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# A Protocol to Generate Germ Free *Drosophila* for Microbial Interaction Studies

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## Abstract

*Drosophila* (fly) gut is used as a model system to study the host microbe interaction. Various developmental, physiological, functional and immunological similarities exist between the gut of the vertebrate and the fly. These similarities advocates their utility as a model system for the study of various gut related diseases. Wild type flies found in its natural environment and those raised in the enclosed laboratory conditions have their own natural microbiota colonizing the gut. This natural gut microbiota may interfere with orally administered microbe-*Drosophila* interaction studies. To overcome this problem various labs are in the process of raising germ free flies. Germ free *Drosophila* is completely devoid of intestinal microbiota or any kind of microbial colonization. These germ free fly would be helpful as powerful model in exploring the role of a particular microbial strain or a specific microbial genera interacting with the host in isolation either in the mono- or poly-associated state unravelling the complex interplay between bacteria and the host. The current paper describes a protocol to generate a germ free fly for use in microbial interaction studies.

**Keywords:** *Drosophila*; Genome; Microbe; Larvae; gut-microbe interaction; probiotic

## Introduction

*Drosophila melanogaster* has been in use for over a century as the most suitable model organism to study genetics. This model organism is not only limited to genetic research anymore but also for diverse research inquires in the field of metabolic, neuronal, behavioural, microbial and immunological related disorders [1]. Apart from these, it has a well-recognized application in drug screening and toxicity, regenerative medicine, biosafety, bioengineering and as a genetic model for human viruses and human microbes studies [2-7]. The availability of complete genome sequence, well studied developmental pathways and up to 75% gene homology with *Homo sapiens* promoted the use of *Drosophila* as a model to decipher many complex pathways associated with diseases [8,9]. Various cause and consequence of disease like Parkinson's, diabetes, sleeping disorders, cancer and many more were checked using *Drosophila* [10].

Recently *Drosophila* is gaining attention as model organism to the study of human gut related disorders [11]. There exist anatomical, structural and functional similarities with the gut lining of the *Drosophila* and the vertebrate [12]. Several studies has been reported using *Drosophila* as model organism in the study of intestinal infection and pathology, recognition and defence mechanisms against intestinal pathogens, natural intestinal microflora and dysbiosis, inflammatory bowel disease, proliferation and pathogenesis of intestinal pathogens like *V. cholera*, *S. marcescens*, *P. aeruginosa* and *Enterococcus faecalis* and intestinal cell differentiation and regeneration [12-22]. Besides these, recently published scientific reports suggested the utilization of *Drosophila* as model to reveal the role of gut microbe in improved life span, intestinal stem cell activity and regulation, metabolic and developmental homeostasis, effect of dietary habits in improvement of beneficiary microflora [22-26]. Screening of potent probiotic strains for administration to humans were also checked using fly model. Growth promoting activity of probiotic bacteria were investigated in *Drosophila* [26-29]. Gut microbiota are known to change the behaviour of the animal by changing the interrelationship between gut-brain axis, protecting against pathogens, stimulating inflammation, activation of the immune system and mid-gut regeneration [13,26,30-36].

Thus *Drosophila* gut model has been widely employed for understanding the host-microbiota interactions [37]. The metagenomic analysis of the human microbiota unravelled the presence of ten different phyla of bacteria and up to a thousand species per individual. Among them less than sixty species are shared among the various human populations [38]. The *Drosophila* microbiota includes phyla Firmicutes (*Lactobacillaceae* and *Enterococcaceae*) and Proteobacteria (*Acetobactereaceae* and *Enterobactereaceae*) with eight main dominating species: *Acetobacter pomorum*, *A. tropicalis*, *Lactobacillus brevis*, *L. plantarum*, *L. fructivorans*, *Enterococcus faecalis*, *Gluconobacter morbifer* and Enterobacteriaceae Group Orbus [13,39-41]. *Gluconobacter morbifer* and *L. brevis* were regarded as colitogenic pathobionts for *Drosophila* as they release uracil as the metabolic intermediate [13,42]. Gut microbiota of *Drosophila* vary with diet, developmental stage and immune status of the fly [43,44]. With the increase of carbohydrate to protein ratio in the fly food augments the proportion of *Acetobacter* versus *Lactobacillus* in adult. Furthermore, flies with healthy microbiome are less susceptible to infection by *Serratia marcescens* and *Pseudomonas aeruginosa* than the germfree flies [26]. The composition of the gut microbiota changes with various developmental stage of the fly. In the larval stages the microbial population shift from *L. fructivorans* to *L. plantarum*, in pupal stage there is predominance of *A. tropicalis*, in early adult stage *L. fructivorans* reappear which is taken over by *A. pomorum* in the later adult stages [40]. The oxidative status of the gut at various developmental stages is thought to be the driving force behind the dynamics in the dominant microflora population of the gut microbiota of the fly [45].

How does the *Drosophila* gut get colonized by the microbiota and

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maintained? To investigate the establishment of the microbiota in the *Drosophila* Blum and co-investigators (2013) evaluated the change in the bacterial community, when the fresh stock of flies were flipped to fresh feed every 3 days and compared them with those flies which were retained in the old feed for up to seven days. It was observed that there was more bacterial population harboured by the fly which was retained in the feed for long time than the quickly replenished ones. This also confirms that the microbiota is established and maintained in the gut of the *Drosophila* from the food which they consume which also suggests the notion that the microbiota could not persist for long in the gut of *Drosophila* [26]. Thus the development stage of the *Drosophila* and frequent replenishment of the feed with the desired microbe plays an important role in the maintenance of the microbiota which has to be taken in to consideration while microbial interaction studies are to be carried out. The newly hatched fly carry less microbiome than the old adult fly, as they develop the microbiota while being exposed to the microbes present in the food [26,46]. The microbiotas are transferred to the offspring by vertical transfer process. The first instar larvae feed on the eggshells which are contaminated with the faecal matter during egg laying by their parents [47]. For this reason some microorganisms gets passed on to the gut of the offspring and maintained as a symbiont from generation to generation.

The microbe has either positive or negative impact on the host depending on the type of microbiota the fly was in contact. The pathogenic microbes affect the total developmental process while the symbiotic microbes promote its systemic growth, few commensals play role in its mating and social behaviour [48,49]. Microbial interaction stimulates immune system by activating Immune Deficiency (Imd) and Toll pathway [50-52]. The Toll pathway is frequently found in fat body and together with Imd it regulates the production of Antimicrobial Peptides (AMPs). Imd pathway is further involved in the formation of barrier epithelial surfaces such as gut and production of Reactive Oxygen Species (ROS) against microbes [53]. Deviation is seen in microbes like *Wolbachia* which stays as a symbiont and help to combat the RNA viruses such as Nora virus and Flock house virus [54].

Presence of various microbial genera in the natural gut microbiota of *Drosophila* often interferes with the host-microbial interaction studies using *Drosophila*. Realising this, the present study is aimed at raising germ free stock for such scientific investigations. Germ free fly are completely devoid of intestinal microbiota or any kind of microbial colonization. Such type of fly would be a powerful model in exploring the role of a particular probiotic strain or a specific microbial genera interacting with the host in isolation either in the mono- or poly-associated state unravelling the complex interplay between microbes and the host. Taking the wide application of germfree flies into consideration the present protocol describes an easy and cost effective method for producing germ free fly.

## Protocol

### Preparation of agar plates

Eggs laid on food vial is difficult to separate, so agar plates supplemented with apple juice is necessary to collect the freshly laid embryos of the flies [55].

**Materials required:** 5 cm petriplate, Agar powder, Mili Q water, Sucrose, Nipagin M (Methyl Paraben), Ethanol, Yeast paste, Apple juice.

- Take 100 ml Mili Q water and dissolve 2 g agar powder by stirring in a magnetic stirrer.
- In another autoclaved flask take 100 ml of freshly prepared apple juice and mix 8-10 g of sucrose. Stir the flask using a magnetic stirrer to dissolve it completely.
- Place both the flaks on a hot plate and boil it. Next combine both the contents together and stir it for few minutes. Care should be taken so that no air bubbles will form during the process (Air bubbles could be a major source of contamination at a later point when the agar plates get solidified with air bubbles).
- Allow the mixture to cool (around 50°C) and then add 10 ml of 20% Nipagin M to it (Nipagin M prevents fungal growth in fly food).
- Pour the media into 5 cm petriplates (sterile) and allow them to solidify.

### Cage preparation

- Cage is a small setup where the flies are trapped with apple juice agar plate to lay embryos. More than 1000-2000 embryos can be collected in a shorter period of time depending upon the quantity of female flies in the cage. This process is widely used for embryo collection [56].

**Materials required:** Apple-juice agar plates, Plastic cup for cage, Baker's yeast, Mili-Q water.

- Take 2 g of yeast in 5 ml water and start stirring, molding it into a paste (add a little more water if necessary).
- The yeast paste is then placed at the centre of the agar plates.
- To prepare a cage we need a plastic cup open at one end, in which holes can be easily made to provide aeration to flies.
- Flies are anaesthetised (using $CO_2$ preferable if not available diethyl ether can be used instead of it) and then transferred inside the cage. The petri plate is placed to close the opening of the plastic cup. Later the cage is placed in upright condition after the flies wake up.
- Cages were set up and the eggs can be collected within 3-4 hrs after setup because further delay may lead to formation of larvae which can also be a source of contamination.
- The laid eggs were collected from the agar plates (Figure 1).

### Collection, separation and dechorionation of embryos

Embryo dechorionation is an important step as it has been observed that the eggshell layer present carries microbes like *Wolbachia* and *Spiroplasma* from the faeces of adults. Removal of this layer or dechorionation by bleaching helps in eliminating major microbes from the embryos but it has been difficult to eliminate the *Wolbachia* completely [52]. Maintaining the sterile conditions after obtaining the bleach treated eggs is the crucial step and all the steps are strictly needed to be performed under laminar hood condition.

**Materials required:** Spatula (autoclaved), MiliQ water, Clean brush, Plastic sieve with pour size 40 µm, 70% Ethanol, 2.7% Sodium hypochlorite.

- To get the agar plates carrying embryos tap the cage upside down and exchange the old plate with a new one (in order to collect more embryos). The embryo containing plate is used for further processing.
- Take out the yeast paste from the plate immediately using a

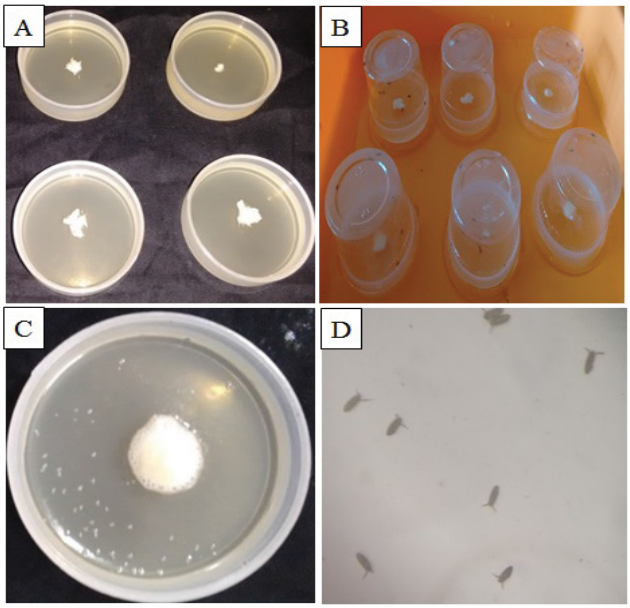


**Figure 1**: Collection of embryos:
A) Apple juice agar plates with yeast paste at the center.
B) Cage setup for collection of embryos.
C) Eggs laid in an apple juice plate by flies after 4hr.
D) Presence of eggs imaged under Stereo microscope indicating the presence of chorion.

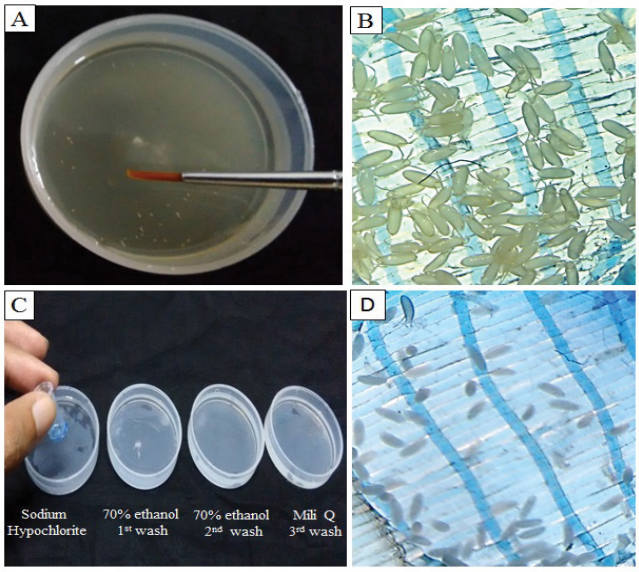


**Figure 2:** Dechorionation of embryos:
A) Detachment of embryos from the agar plates.
B) Chorionated embryos collected after detachment from the agar plate.
C) Treatment with Sodium hypochlorite and washing with ethanol to dechorionate the embryo.
D) Note the loss of chorion after the dechorionation step.

sterile spatula. This step is essential as it causes clogging during the filtration of the embryos.

- To obtain the embryos add 1 ml of sterile water onto the plate and wipe the surface with a clean brush. This step helps in the detachment of the embryos from the agar surface (Figure 2A). Embryos with chorion are shown in Figure 1D, 2B.
- Dechorionation of the embryos was done by treating the embryos with 2.7% sodium hypochlorite solution for of 2-3 minutes (Figure 2C) (Please note excess treatment can cause damage and can produce developmental defects).
- Next embryos are washed twice with 70% ethanol followed by three washes with water for 10 min (Figure 2C) until there is no bleach smell.
- The water carrying the embryos is now filtered through a plastic/metal sieve of less than 40 µm. One end of the 1.5 ml eppendorff tube was cut and the metal or plastic sieve was added to it.
- Eggs were monitored under the stereoscope to verify dechorionation (Figure 2D).

## Transfer of embryos to axenic media and amplification to next generation

Axenic media is microbe free media required for generation of a germ free fly as food is the sole source of microbial contamination. Propionic acid helps to eliminate the mold and Nipagin M prevents the fungus growth in the media (Figure S1). The axenic media contains the normal food materials along with tetracycline antibiotic because bleaching fails to remove endosymbionts like *Wolbachia* and *Spiroplasma*, hence tetracycline treatment will help in removal with its subsequent generations. The flies hatched from the bleached embryos are then transferred to the axenic media. These flies are considered as $F_0$ generation flies. The flies obtained from the $F_0$ generation were maintained in antibiotic containing food for next three generation to get a complete germ free fly.

**Materials required:** Whatman filter paper, Forceps, Axenic media, Tetracycline.

- The dechorionated embryos obtained from the above step are now transferred to the axenic media.
- The embryos from the plastic filter are transferred to a small cut sterile Whatman paper, and then the paper carrying embryos is carefully transferred to the axenic media using forceps (Figures 3A-C).
- The axenic media carries the normal ingredients of fly food, i.e., corn meal, agar, sucrose, propionic acid, yeast and Nipagin M. Live yeast can be a source of contamination [57]. To avoid contamination the yeast is mixed with corn meal, agar, and sucrose before it is autoclaved. Later propionic acid, Nipagin

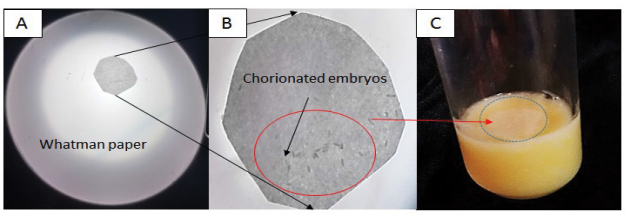


**Figure 3:** Implantation of dechorionated embryos in Axenic media:
A) The embryos were collected from the net are transferred to whatman paper.
B) Red circle indicating the presence of embryos in the filter paper.
C) Transfer of Whatman paper carrying embryos to the axenic media present in the food vial.

M, tetracycline (50 µg/ml) are added inside laminar conditions as autoclave destroy the properties of the chemicals.

- Transfer the freshly hatched flies from the above vial to a new vial with axenic food, maintaining the same growth conditions. This step is repeated for next three generation to get a germ free fly.

## Monitoring the development of lifecycle

The fly obtained after third generation was subjected to check the life cycle. The lifecycle is an indicator of the physiological status of the fly. Flies were maintained at 25°C with 70% Relative Humidity (RH) with 12 hr light/dark cycle and the development was monitored. No such developmental change was observed between the normal and germ free flies (Figure 4).

## Validation of the germ free state of *Drosophila*

The germ free status of the *Drosophila* stock can be validated by homogenizing the fly under aseptic conditions and introducing them to sterile Brain Heart Infusion agar (BHI) and incubated for overnight and observed for any sign of microbial growth in the medium which indicates the presence of bacterial contamination in the germ free fly [58-60]. This is a crude experiment to check for the presence of microbes if any.

In order to study the effect of microbe such as *Lactobacillus* in gut of *Drosophila*, the gut is dissected out first to check for its presence. Selective media like MRS media is used to detect specifically

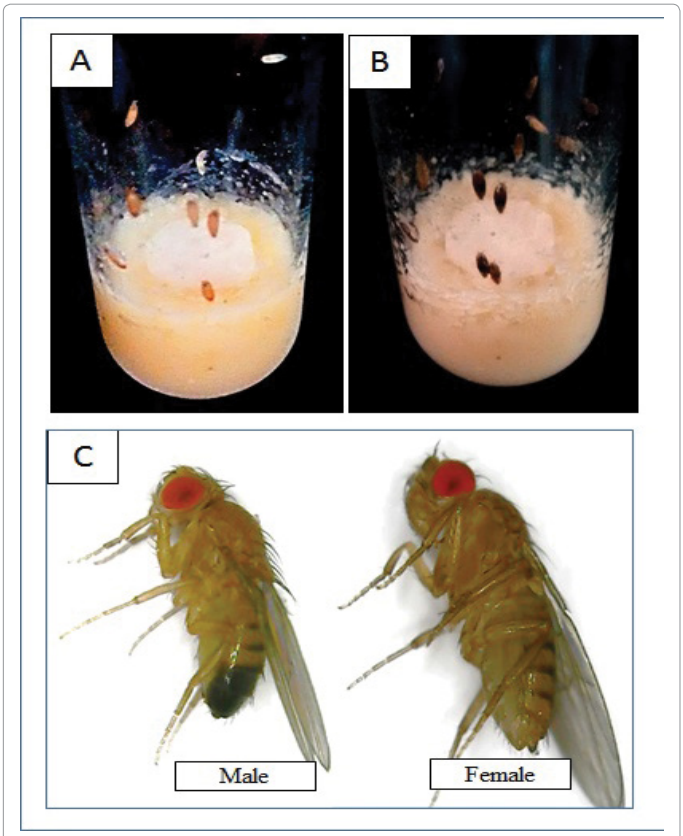


**Figure 4:** Development of germ free fly:
A) Early pupa formed after 5 days.
B) Late pupa at 7th day.
C) Male and female fly hatched from the implanted germ free embryo. No obvious difference was noted between the wild type and germ free flies.

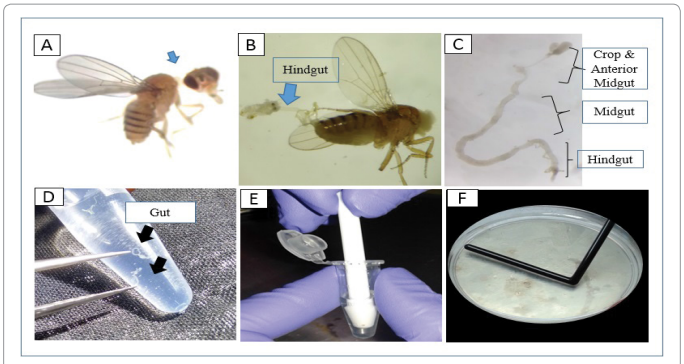


**Figure 5:** Fly gut preparation to check the presence of microbes inside it:
A) Head incised using sterile scalpel (indicated with arrow).
B) Hindgut pulled out with forceps from fly posterior end after the head is decapitated.
C) The gut pulled out from the fly (foregut lies within the head region).
D-E) Gut processed in PBS solution using micro pestle.
F) Spread plate of the gut homogenate in an agar plate.

*Lactobacillus* species and after confirmation the flies are infected with the microbes of our interest and then checked for its effect. There are ranges of other selective medias to analyse the presence of the specific microbes such as Enterococci confirmatory agar for *Enterococci* SP., Eosin Methylene Blue agar for *Enterobacter* sp., Dextrose Sorbitol Mannitol supplemented with Sodium deoxycholate for differentiating between *Gluconobacter* and *Acetobacter* that can be used to check its presence before testing the microbe of interest.

**Materials required:** MRS Agar Plates (selective media), Sterile forceps, Scalpel, Stereo microscope, 1x PBS, Sterile Mili-Q water, Micropestle, Eppendorff tubes, L-shaped rod.

- MRS Agar plates were prepared beforehand and kept inside laminar hood.
- The surface of the stereo microscope is cleaned with 70% ethanol and then placed inside laminar hood.
- The flies are now transferred to a small petri dish carrying 2-5 ml of PBS in it.
- Transfer the fly to the petri dish and dissect it under microscope (Figure 5A).
- Pull the gut out by holding the abdomen of the fly. Pull out the gut in a single strike because slow pull results in a discontinuous gut (Figure 5B,C).
- After successful dissection, the intact gut is transferred to an Eppendorff tube carrying 1 ml of 1X PBS in it (Figure 5D).
- With the help of a micropestle homogenise the gut (Figure 5E).
- Transfer100 µl of the homogenate to the agar plates and gently spread it using an L-shaped glass rod (Figure 5F).
- The plates are kept at 37°C in the incubator for 12 hours for the growth of microbe if there is any.
- Absence of colony in the plate represents the germ free status of the fly.

## Authenticating the mono-associated or poly-associated state of the germ free *Drosophila* fed with the microbe of interest

The microbiota of adult fly/larvae under investigation can be

validated for the presence of the microbe of interest by initial enrichment in BHI broth followed by plating on to selective media. Specific analysis like 16S ARDRA (Amplified Ribosomal DNA Restriction Analysis) or 16S r-DNA sequencing can be done with the recovered axenic cultures or amplicons from the *Drosophila* homogenate for authenticating the identity of the recovered bacteria with that of our test bacteria (which are fed to the *Drosophila* larvae) [61]. Quantitative determination of the bacteria colonizing the *Drosophila* can be done by FISH (Fluorescent In Situ Hybridisation) or by using DGGE or 16S rDNA targeted quantitative real-time PCR for community analysis of the colonized microbiota if a consortium of bacteria is fed to the *Drosophila* or to determine the abundance of a particular bacterial species among the natural microbiota present in the gut of the *Drosophila* [62-64].

We here in our review follow the basic way to show the germ free fly mono-associated to the microbe of interest by culturing the *Drosophila* gut homogenate on the selective media plates.

After generation of germ free fly we use them to check the effect of probiotic bacteria on the *Drosophila*'s general physiology and development. For this step the microbe of interest are inoculated on to the fly feed which is modified with the exclusion of yeast and allowed to grow by incubating it overnight at 35°C. The previously parted male and females are now introduced in to the incubated fly food and allowed to mate. The females lay their eggs and the larvae hatch from the eggs. The larvae are voracious feeders and hence they consume the food along with the microbes present in it. Thus the microbe of interest is introduced to the gut of the *Drosophila* larvae.

**Materials required:** Phosphate Buffered Saline pH 7, McFarland standard 3, Test tubes with axenic food without live-yeast.

**Preparation of the inoculum for the fly feed:**

- The microbe of interest at its active log phase of growth is obtained by growing them in the respective liquid media at their optimal physico-chemical condition.
- The microbial cells are centrifuged and the pellet is washed twice with sterile Phosphate Buffered Saline (PBS) of pH 7 under aseptic condition.
- The pellet was re-suspended in PBS of pH 7 and then diluted with PBS to obtain the final cell suspension with a minimum of at least McFarland standard 3.
- The bacterial inoculum of 0.2% is added to the fly food, and incubated for a day at 35°C to allow the bacteria to grow and establish themselves in the fly food. Check the surface of the media to be dry properly before transferring the flies to it.
- The germ free male and female flies are flipped in to the inoculated fly feed aseptically and allowed to mate and lay eggs on the fly feed.
- The vials containing the eggs are then incubated in the growth chamber maintaining 25°C temperature and 70% RH for promoting the hatching of the eggs and development of the larvae and adult.

The fly life cycle is monitored to check the effect of probiotics on growth and development of fly.

## Conclusion

The current protocol describes how to generate germ free flies in a laboratory. Although this is an easy method, sterile condition is required at each step which can achieved by performing maximum steps under laminar hood, autoclaving the reagents used in this study, wiping the microscope and dissecting tools with 70% ethanol. With appropriate precaution the germ-free fly can be generated within a short time period and various probiotics and molecular mechanism of host-microbial interaction taking place in the gut can be studied using the resultant germ free fly.

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