CHAPTER 3

3. MATERIALS AND METHODS

Following a detailed survey of agricultural fields in and around Vadodara, rearing the pest *Spodoptera frugiperda* Smith inside the lab on an artificial diet was standardized. After that, insecticides were chosen for testing. The basis for the selection of two insecticides, Chlorantraniliprole and Emamectin benzoate, were:

A. In terms of toxicity, they are "green label": significantly less toxic to non-targets.
B. They are recommended for controlling lepidopteran pests by national and international agencies.
C. Only commercial grade has been tested with preliminary data in India.
D. A detailed study on them, including resistance in the Indian scenario, is insufficient.

The work aims to investigate the efficacy of two new-generation insecticides and determine if their use against *Spodoptera frugiperda* Smith, 1797 can get controlled or stop responding to these insecticides in the future. Hence, lab testing of both insecticides was done, for which agricultural fields were surveyed, pests were collected, and lab rearing on a diet was done.

3.1. METHODOLOGY FOR OBJECTIVE 1

Survey of agricultural fields: A thorough survey of agriculture fields in the Vadodara district was done **(Figure 3.1)**. Various fields are in different directions where several crops are grown **(Table 3.1)**. These agricultural fields were surrounded in and around Vadodara. This included various necessary fields where farming is done to a large extent, and vast areas of farms are present. Different types of crops are grown in various fields **(Figure 3.2)**. The presence of fall armyworms was checked in the fields. Covering the maize fields was the prime focus of the survey **(Figures 3.3 & 3.4)**. Other than the agricultural fields of Vadodara, some other fields in Gujarat were also visited to check the presence of FAW; these were Anand (46 km NW), Navsari (168 km S), Zanzad (57 km N).

Figure 3.1: Surveying in the fields of Savli and Waghodia

Figure 3.2: Cauliflower and Cotton fields in Waghodia

Figure 3.3: Maize field in the Channi and Sherkhi regions of Vadodara

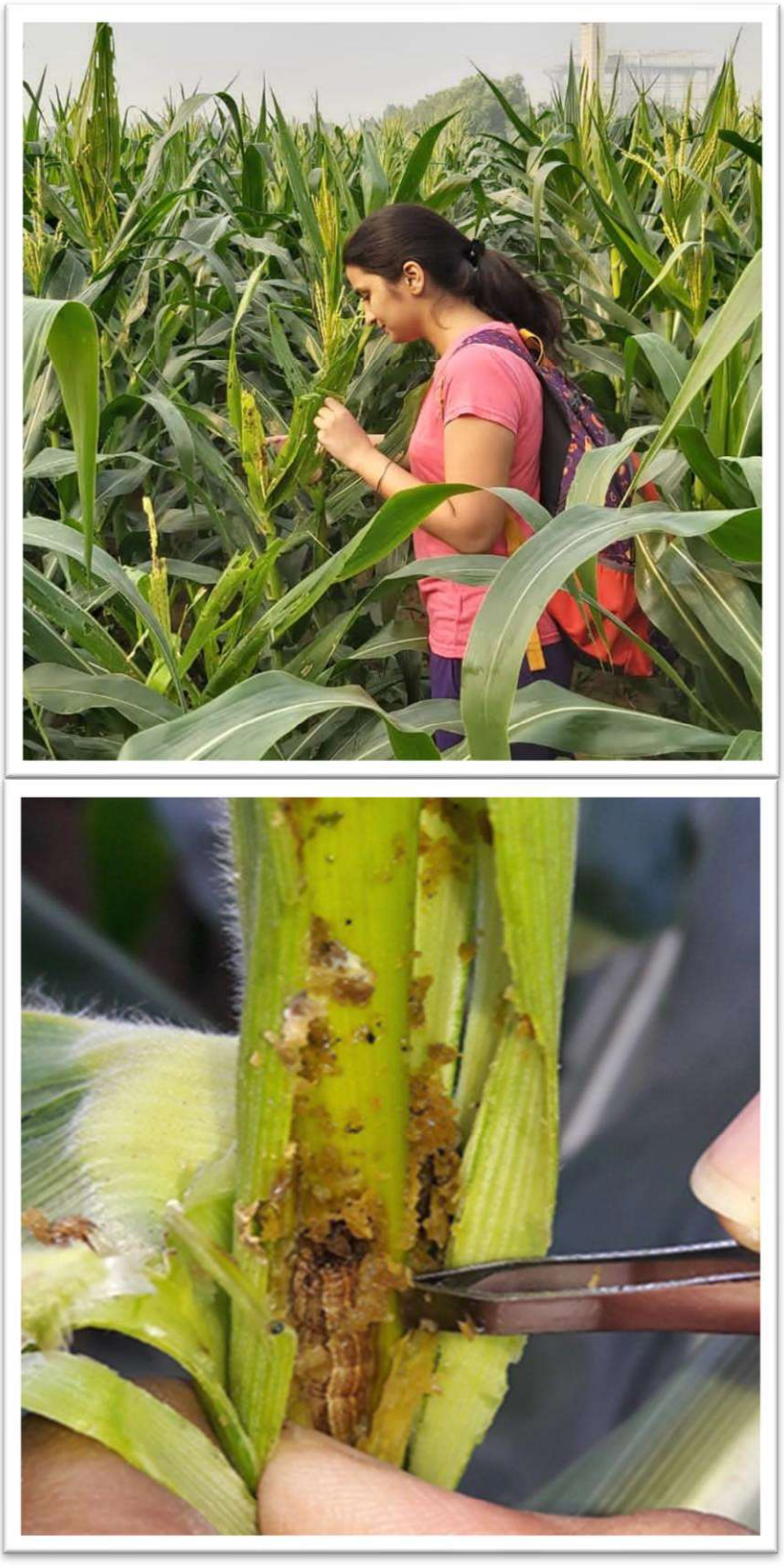

Figure 3.4: Collection of Fall Armyworm from Agricultural Fields

Table 3.1: Analysis of the agricultural areas in and around the district of Vadodara

Study sites	Location	Types of crops
Chhani (locality in city of Vadodara)	9 kms towards North from The M.S. University campus	Millets, Sorghum, Maize, Cotton, Castor, Brinjal, Pigeon pea, Ladies finger, Potato, Radish & Cauliflower
Sherkhi (village near Vadodara city)	11 kms in the North West direction from The M.S.University campus	Maize, Cotton, Castor, Pigeon pea, Sugarcane, Cauliflower
Waghodia (One of the taluka headquarters of Vadodara District)	8 kms South East from The campus of The M.S.University of Baroda	Maize, Cotton, Castor, Sugarcane & Brinjal
Karmaliyapura (village in Waghodia taluka,Vadodara district)	35 kms towards South East from The M.S.University of Baroda	Maize, Wheat, Sorghum, Chickpea, Cotton
Padra (Town and a municipality in Vadodara district)	16 kms South West from The Universitycampus	Paddy, Maize, Cotton, Castor, Pigeon pea, Cabbage
Savli (Village in Vadodara district)	25 kms towards North from The campus of The M.S.University of Baroda	Maize, Cotton, Castor, Rice, Banana, Cauliflower
Tavra (Village in Waghodia taluka , district Vadodara)	26 kms towards West from The University	Maize, Paddy, Wheat , Cotton, Castor,
Chapad (Village located in Vadodara taluka of district Vadodara)	11 kms towards South East from the University campus	Maize, Cotton, Castor, Banana
Golden Chokdi (National Highway no.8,Vadodara)	10 kms towards North East from The University campus	Castor, Cabbage , Cauliflower

Maize: Maize (*Zea mays* L.) is a crucial crop most affected by *S. frugiperda.* Maize is utilized as an essential cereal after wheat and rice. In Gujarat, agricultural fields have many crops, including Cotton, Potato, Brinjal, Castor, Pigeon pea, Cabbage, Radish, Wheat, Pearl millet, Paddy, Jowar, Spinach, Sugarcane, Maize, Sorghum, Beans, and Jowar. Gujarat is one of the significant maize producing states in the country **(Figure 3.5)**. Several insect pests from various orders are present in the fields of Gujarat **(Table 3.2)**. We saw an infestation of *S. frugiperda,* damaging the maize fields primarily, followed by other fields like sorghum and millet. The typical maize plant grows 18-22 leaves; silk develops post 55 days emergence and matures in about 125 days **(Ritchie et al., 1993)**.

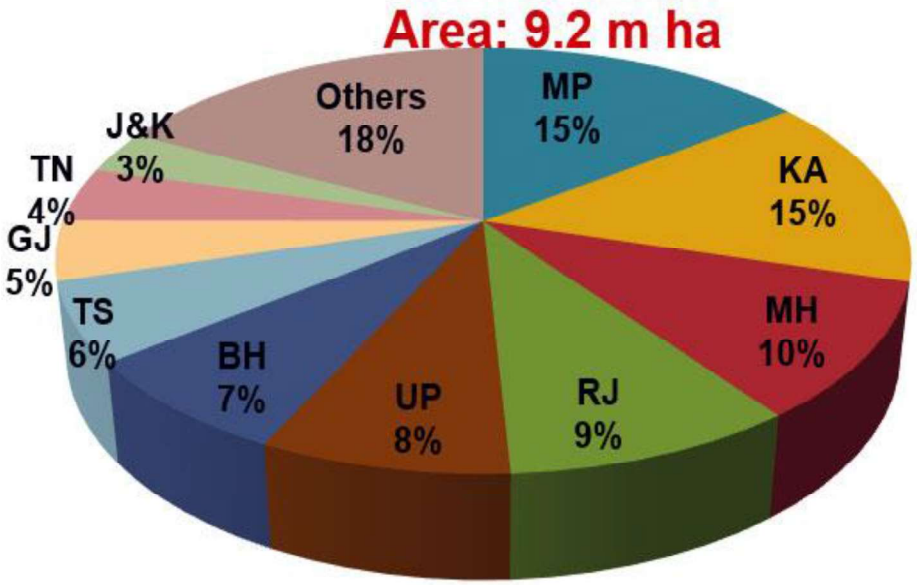

Figure 3.5: Maize-producing states in India (ICAR)

Table 3.2: Assessment and severity of insect pests and the crops they damage in the agricultural fields of Vadodara **(Kataria & Kumar, 2020)**

Crop	Order	Family	Pest name
Paddy	**Orthoptera**	Gryllotalpidae	*Gryllotalpa fossor*
Cotton, Wheat		Acrididae	*Schistocerca gregaria*
Cotton, Wheat, & Sugarcane	**Isoptera**	Termitidae	*Microtermes obesi*
Castor, Cotton, & Sugarcane			*Microtermesmycophagus*
Wheat			*Odontotermes redemanni*
Cotton, Wheat, & Castor			*Odontotermes obesus*
Sugarcane, Cotton, & Castor			*Odontotermes guptai*
Sugarcane, & Castor			*Odontotermes bhagwathi*
Brinjal	**Hemiptera**	Aphididae	*Leptocentrus taurus*
Paddy			*Nephotettix nigropictus*
Cotton, Brinjal			*Aphis gossypii*
Chickpea, Bean			*Aphis crassivora*
Maize, Wheat			*Rhopalosiphum maidis*
Radish, Brinjal, Cabbage,			*Myzus persicae*
Tobacco, Spinach, Brinjal		Aleyrodidae	*Bemisia tabaci*
Maize, Wheat, Sugarcane, Pea.		Lophopidae	*Pyrilla perpusilla*
Tomato, Cotton, Ladies' finger		Pseudococcidae	*Maconellicoccus hirsutus*
Sugarcane		Lygaeidae	*Blissus gibbus*
Cotton			*Lygaeus militaris*
Cotton			*Lygaeus hospes*
Bean, Chick pea			*Riptortus linearis*

Identification: Its specific morphological characters in the larval stage were checked to identify the fall armyworm. Since the larval stage is the one causing damage, it was selected for identification. The insects were collected with the help of blunt forceps to avoid damaging them. They were put on a plain surface and were confirmed with the help of identifying features and characters given by **Sharanabasappa et al., 2018**.

Collection of different stages of larvae of *S. frugiperda*: Following a detailed survey of various agricultural fields in and around Vadodara, the collection was done **(Figure 3.4)**. It was among the initial observations of fall armyworms in a few agricultural fields in Vadodara in June 2019. The insects were collected from the agricultural fields where the FAW attack was observed. The stage selected for the collection was the larval stage (in caterpillar form). The identifying features of the FAW caterpillar are the Y-shaped white line on the front in the anterior region and the four dots, which form a square at the posterior end. The colour is shades of brown with black lines on its body. The caterpillars were allowed to feed initially on the natural leaves after the collection.

3.2. METHODOLOGY FOR OBJECTIVE 2

Rearing in the Lab: The insects were reared on a natural diet for a complete life cycle. The larvae were reared in insect culture trays containing the maize-based artificial diet and natural diet in separate trays, closed with a lid, and maintained at 25±4˚C, 70±10% RH **(Figure 3.6)**. Each insect was kept in separate cells to avoid cannibalism. The adults were allowed to mate in adult cages. The first rearing started from the $3^{rd}/4^{th}$ instar stage brought from fields, fed on a natural diet, i.e., maize leaves to the last instar, pupa, and adult. The adults were allowed to mate. The hatching of the eggs resulted in neonates being fed a maize-based artificial diet in one set and a natural diet (maize leaves) in another set until they entered the pre-pupation phase. Pupae that had formed were kept in different plastic boxes. The pupae were washed with 1% sodium hypochlorite to avoid microbial infections. Adults who emerged from these were fed on a honey-sucrose-based diet with the help of cotton for diet soaking. Different life stages were studied. The biology of fall armyworms was studied to elicit information on the first, second, third, fourth, fifth, pupa, and adult emergence.

Figure 3.6: Larvae Reared on Natural and Artificial Diets

The various ingredients in the diet are essential to provide various nutritional requirements for the insect to grow. For further diet analyzing studies, the larvae were taken from the fields to the laboratory and placed into different trays for each field; every insect was reared in individual cells of the 32-cell insect culture plastic trays (Zenith Plastics, Ahmedabad). Each insect was kept in separate cells to avoid cannibalism. The insects brought directly from the fields were termed as "F0-generation". These larvae were fed a natural diet from freshly cut maize leaves. The larvae were allowed to grow into pupa and adults. Emerged adult males and females were allowed to mate and lay eggs. The neonates hatched from these eggs were termed the "F1-generation". This generation was taken into consideration for the study. A maize-based artificial diet consisted of various ingredients, as mentioned in **Table 3.3**. The newly hatched larvae were fed on a maize-based artificial diet until the pre-pupation stage. The entire rearing was done in controlled conditions of 25±3˚C Temperature and 70±5% Relative Humidity with the help of a BOD incubator. The number and duration of each instar stage were noted. Pupae formed were sterilized with the help of a 1% sodium hypochlorite solution. Adults emerged and were then placed in cages of plastic containers covered with the help of muslin cloth and rubber bands. Equal males and females were placed in each cage (5 males: 5 females). The honey-based artificial diet **(Table 3.4)** was provided through cotton rolls dipped and tied with a thread. The butter paper was folded to make rough edges for egg laying.

Rearing on different Artificial Diets: A larval diet was prepared with the minimum ingredients possible to make it economically viable, as shown in **Table 3.3**. Previously work in the lab has been done on the rearing of Lepidoptera. The diet for *Spodoptera litura* **(Sharma & Kumar, 2020)** and *Plutella xylostella* **(Kumar & Sharma, 2022)** has been standardized. The diet studies and ingredients served as the basis for diet studies. The various ingredients in the diet are essential to provide various nutritional requirements for the insect to grow. Modifications also inspired the diets in the diet of *Spodoptera litua* **(Gupta et al., 2005)**. The pupae formed were sterilized with 1% sodium hypochlorite and released in adult cages. Many ingredients are used to make artificial diets for lepidopterans. Components like cholesterol and casein are also added besides the mentioned ingredients. However, we tried to make the diet economical and selected the necessary ingredients for proper growth and development.

Diet preference: A preliminary investigation was conducted to determine the fall armyworms' preferred artificial diet when different host crop elements were present. Four diets with different flours—Maize, Soy, Chickpea, and Jowar—were kept evenly spaced from one

another and from the centre to examine preferences. Ten neonates were released in the centre, and their locations were discovered an hour and a day later, respectively.

Four diets were created to test how varied diets affected FAW survivorship **(Table 3.5)**. Rearing was done in culture trays. Diets were changed as and when required. The BOD incubator was maintained at a constant temperature and humidity. With a 12:12 D:L photoperiod, temperature and humidity are maintained at 26°C and 70%, respectively, for diet optimization. The diet was changed, and the trays were cleaned regularly. Photography was done regularly. The percentage of survival was noted. Observations were kept till the emergence of adults.

DIET PREPARATION PROCESS

The diet's components are all combined with water. The diet's water content is split into two categories, A and B. Each portion of a 500-ml diet is consumed in 250 ml. The first step involves heating the water until bubbles start to form. Next, agar is added, and the mixture is continuously heated and mixed until it has a thick consistency. After that, yeast is added and the mixture is cooked for one minute. The remaining diet elements as well as liquid substances like becosule are introduced in the second section. Formaldehyde is added immediately, and solid components like flour come afterwards. Using a hand blender, it is blended. The first component is introduced and properly blended after that. The new diet is made, portioned, and placed in the ketchup bottles. Certain portions may also be placed in the broad trays for the purpose of creating diet pieces. Insects of any stage can be transferred to the "semisolid diet" in the tray, which can then be covered with stickers (transparent covers that stick at the edges and allow air exchange through its tiny holes) or pierced lids (allowing air exchange) to prevent insects from moving out of the trays after the "semisolid diet" has cooled and solidified.

Table 3.3: Artificial maize-flour-based diet ingredients for larvae

Sr. No.	Ingredients	Amount
1.	Agar-agar powder	18g
2.	Ascorbic acid	5 g
3.	Becosule	6 ml
4.	Formaldehyde (10%)	20 ml
5.	Maize powder	150 g
6	Wheat germ	50 g
7.	Methyl-paraben	3 g
8.	Propionic acid	2 ml
9.	Sorbic acid	2 g
10.	Yeast powder	50 g
11.	Water	1000ml

Table 3.4: Artificial diet for adults

Sr.No.	Ingredients	Amount
1.	Honey	100 g
2.	Sucrose	100 g
3.	Becosule	4 g
4.	Methyl-paraben	4 g
5.	Ascorbic acid	40 g
6.	Water	1000 ml

Table 3.5: Different diet composition in gm or ml for *Spodoptera frugiperda* (1:l diet)

Sr. No.	Ingredients	Diet 1	Diet 2	Diet 3	Diet 4
1	Yeast	53g	53g	53g	53g
2	Agar	20g	20g	20g	20g
3	Sucrose	36.36g	36.36g	36.36g	36.36g
4	Methyl-p hydroxy-benzoate	3.3g	3.3g	3.3g	3.3g
5	Wheat germ	60g	60g	60g	60g
6	Ascorbic acid	5.3g	5.3g	5.3g	5.3g
7	Sorbic acid	1.7g	1.7g	1.7g	1.7g
8	Formaldehyde	20 ml	20 ml	20 ml	20 ml
9	Becosule	12ml	12ml	12ml	12ml
10	Propionic acid	2ml	2ml	2ml	2ml
11	Maize flour	160g	-	-	-
12	Soya flour	-	160g	-	-
13	Chickpea flour	-	-	160g	-
14	Jowar (Sorghum) flour	-	-	-	160g
15	Water	1000 ml	1000 ml	1000 ml	1000 ml

3.3. METHODOLOGY FOR OBJECTIVE 3

Efficacy studies-

Larvae selection: The insects used for the study were first collected from the fields and made to acclimatize in the laboratory for a few generations (three generations). They were fed an artificial diet and kept at constant temperature and humidity (26˚C, 70%, 12:12 D:L). This period is crucial as it is required to make the insects susceptible. Once a few generations started surviving well in laboratory conditions, they were considered for experimental purposes. The larvae stage selected for insecticide efficacy experiments was of the fourth instar stage. The reason behind selecting this stage is the voracious feeding nature, which supports the experiment ensuring experimental doses reach inside with diet. These larvae were healthy i.e. free from any kind of infection which could be used for experimental set ups. Such insects kept for experiments were not fed four hours before the experiment.

Insecticide selection: The efficacy of an insecticide depends on its effectiveness in controlling the target insect pest. The insecticides selected for the study (Chlorantraniliprole and Emamectin Benzoate) are checked here for their efficacy in controlling the insect pest *Spodoptera frugiperda.* We observed no insecticides from the older classes of chemicals like Organophosphate, carbamates, and synthetic pyrethroids, single or combined, effectively control this insect pest. Interviews with farmers at all the surveyed agriculture fields mentioned the inefficacy of the available insecticides. A typical insecticide they sprayed in most fields to control fall armyworms was a combination of Cypermethrin and Chlorpyriphos (Synthetic Pyrethroid + Organophosphate). There was no control provided by this insecticide **(Figure 3.7)**. Other than that, many health hazards to non-target organisms are caused by it.

Figure 3.7: Insecticides found in an agricultural field (Cypermethrin + Chlorpyriphos)

While starting the survey of agriculture fields in and around Vadodara, there was a trend toward using older insecticides against fall armyworms. Because the pest was new and there was no recommended insecticide treatment, farmers mostly sprayed Organophosphate, carbamates, and synthetic pyrethroids as single agents or in combination. Farmers complained about not getting any control using the insecticides against the fall armyworm. Going through the literature survey, I found that *Spodoptera frugiperda* has already developed resistance to those insecticides. This led to my choosing and finalizing the two new-generation insecticides, which have proven effective against Lepidoptera. Experiments began, and by 2020, government agencies like CIB also enlisted recommendations for control against fall armyworms, and our insecticides were mentioned in that list. The technical grades of these insecticides have not been thoroughly tested in the Gujarat population or the Indian scenario. Therefore, finding the doses for control of these insecticides was essential.

Meanwhile, a field survey was also going on simultaneously. In 2021 and 2022, the maximum number of fields in Vadodara were surveyed where maize was grown, or *Spodoptera frugiperda* infestations were seen. It was observed that the usage of Emamectin benzoate **(Figure 3.8)**, commercial grade, under different names (Proclaim, Jakpot), has increased to an extreme level. This calls attention to the danger of resistance development, which might occur shortly. Such a scenario urges the need for a resistance study wherein the molecular mechanism in Emamectin benzoate-treated *Spodoptera frugiperda* should be known beforehand to allow for its rescue.

Figure 3.8: Commercial grade Emamectin benzoate

Diet incorporation assay: The different concentrations of both pesticides were tested through the "Diet Incorporation Assay" (Insecticide Resistance Action Committee), selected for performing the bioassays. The initial stock solutions of insecticides were calculated and later used to make different concentrations, or ppm, of insecticides. The stock solutions for both insecticides were prepared in acetone, later diluted in water.

The technical grade of insecticides Chlorantraniliprole and Emamectin benzoate were purchased from Sigma-Aldrich **(Figure 3.9 & 3.10; Table 3.6, 3.7)**. The properties of the compounds were provided. A 100 ppm stock solution was initially prepared for both insecticides **(Figure 3.11)**. For that, technical-grade insecticides were used. The insecticide solution: and water were in a ratio of 2:8. For example, to prepare a 100-ml diet, 20 ml of insecticide solution is added to 80 ml of water to make the artificial diet. Different concentrations of both insecticides were made from the stock solution. While the stock solution was prepared in acetone to dissolve the technical-grade insecticide salt, further dilutions were done in distilled water. The diet preparation process included weighing ingredients, boiling Part A, mixing parts A & B, adding insecticide solutions of various dilutions, stirring/mixing, filling bottles, pouring in trays, and releasing insects **(Figure 3.12)**.

An artificial diet was made, cut into pieces, and placed in every tray cell. Third instar larvae (F1 generation) were released in each cell using a fine camel hair brush. The conditions were adequately monitored to ensure the proper conditions for the rearing. Testing trays consisting of larvae were kept at a temperature of 25±2°C and 70±10% RH. A control was always maintained (diet without insecticides). The setup was checked every day. After 72 hours, observations were taken. Each cell of test trays was inspected to observe the larval condition. Observations were made to check the survival rate of larvae. A movement on stimulation means alive, while no movement means the insect is dead. Cleanliness and a sterile environment were tried to be maintained to avoid any mixing and infection. The insect culture trays were cleaned on a daily basis. Observations were done on the third day of the experiment. The per cent mortality for all the concentrations of both insecticides was noted. The experiment waste was properly disposed of as it constituted hazardous chemicals. In all the observations, anything unusual was noted down. Sterilized forceps were used during experiments. Comparison of the treated and controlled data was always observed. In treatment and control both, n=10 were maintained and replicated thrice.

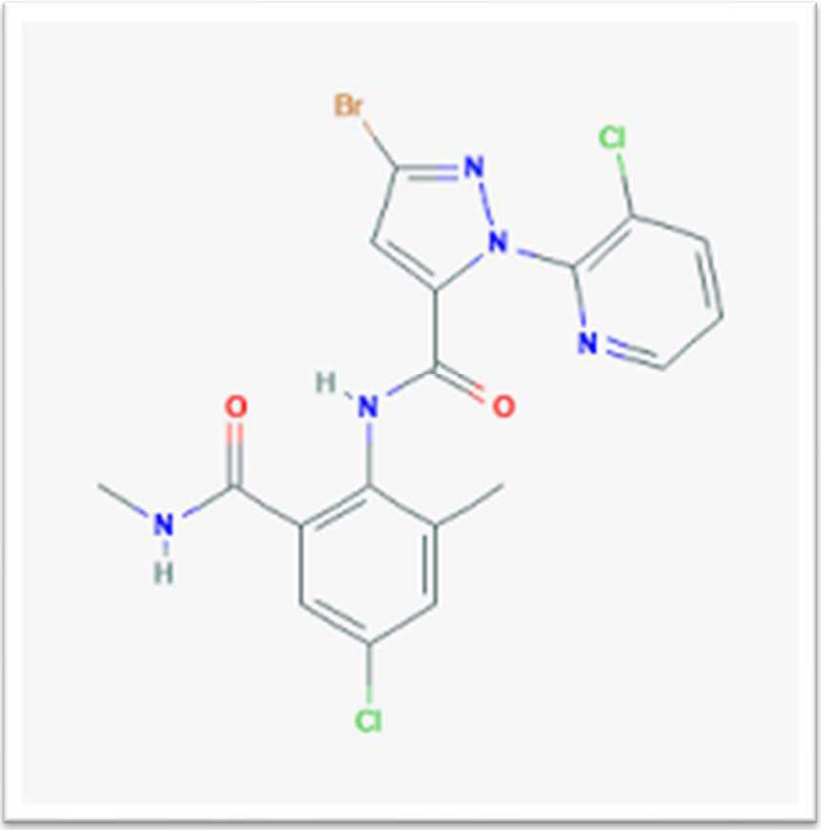

Figure 3.9: Technical grade product and structural formula of Chlorantraniliprole

Table 3.6: Technical details of Chlorantraniliprole

Specifications	Chlorantraniliprole PESTANAL, analytical standard
Brand	Sigma-Aldrich
Appearance (colour)	White to off-white
Appearance (form)	Powder or Crystal
Purity	≥95%
Melting point	223-228 C
Water	≤1%
Purity	Confirms to structure
Formula	C18H14BrCl2N5O2

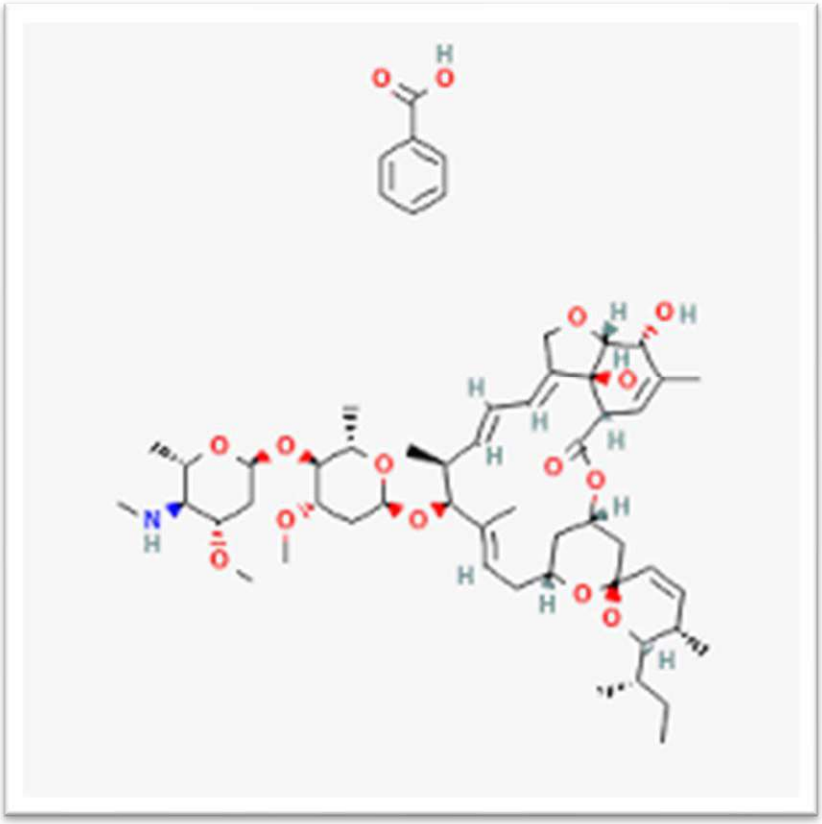

Figure 3.10: Technical details of Emamectin benzoate

Table 3.7: Technical details of Emamectin benzoate

Specifications	Emamectin benzoate PESTANAL, analytical standard
Brand	Sigma-Aldrich
Appearance (colour)	White to off-white
Appearance (form)	Powder or Crystal
Purity	≥85%
Melting point	150-155 C
Water	≤3.0%
Purity	Conforms to structure
Formula	C56H81NO15

Figure 3.11: Stock solutions and preparing different concentrations of insecticide solutions.

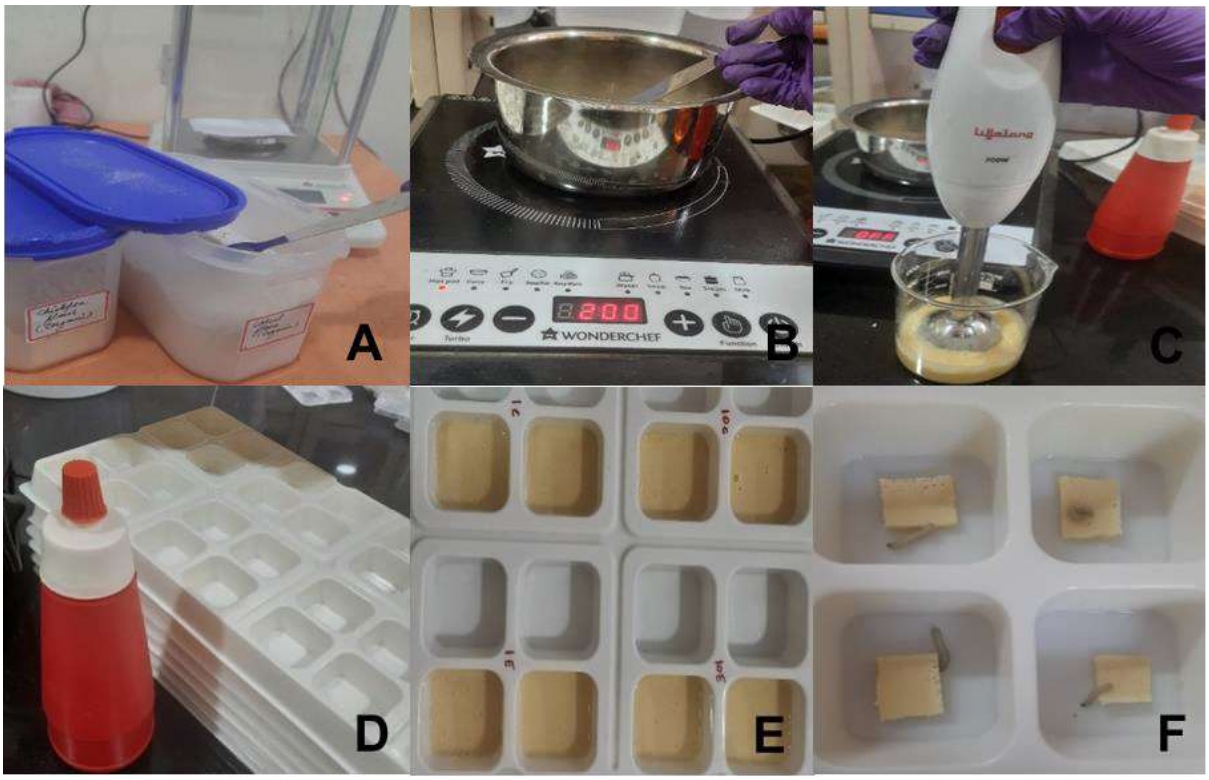

Figure 3.12 A-F: Diet preparation process (weighing ingredients, boiling Part A, mixing parts A and B, filling in bottles, pouring in trays, releasing insects)

3.4. METHODOLOGY FOR OBJECTIVE 4

Histology

Histological studies were conducted for insect control, treated, and resistant *Spodoptera frugiperda* Smith. Midguts from all three types were taken for analysis. The differences between the control and resistant insects were attempted to be observed through histological analysis. Since the midgut is essential to detoxification, it was selected for histological studies. The control population was kept insecticide-free throughout the study, the resistant (treated population at LD_{50} concentration (0.1 ppm)) population had undergone insecticide testing for some generations. A surviving insect was taken for the studies from each of these types. After proper sectioning, staining with hematoxylin-eosin was done and observed under a bright-field microscope, the Leica DM750 **(Figure 3.13)**.

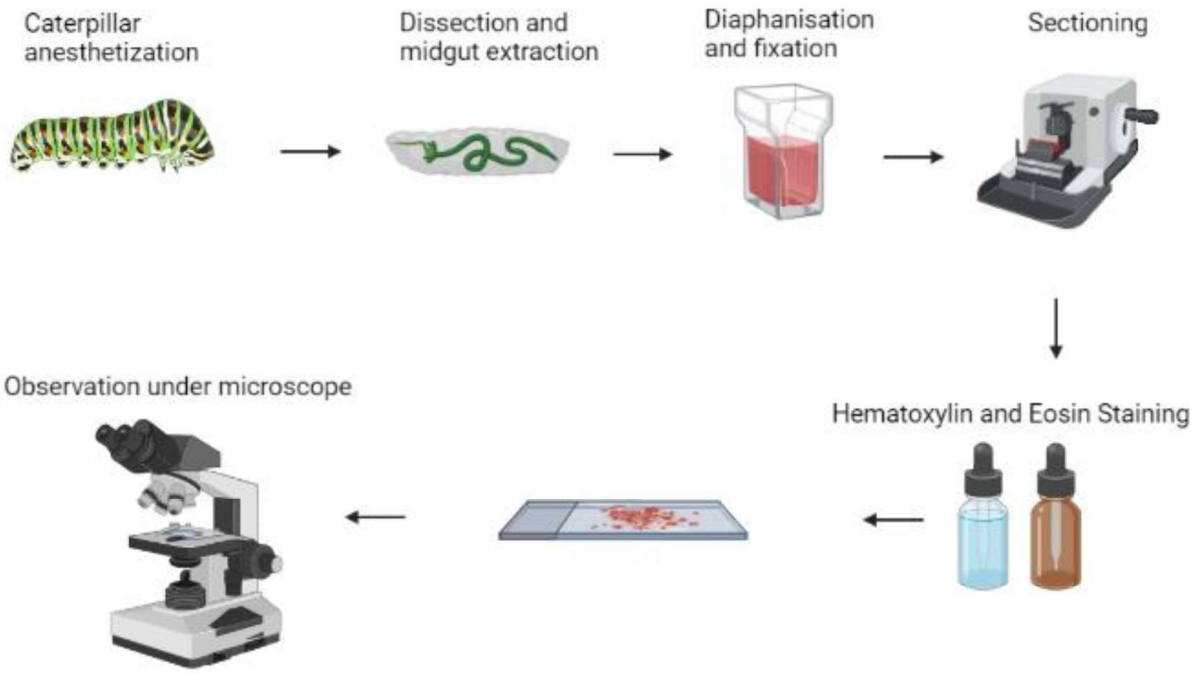

Figure 3.13: Flow of histological studies

3.5. METHODOLOGY FOR OBJECTIVE 5

Resistance Study at the Molecular Level/ Molecular Basis of Resistance

It means finding the cause of resistance at a molecular level. The reasons behind the resistance can be better understood by looking at what is happening at the gene level. It can be summarised in a few steps **(Figure 3.14)**.

The development of resistance occurs after the target population develops some internal mechanism to fight and inhibit the effect of the insecticides. These internal mechanisms need to be known to understand the cause of resistance. Midgut samples were collected from the control (untreated throughout) and test populations (the ones exposed to Emamectin benzoate treatment for some generations) to identify the genes responsible for resistance development. The reason behind the selection of the Emamectin benzoate-treated insects for molecular studies was the extensive increase in the use of insecticides against fall armyworms in Vadodara fields over the last two years. The difference between the control and treatment profiles would reveal the genes responsible for causing resistance. This can be achieved through the transcriptome profiling of the samples. Further looking for differential gene expression.

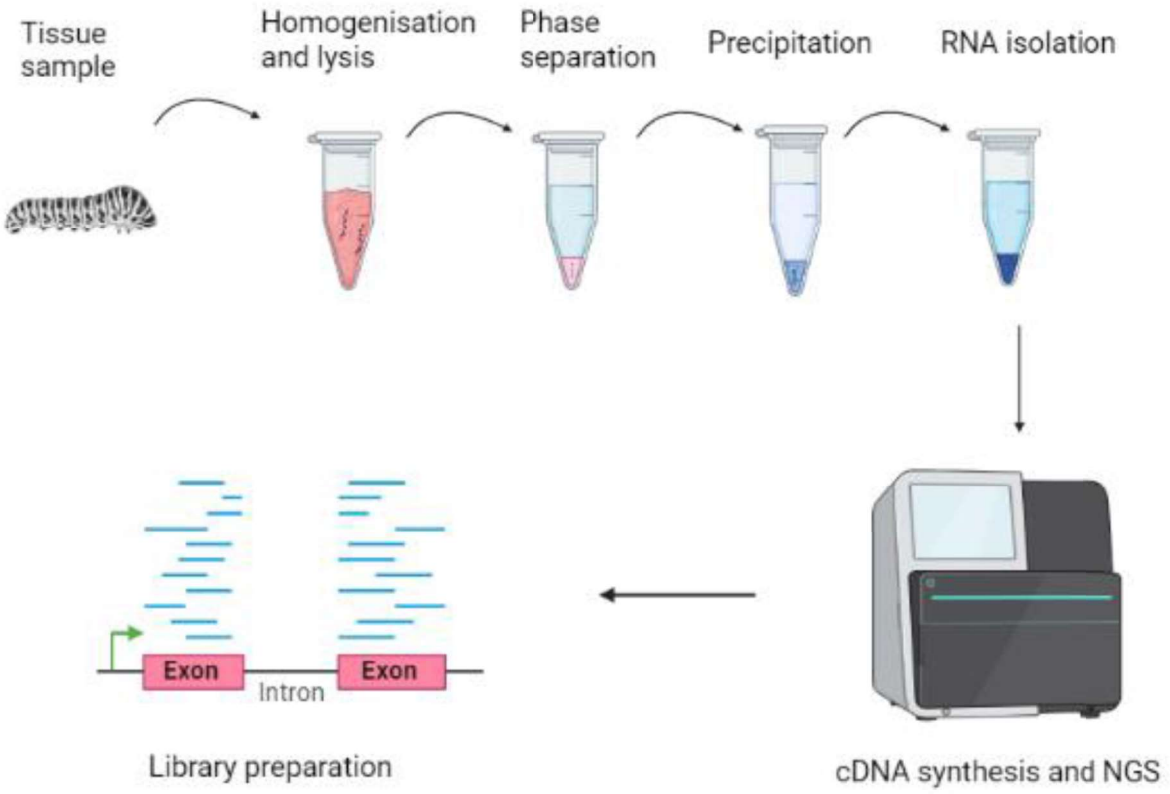

Figure 3.14: Flow of molecular work

Although high mortality with less concentration was observed in Chlorantraniliprole treatment, Emamectin benzoate was further taken for NGS as fields in and around Vadodara affected by fall armyworm infestation were spraying Emamectin for the control.

Selection of the concentration: For the conduct of the resistance studies, there is a significant role in the selection of the concentration for testing. The concentration of prime importance is the one at which 50% of the population results in death. Concentrations above and below this concentration were also considered for the study. A range of concentrations was chosen for testing throughout the experiments. This was done to observe the trend of mortality in every generation.

Sets of cultures: A total of three sets of insect cultures were maintained throughout the experiments related to the generation study. These are-

1. Control or Susceptible Culture: This culture is never exposed to any insecticide.

2. Chlorantraniliprole Culture: This culture is exposed to Chlorantraniliprole insecticide.

3. Emamectin Culture: This culture is exposed to Emamectin benzoate insecticide.

Generation Study: A range of concentrations was tested in every generation. The insects surviving from the first insecticide testing generation, where 50% mortality was observed, were made to grow into pupae and adults. These adults were allowed to mate and lay eggs. Some were taken for further testing when the hatched larvae reached the third instar stage. The concentration giving the LD_{50} value was again given to them through a similar diet incorporation assay. The experiment was repeated until a generation was obtained where the LD_{50} value was not observed with the same concentration. Resistance development occurred when the insecticide was not given the expected number of mortalities, and relatively fewer pests were sacrificed. This generation number was noted for both insecticides.

Mechanism of resistance: To check the genes responsible for the development of resistance, a sample (midgut) was taken from the control and test populations subjected to the drug Emamectin benzoate, along with the tissues from the resistant population of the drug. These samples were checked for gene expression along various fundamental pathways. The difference in control and treatment profiles revealed the genes responsible for causing resistance. The pest's mechanism needs to be examined to understand the reason for resistance. For that, two populations were required:

1. Susceptible population: The susceptible population was made in the laboratory. The FAW were reared inside the lab for several generations (five generations) and maintained at optimum temperature of 25±4˚C, 70-80% RH, and photoperiodism of 12:12 Light: Dark.

2. Resistant population: The internal mechanism behind resistance was investigated through the insects exposed to the insecticide for some generations and have developed resistance. Here, we selected Emamectin benzoate-treated insects. The reason behind selecting this chemical was its most widespread usage in agriculture in recent times. Besides being recommended by the CIB, i.e., the Central Insecticide Board, they are also currently the most prevalent chemical for controlling the FAW in Gujarat and throughout India.

Transcriptome analysis of midgut: In both the populations, FAW late instar larvae were taken (4^{th} instar). The larvae were sacrificed after being anaesthetized by putting them in a deep freezer for 1 hour. After proper dissection, midgut tissue was stored at at -20 degrees. The midgut from both populations was collected in separate vials containing TRIzol (Sigma-Aldrich), labelled R and S for resistant and susceptible samples. Samples were checked for differential gene expression by transcriptome analysis.

Detailed procedure for molecular analysis

Extraction of RNA and quality assessment:

Tissues from insect midguts were collected from control and insecticide-treated pests in the TRIzol reagent (Sigma Aldrich, USA) and stored at -80°C till requirement. The TRIzol technique was used to extract the RNA. To determine the purity of the extraction, the Nanodrop 1000 concentration was evaluated. The quality of the extracted RNA was evaluated using an RNAHS test kit (Thermofisher #Q32851) & Qubit 4.0 fluorometer (Thermofisher #Q33238). We tested the Nanodrop 1000 concentration in addition to the purity of the extraction. Finally, RIN values were obtained by checking the RNA on the TapeStation using HS RNA ScreenTape (Agilent). In accordance with the manufacturers' methodology, the library was queried on the Tapestation 4150 (Agilent) using vulnerable D1000 screen tapes (Agilent #5067-5582). After passing on, all the libraries were collected for library sequencing.

RNA-Seq Analysis: Using FastQC v.0.11.9, the sample's raw fastq readings' quality was evaluated (default parameters). Fastp v.0.20.1 (parameters: —trim front1 8 —trim front2 5 — length required) was used to preprocess the raw fastq reads—correction: 50 30), followed by quality re-evaluation using FastQC and summarisation using MultiQC. — trim poly g — qualified quality phred 30). Utilizing HISAT2 v2.2.1, the *Spodoptera frugiperda* genome (GCF 011064685.2) was indexed. HISAT2 v2.2.1 was used to map the processed reads to the *Spodoptera frugiperda* reference genome. The *Spodoptera frugiperda* GTF file no longer contains the rRNA and tRNA characteristics. Using featureCounts v. 0.46.1 based on the filtered GTF file, each sample's alignment file (sorted BAM) was quantified to produce transcript counts. These *Spodoptera frugiperda* transcript counts were inputs to EdgeR with exactTest for estimating differential expression (parameters: dispersion = 0.1). PANNZER2 was used to annotate protein sequences for GO using transcript ids from edgeR findings acquired from NCBI. Using the GTF file and GO annotation, the transcript ids from the EdgeR result were annotated. Filtering was done on the EdgeR exactest annotated file using an Unadjusted p-value of 0.05 and LogFoldChange 1.5. These filtered annotated files provided the protein ids, which were then used to extract the relevant protein sequences and subject them to the KEGG annotation programme BlastKOALA. The work is summarised as a flowchart **(Figure 3.15)**. Differentially expressed genes will be analyzed to investigate mode of action of the insect pest *S. frugiperda* towards Emamectin benzoate.

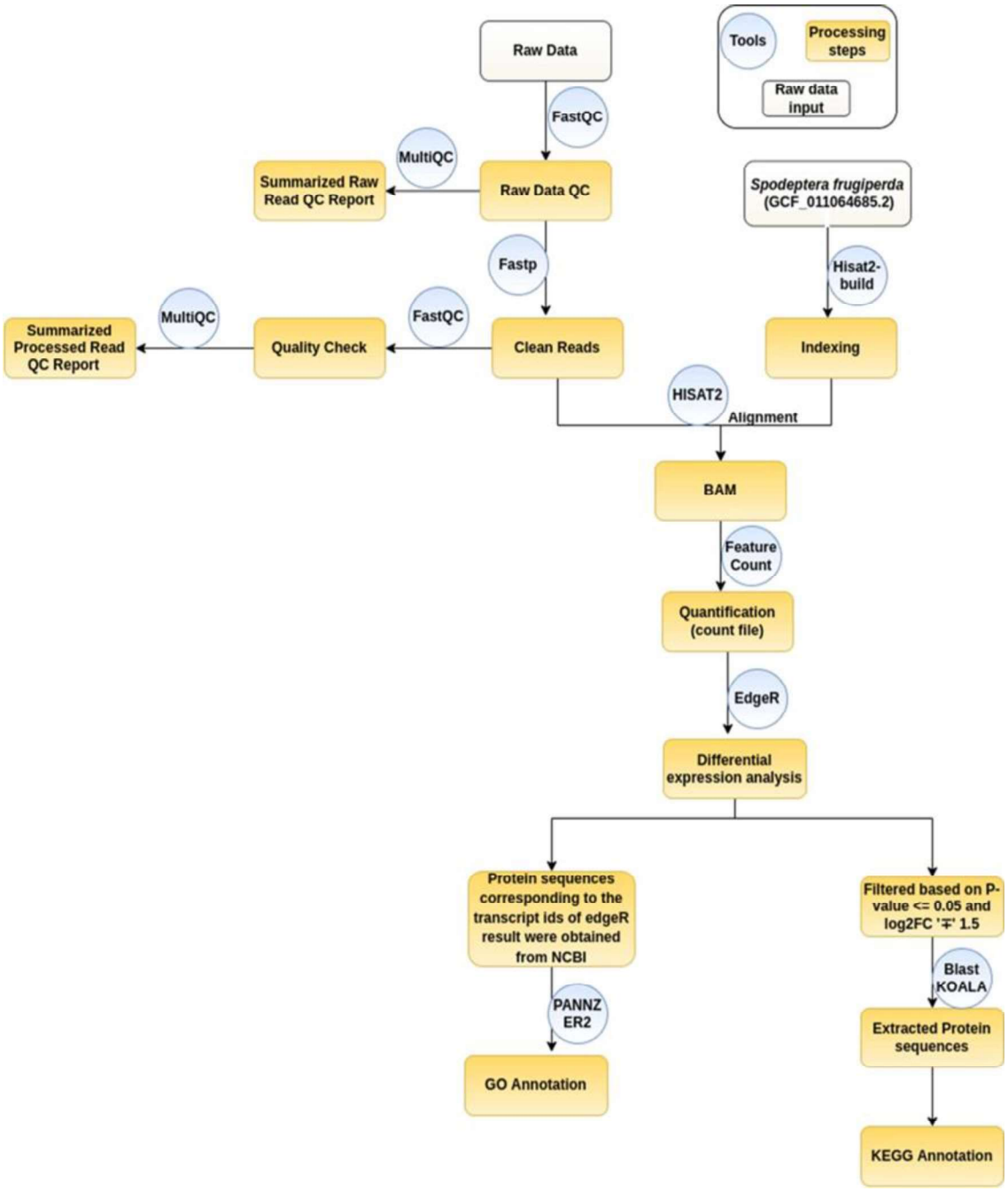

Figure 3.15: Flow of transcriptome analysis

Overall Statistics: Percent survival on various diets was checked. Larval growth was compared for the diets using the formula of larval growth index. The statistics used for comparing natural and artificial diet rearing were through the descriptive statistic feature of the statistical software Minitab19. A graphical comparison was made through the statistical software GraphPad prism 9. Both insecticides' toxicity at different concentrations against Fall armyworm was evaluated. The LD50 was checked with the help of Probit analysis of SPSS software. The generation-wise results in both insecticides were observed in tabular form. Various plots represented

transcriptome analyses. DGE was analyzed by graphical comparison between the control and treated sample. A total of three plots were made, including the MA plot, Volcano plot and Heat map.

Good laboratory practices and precautions

1. The temperature and humidity should be well maintained for effective and precise results.

2. Measurements and calculations should be done correctly, and all the ingredients should be weighed accurately.

3. While taking observations of the treated larvae, they should be checked adequately with the help of a brush for being live, dead, or moribund.

3. Proper care should be taken while dealing with a flame, microwave, blender, and other electronic items.

4. During diet preparation, all the ingredients should be mixed appropriately. Also, the consistency of the diet should be taken care of.

5. Always use PPE, or personal protective equipment, while performing the experiments in the lab, including a lab coat, hand gloves, and protective glasses.

6. All xenobiotics and other living and non-living wastes should be properly disposed of.

7. The insects should be maintained hygienically by regularly cleaning, transferring, and changing their diet.

8. Dissections should be performed with utmost alertness to avoid any breakage of the required tissue sample.