cerana indica* Fab.) suffering from various stressors (pathogens and insecticides) and, after being fed with probiotics.

Purposive sampling was conducted at Thiruvananthapuram, Kollam, Pathanamthitta and Idukki districts for the present study. Twelve larvae (3 to 4 days old) each from naturally occurring feral colony, domesticated colonies which were fed with and without sugar syrup, naturally infected ones with bacterial brood disease as well as larvae fed with insecticide treated royal jelly (Thiamethoxam 25% WG) were collected and subjected to histomorphological evaluation. Also, the effect of probiotics on larvae under different stress conditions were also evaluated.

The study mainly focused on the variations in the midgut histology of the larvae. The midgut of the honey bee larvae from feral colony had high epithelial integrity, well defined brush bordered microvilli which probably helps in the easy absorption of nutrients, prominent regenerative cells that is responsible for epithelial cell replacement and copious secretion of peritrophic membrane that are intented for the protection of the midgut from abrasive food particles, pathogen and toxins. The larvae of the colonies which were not fed with sugar also had similar histology. Histomorphological studies of honey bee larvae fed with sugar syrup, revealed the adverse effect of sugar on midgut cells *viz.*, presence of vacuoles in the epithelium, absence of microvilli, reduced number of regenerative cells and uneven peritrophic membrane secretion.

Studies on the midgut histomorphology of bacterial brood disease infected larvae indicated necrosis with picnotic or karyorrhectic nuclei, moderate to severely vacuolated cytoplasm, inconspicuous microvilli and distorted peritrophic membrane. Only occasional regenerative cells were apparent. The midgut epithelial cells of the larvae fed with insecticide, thiamethoxam 25% WG @ 0.05g $L^{-1}$ exhibited only the presence of vacuoles of variable sizes.

The midgut histomorphology of honey bee larvae treated with the probiotic, Darolac (1.25 x $10^9$ cells of *Lactobacillus acidophilus, L. rhamnosus, Bifidobacterium longum* and *Saccharomyces boulardi* $g^{-1}$ @ 1.2 g $L^{-1}$ of water and sugar syrup) at weekly intervals for a period of four weeks was also evaluated. An increased cell activity which was indicated by the prominent aposematic secretion towards the gut lumen was observed in those larvae supplemented with probiotics when compared with that of the normal larvae from feral colony. Apart from these, enhanced integrity of the epithelial columnar cell, even distribution of microvilli and augmentation in the secretion of peritrophic membrane was also recorded.

The present investigation revealed that the larvae fed with sugar syrup or those exposed to various stressors (disease and insecticide) had adverse effect on the midgut where they exhibited necrosis of epithelial cells, broken and unclear microvilli and distorted peritrophic membrane. Thus, the disruption of epithelial cells hindered the absorption of nutrients and water which led to the death of honey bee larvae. When probiotic (Darolac @ 1.2 g $L^{-1}$) was supplemented to honey bee larvae under different stressors, it helped in mitigating the intensity of cell necrosis by restoration of regenerative cells and the larval health was found to be recovered which was indicated by the even distribution of microvilli.