Understanding the impact of High-Sugar Diet on the gut homeostasis of *Drosophila melanogaster*

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Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled "**Understanding the impact of High-Sugar Diet on the gut homeostasis of *Drosophila melanogaster***" submitted by **Ashitha V. V. (Reg. No. MS14183)** for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: April 26, 2019

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Dr. Sudip Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institution. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Ashitha V. V.
(Candidate)

Dated: 26-04-2019

In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr.Sudip Mandal
(Supervisor)

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1. Results

Abstract

Diet of an organism has a great influence on its health and homeostasis. Considering that diabetes and related metabolic disorders are growing health problems worldwide, we are trying to understand the effects of High Sugar Diet on intestine using the model organism *Drosophila melanogaster*. In this study, we fed flies on high sugar diet (1M-sugar) sugar to provide a condition that resembles hyperglycemia. Our results demonstrate the effect of high sugar diet in the gut morphology, cell size, cell death, and some signalling pathways that are critical for maintaining gut homeostasis -We conducted parallel experiments to study the impact of HSD on gut commensal bacteria and observed a significant decrease in the number of gut commensal bacteria in the gut of high sugar diet fed flies. This had a natural consequence on the expression of genes involved in immune pathways.

Chapter 1- Introduction and Objectives

1. Introduction

Mammalian and *Drosophila* intestines share many similarities structurally and functionally; also the ISC lineage and the common pathways that control their proliferation and differentiation are similar[1]. The main circulating energy sources are also same in both (sugars). Here, we are trying to understand the impact of High Sugar Diet (HSD) on the structure and function of gut using the model organism *Drosophila melanogaster*.

1.1 Diet and Diseases – High Sugar Diet and its consequences

Diet plays a central role as a risk factor for chronic diseases. In 1989 a Consultation followed by the work of a World Health Organization Study Group acknowledged the significance of dietary and lifestyle changes in the increase of chronic disease (both in developed and developing nations[2]. They identified some particular dietary constituents can enhance the chances of these diseases and their impact can be altered with interventions.

Through more basic research on some aspects of mechanisms that connect health and diet, chronic diseases can be prevented. In current dietary habits, traditional plant-based diets have been replaced with energy dense and high-fat diets. Because of these variations, Chronic NCDs (non-communicable diseases) such as diabetes mellitus (T2DM), obesity, cardiovascular diseases (CVD), some kinds of cancer/tumor, etc. are becoming growingly significant.[2]

In all parts of the world, sugar is one of the main ingredients on Diet[3][4]. Eating too much added sugar could give rise to many negative health effects[5]. Prolonged

hyperglycemia drives resistance to insulin-a hormone that regulates blood-sugar-levels. This further cause blood-sugar-levels to rise and in a strong manner promotes the peril of diabetes[6]. There was a world-wide (comprising more than 175 countries) population study, which demonstrated the growth of diabetics and related risks by 1.1% for every 0.15 kilo calories of sugar (or about 1 tin of sweetened soda) ingested per day[7][8]. Other dangerous conditions include weight gain (obesity is another potential risk factor for diabetes), blood-sugar level riskiness and an elevated risk of heart-disease.

So, in summary, High Sugar Diet could cause obesity and resistance to insulin- which are risk factors for diabetes. Eating an excessive amount of sugar can even induce chances of developing certain cancers[9][10][11]. There are many other risk factors such as increased kidney disease[12], fat deposition in the liver[13], tooth demineralization[14], increased chance of developing gout[15], impaired memory or dementia[16], etc.

1.2 *Drosophila* as a model system

The fruit fly, *Drosophila* melanogaster is a small insect (dipterid) primarily used for genetic and developmental studies for nearly over a century in research laboratories. William Ernest Castle (1867-1962) was the first person to bring the fly to workbenches from its ancestral home in Africa. But Thomas Hunt Morgan (1866-1945) and his students at Columbia University made the scientists and the scientific world realize the potential of this model system (Morgan, 1910). Till date, various discoveries were achieved using this animal model in behavior, human genetic diseases, neuroscience, cell, and developmental biology studies.[17]

Drosophila has a short life cycle, high fecundity, fully sequenced genome spread over only four chromosomes, cheap maintenance and needs simple instruments and less human resources for its rearing and maintenance compared to vertebrate model organisms.

Drosophila genome can be easily manipulated, and there are many *Drosophila* transgenic lines available, which are developed using different strategies like- transposons (mobile genetic elements), transposon insertion. Transposon insertions usually generate a loss of function mutant. It can also be used to insert transgenes into the genome. CRISPR/Cas9 mutagenesis, FLP/FRT recombination, phiC31 integrase-mediated target insertion, etc. are some other commonly used genetic manipulation tools. Tools like UAS GAL4

system- for spatiotemporal regulation over gene expression, P element mutagenesis- for targeted mutagenesis also simplified the life of *Drosophila* researchers.[18][19]

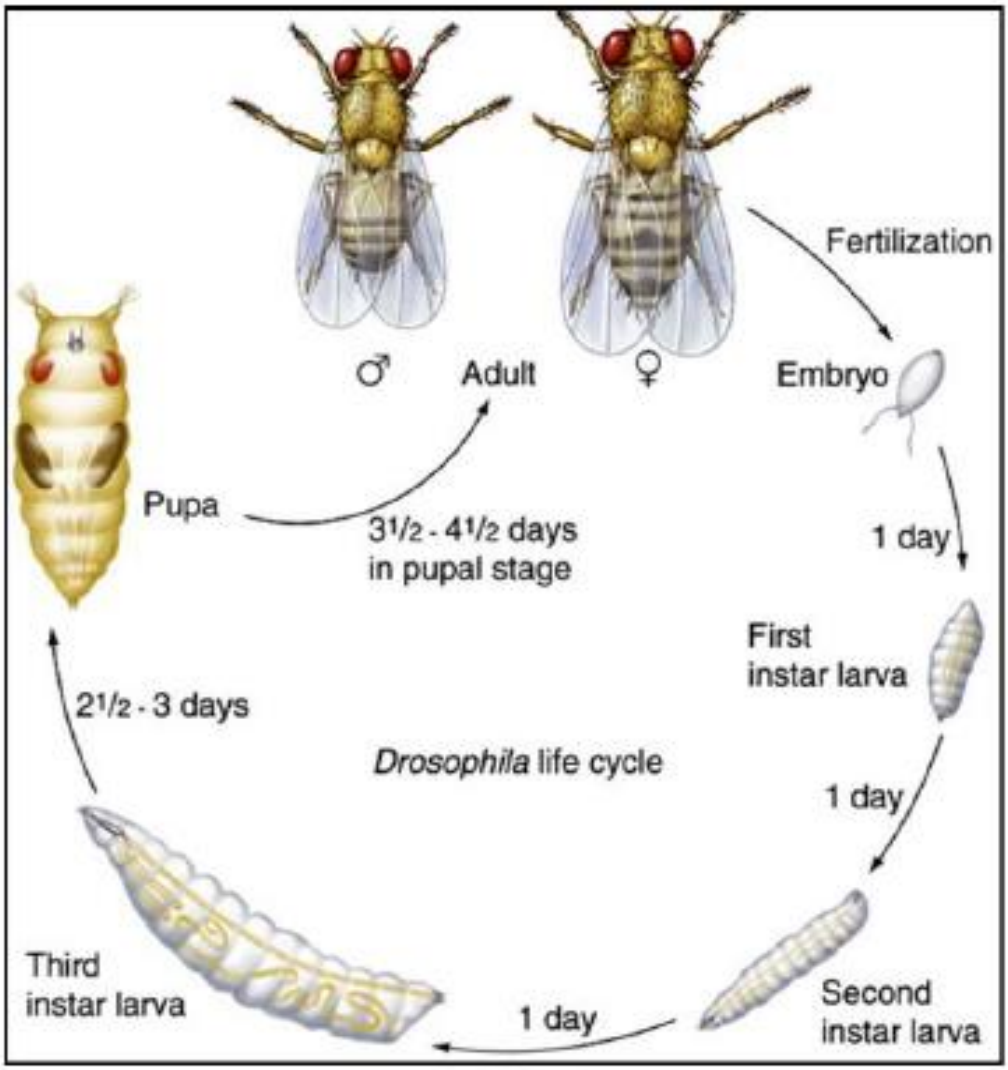

Figure 1: Life-cycle of *Drosophila melanogaster*

Drosophila has four distinct developmental stages-embryos, larvae, pupae, and the adult fly. Larval stages are significantly different from adult stages. After growing and undergoing several molts, larvae become pupae. Pupae undergo metamorphosis and after 3-4 days, fly emerges out of the pupal case.

Drosophila is a holometabolous insect with distinct developmental stages (-embryo-larvae-pupae-adult fly-). Drosophila has internal fertilization. Soon after fertilization females lay eggs (around one and a half mm length) at 50-60 eggs per day. [20]

Temperature influences the growth and development of *Drosophila*. Embryos hatch out to the first instar larvae within 24 hours of egg laying at 25ºC and the tiny larvae that emerges are called the first instar. It molts to develop into the second instar in 24 hours and then into third instar (largest of all larval forms) larvae in subsequent 24 hours. Larvae feed on a given food and third instar continue feeding for around 48 hours, later climb upward and ultimately become sedentary and start preparing for the pupal stage and eventually pupated on the wall of the vial or bottle where we provide food resources. During this pupal stage, adult structures replace the larval structures (metamorphosis), and the adult flies emerge out of the pupal case in 3-4 days. The adult flies have an average lifespan of six weeks (approximately 45 days). The life cycle illustrated in **figure 1**

1.2.1 GAL4 - UAS system

GAL4-UAS system is a genetic tool which allows the ectopic expression of any given sequence, in the tissue or cells of interest and enables the users to have spatiotemporal control over the expression of a gene of interest. GAL4 is a transcription activator identified in yeast *Saccharomyces cerevisiae*, which binds to a DNA sequence called Upstream Activator Sequence (UAS) and activates transcription of GAL4 targeted genes. GAL4 with the tissue-specific promoter is called driver line, and UAS with the specific gene of interest is called the responder line. These two transgenic lines are crossed to obtain desired responder line containing both GAL4 and UAS elements in a single F1 progeny. As a result, GAL4 protein will go and bind to UAS and activates transcription of the target gene which downstream of UAS element (**Figure 2**). [21][22].

To gain temporal control, a temperature sensitive variant of gal80 protein is used. This protein potentially binds to activating domains of Gal4 protein, preventing its binding to UAS under restricted temperature conditions. Another advantage of the GAL4-UAS system is that it allows expression of lethal genes for a short window of time in specific tissues and excludes its effects in early developmental stages

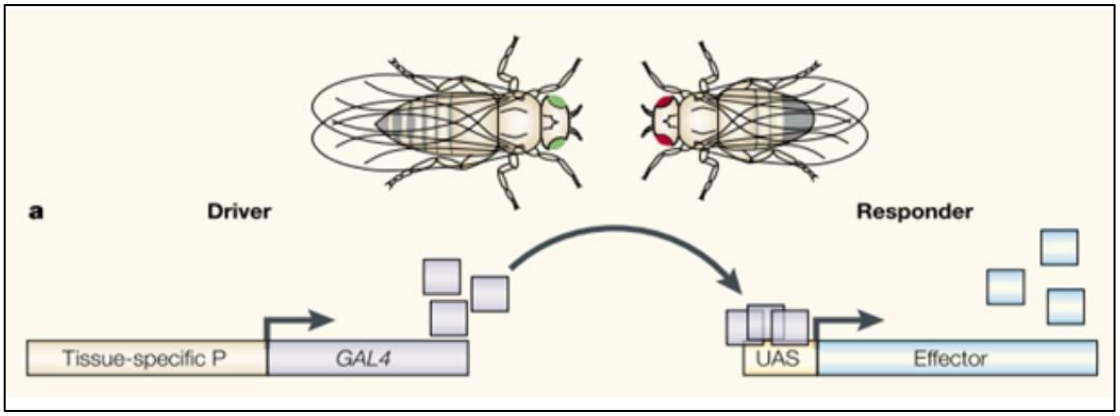

Figure 2: The GAL4-UAS system in Drosophila

The GAL4- UAS system in *Drosophila* allows targeted gene expression. Driver line has GAL4 with tissue-specific promoter whereas responder line has Upstream Activator Sequence (UAS) with a specific gene of interest. When these lines are crossed, F1 progeny will have both elements and this allows expression of our gene of interest, in a tissue-specific manner.

(Copyright from http://hawaiireedlab.com/wpress/? p=477)

1.2.2 Drosophila as a model for studying alimentary canal

The intestine is one of the largest organs in the body cavity having a primary role in digesting and absorbing nutrients. It also provides the first line of defense against a wide variety of pathogens entry.

The *Drosophila* gut consists of simple epithelium, and it is surrounded by visceral muscles, tracheae, and nerves. The alimentary canal of *Drosophila* is majorly divided into three parts- foregut, midgut, and hindgut. Foregut and hindgut have originated from distal layer (ectoderm of germ layers) while midgut has a middle layer (endoderm of germ layers) origin. The adult midgut is further divided into different regions according to variations in function.[23] As an invertebrate model for studying gut *Drosophila* shares many similarities with the mammalian alimentary canal.[24]

1.2.2.a Structural and functional level:

The primary function of both systems is the absorption of nutrients and secretion of enzymes or hormones. They also serve as a barrier against pathogens and microenvironment for variety of commensal microbes.

The crop of *Drosophila* is similar to the stomach of mammals structurally and functionally. The midgut is similar to the small intestine, and the hindgut is similar to the large intestine and rectum. Also, some accessory glands associated with the human alimentary canal has (partial) counterparts in *Drosophila* (e.g., salivary gland- salivary duct, liver- fat body). (**Figure 3**)[24][25]

1.2.2.b Cellular and Signalling level:

The *Drosophila* midgut is comprised of a single layer of epithelial cells and deficient of the villus or crypt structure in the mammalian intestine. Intestinal Stem Cells in *Drosophila* gut give rise to enteroblasts (EBs) by dividing symmetrically or asymmetrically[26].

The (adult) midgut epithelium contains two types of differentiated cells namely enterocytes (ECs), and enteroendocrine cells (EECs) derived from EBs. Enterocytes mainly absorb nutrients, and EECs secrete hormones.

Several conserved pathways are involved in this ISC self-renewal and differentiation processes, including Notch, JNK, EGFR, Wnt, and JAK/STAT (**Figure 5**) to sustain gut homeostasis. [27]. Diseased conditions will cause an over-accumulation of cells, which can further cause inflammation and even cancer (**Figure 4**). Similar types of cells, signaling pathways, and differentiation pattern are observed in mammalian intestine too (**Figure 5**).

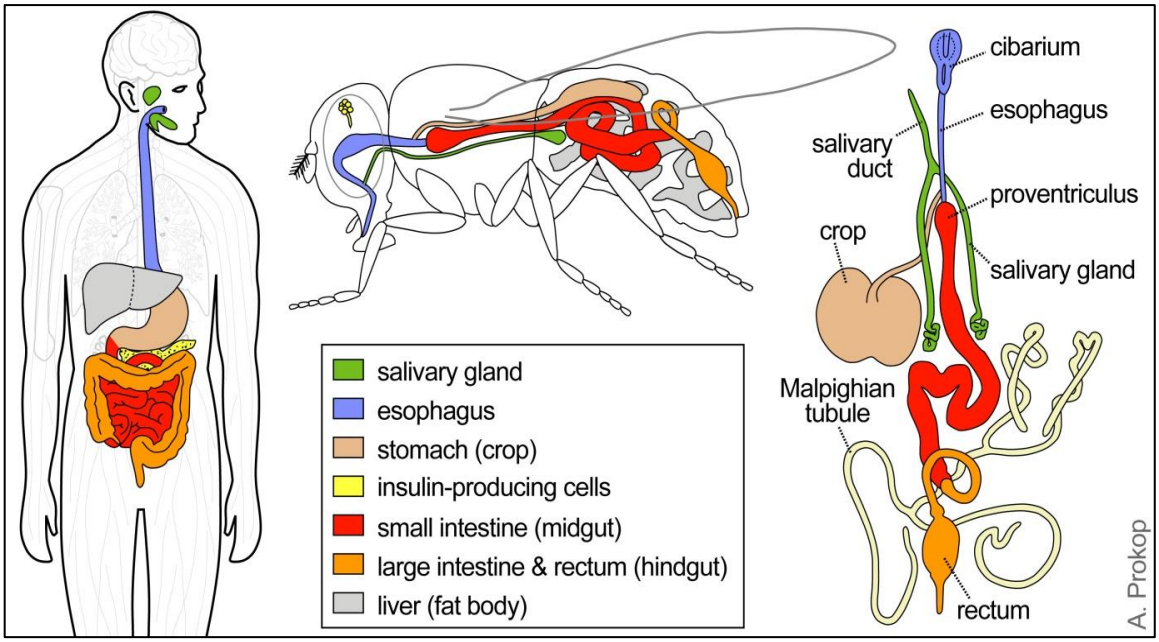

Figure 3: Comparison of the alimentary canal of *Drosophila melanogaster* and Human.

Colours indicate homologous and functionally related structures of the alimentary canal. Crop is similar to stomach, foregut to esophagus, midgut to small intestine, and hindgut to large intestine & rectum.

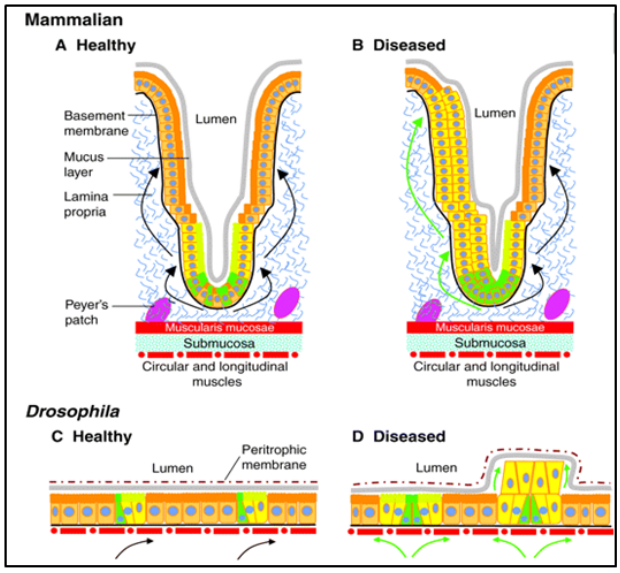

Figure 4: Mammalian intestinal crypts and villi, and the *Drosophila* midgut, in healthy and diseased animals

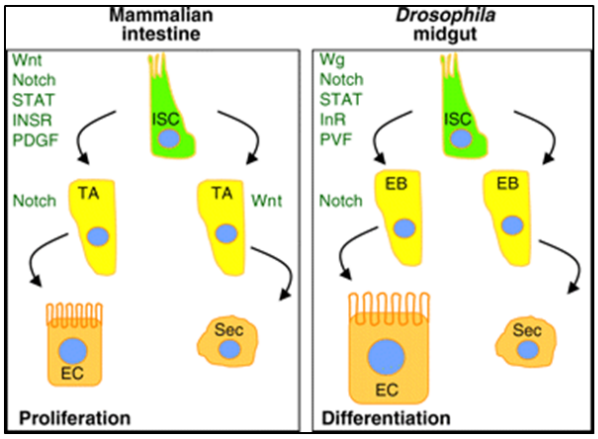

Figure 5: ISC lineages of mammalian intestine and *Drosophila* midgut, and the common pathways that control them

1.2.2.c Significance of Notch and JAK/STAT signaling

Both Notch and JAK/STAT signaling pathways are conserved in *Drosophila* and mammalian models in maintaining homeostasis of the intestine.[1] Notch signaling is known to have an essential role in Intestinal Stem Cell (ISC) fate establishment and suppresses ISC self-renewal. It also promotes ISCs to differentiate into enteroblasts in the enterocyte lineage. In the absence of Notch signaling, ISCs divide symmetrically and forms intestinal tumors consisting more number of EE-like cells and ISCs. Studies also show similar occurrence in aging intestines because of mutations that deactivate Notch in ISCs. While activation of Notch in ISCs stimulate ectopic differentiation of EBs to become matured ECs

JAK/STAT pathway has been reported as one of the main mitogenic pathways. It is essential for the ISC division activation throughout regeneration[28]. Ectopic expression of JAK/STAT in midgut induces dramatic ISC proliferation. *Drosophila* cytokines (Upd1, Upd2, and Upd3) are upstream ligands for the activation JAK/STAT signaling. Upd1 is only expressed in progenitors; Upd 2 is expressed in both progenitor-cells and mature enterocytes, and Upd3 majorly in matured enterocytes[29]. Midgut stress due to damage induces these ligands and results in dramatic activation of JAK/STAT to promote ISC proliferation.

1.3 Innate immune defenses in the *Drosophila* intestine- Toll and Imd pathways

The Fruit fly *Drosophila melanogaster* alimentary canal is regionalized into ectodermally derived foregut, endodermally derived midgut, and ectodermally derived hindgut. Gut epithelia itself provides a primary and efficient barrier, and during pathogen infection, innate immunity gets activated.[30][31] Toll and Immune Deficiency (Imd) are two classical signaling pathways (**Figure 6**) control inducible immune responses in Drosophila melanogaster against bacteria and fungi. In case of infection, they recognize specific patterns in pathogen or pathogen cell products and expresses antimicrobial peptides (AMPs). In common *drosomycin* and *diptericin* expressions are used as read-outs for Toll and Imd pathways respectively.[32]

Drosophila deficient of the Toll pathway is prone to infections by fungi and Gram-positive bacteria while Drosophila defective of the Imd pathway is susceptible to infections by Gram-negative bacteria.[33][34][35]

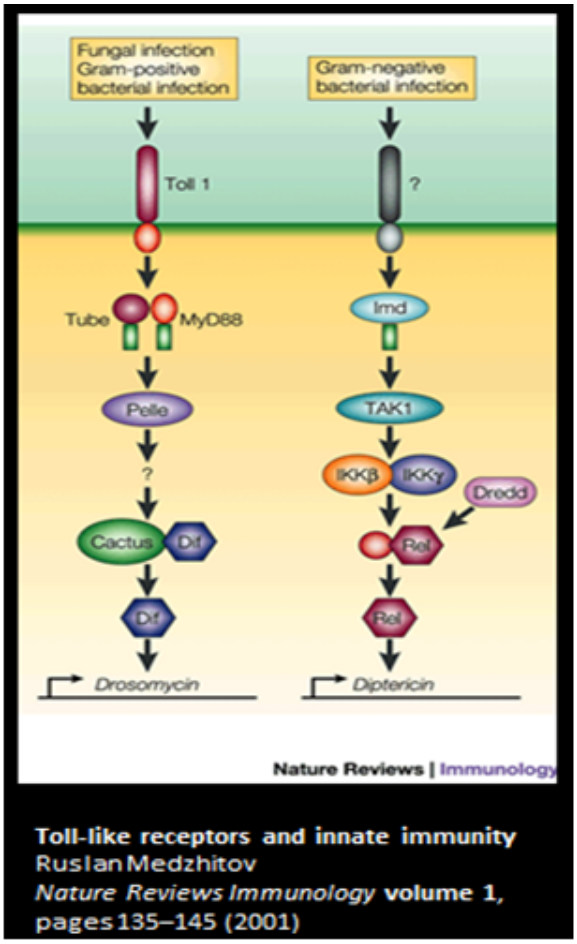

Figure 6: Innate immunity in *Drosophila melanogaster*- The Toll and Imd pathways

Toll and Immune Deficiency (Imd) are two classical signalling pathways control inducible immune responses in *Drosophila melanogaster* against bacteria and fungi. The Toll pathway is active only in the foregut and hindgut. Imd pathway mostly regulates the immune response in the midgut. Gut produces different antimicrobial peptides (AMPs) when Imd gets activated. Recognition of pathogens in the midgut occurs either by membrane-bound peptidoglycan recognition protein LC (PGRP-LC) or by intracellular PGRP-LE.

The AMP end product of Toll and Imd pathways are *Drosomycin* and *Diptericin* respectively.

1.3.1 Microorganisms in the *Drosophila melanogaster* intestine:

Drosophila is a simple and better model to study the gut microbiome and dietary effects on gut microbiome[36][37]. Recent studies in both wild type and laboratory-reared flies shows that gut microbiome of Drosophila only consists of about 30 species in maximum[30][38]. It is quite simpler compared to a complex organization in vertebrate models. The fact that all these species are culturable makes this model better for functional analyses efficiently. As in higher organisms, gut microbiome and immunity are correlated in Drosophila. It also affects the development of Drosophila and dysbiosis can lead to impaired systemic growth and metabolic disorders, which is similar in humans. The bacterial cell wall components also serve as ligands to Toll and Imd immune pathways. This helps to keep a basal level expression of AMPs even in the absence of infection. The microbiome also has positive effects on ISC proliferation and intestinal regeneration.[38]

1.4 Experimental Model – As a powerful system to study the consequences of High Sugar Diet.

The experimental model I have adopted for my experiments were developed by Ms. Jayati Gera, one of my colleagues (Ph.D. scholar) in the laboratory. The experimental strategy is to feed the experimental flies (with 2:1 Female to Male ratio) on High Sugar Diet (HSD: 1M sugar) two days after eclosion while control flies reared on Normal Diet (ND: 0.15 sugar). Two days after eclosion healthy flies were randomly chosen to prepare both control and experimental batches.

It has been already studied in our laboratory that this experimental model resembles Type 2 Diabetes Mellitus (T2DM) model in Humans from 13 Days (threshold) of HSD feeding. It shows T2DM characteristics such as increased Glucose level in hemolymph (fluid equivalent to blood), increased Trehalose levels (sugar consisting of 2 molecules of Glucose, the principal source of energy in fruitfly), and tissues develop insulin resistance while their production is not affected. This experimental model itself serves as a powerful tool to understand the effect of HSD in the gut and correlate the effects with T2D condition in humans.

2. Objectives

The main focus of this project is to understand and study the effects of High Sugar Diet on the Female Adult Gut of *Drosophila melanogaster* by:-

1. Analyzing gut structure and function
2. Studying individual cell size, shape, integrity and survival
3. Determining the status of signaling pathways involved in maintaining intestinal homeostasis
4. Finding the effects on gut-associated bacterial population and immune pathways

Chapter 2: Materials and Methods

1. Materials and methods

1.1 Rearing of flies and maintenance

Most of the *Drosophila* stocks used for this study were obtained from different stock centers. Two stocks (*upd3*) used were a gift from Dr. Erica Bach. The flies were reared on food made from cornmeal, agar, yeast, and fungicides. Eclosed flies were collected and fly batches, with 2:1 female to male ratio, were made two days after eclosion. The concentration of sugar in the Normal Diet (control) and High Sugar Diet (HSD-experimental) were 0.15 M and 1 M respectively. The flies were maintained at 25ºC in standard bottles if not mentioned otherwise. Female flies were dissected at 3-time points, Day 5, Day 10 and Day 15 after eclosion for most of the experiments. Some dissections were done only on Day 15. The experimental strategy is demonstrated in **Figure 7**.

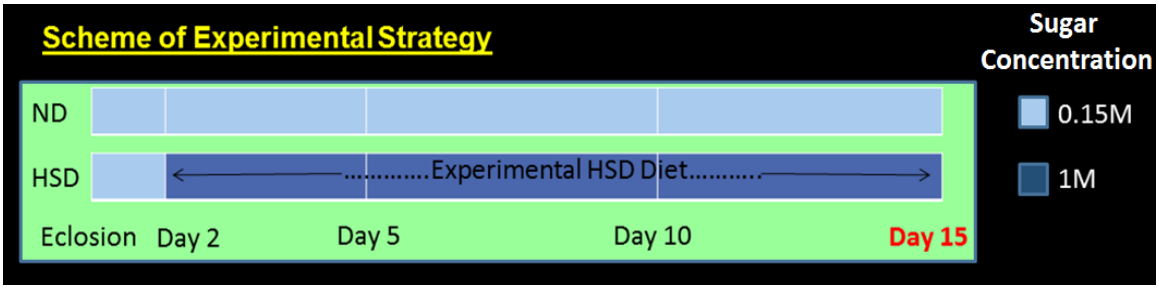

Figure 7: Schematic Representation of experimental strategy

High Sugar Diet (HSD) and Normal Diet (ND) batches were made on Day 2 after eclosion. Dissections were done on Day 5, Day 10 and, Day 15. In this diagram **Light blue** boxes represent Normal Diet and **Dark blue** boxes represent High Sugar Diet.

1.2 Fly stocks and genotypes

Oregon R: Wild-type laboratory stock of *Drosophila.*

Transgenic lines used:

P{ry[+t7.2]=*Dipt*2.2-lacZ}1, P{w[+mC]=*Drs*-GFP: This transgenic line component P{Drs-GFP.JM804}1 expresses GFP-tagged drosomycin under the control of its native promoter and component P{Dipt2.2-lacZ}1 Expresses lacZ in the fat body under the control of the Dpt on the X chromosome.

y[1] w[1118]; P{w[+mC]=GAL4-ninaE.GMR}12/CyO: This transgenic line has Gal4 insertion downstream to *Glass* enhancer. It expresses Gal4 in all cells behind the morphogenetic furrow in the eye imaginal disc at third instar larval stage. This transgenic insertion is on the second chromosome. This fly stock is not homozygous viable, therefore it is balanced by CyO balancer.

P{w[+mC]=UAS-*hid.Z*}2/CyO: This fly line expresses *hid* under the control of UAS on the second chromosome.

w[1118]; P{w[+m*]=NRE-EGFP.S}5A: This transgenic fly line expresses EGFP under the control of a Notch Response Element (NRE) on the second chromosome.

***upd3*-LacZ/Cyo; Δr/TM6B, Tb** and ***upd3*-Gal4-UAS-GFP/Cyo**: Upd3 reporter (on the second chromosome) transgenic lines gifted by Dr. Erica Bach.

1.3 Immunohistochemistry

Entire guts were dissected from the *Drosophila* adult flies in 1X PBS followed by fixation in 5% paraformaldehyde for 60 minutes on 60 rpm shaker. After removing the fixative, three times 0.3% PBT washes of 15 minutes each were given for permeabilization (60 rpm shaker). Gut samples incubated in blocking solution, 10% NGS (Normal Goat Serum) for 1 hour (60 rpm shaker). Once blocking is done, the sample was incubated with primary antibody for 18-20 hours at 4ºC in Nunc multiwell plates (All primary antibodies, source, and its dilution mentioned below).

Then samples were washed three times for 15 minutes each using 0.3%PBT at room temperature (60 rpm shaker). Before secondary antibody guts were incubated in 10% NGS for 30 minutes (Secondary antibody, source, and its dilution mentioned below). Samples were incubated in secondary antibody for 16-18 hours at 4ºC followed by three 0.3% PBT washes for 15minutes each. Then samples were incubated with DAPI and Phalloidin (10% Red/Green) for 1 hour. After DAPI and Phalloidin treatment, samples were washed in 1X PBS for two times of 5 minutes each and mounted (Bridge mounting) in vectashield.

Imaging

Images of the mounted samples were taken by using confocal microscope (Zeiss LSM 780) and processed using Fiji or Image J software.

Statistical Analyses

Statistical analyses for gut width quantification and Bacterial plating and culturing were done using Microsoft Excel 2010, and the significance was determined using students t-test (two- tailed distribution of unequal variance).

1.3a Primary antibodies used

Immunostaining-primary antibodies used:

Antibody	Developed in	Source	Antibody number	Dilution used
Anti-β-gal	Mouse	DSHB, lowa	Z3781	1: 100
Anti-fas3	Mouse	DSHB, lowa	7G10	1: 3
Anti-Dlg	Mouse	DSHB, lowa	4F3	1: 5

1.3b Secondary antibody used

Antibody	Source	Details
Cy™3-Conjugated AffiniPure Goat Anti-Mouse IgG(H+L)	Jacksons Immuno Search laboratories the USA Code-711-165-152	Conjugated with cyanine Cy™3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) working dilution: 1:500 Detect primary antibodies raised in mouse.

1.3c Stains used

- **DAPI:** (4',6-diamidino-2-phenylindole dihydrochloride). This is a blue fluorescent dye that binds to A-T rich region in double-stranded DNA. It is used to stain nuclei in live (less efficient) as well as fixed tissues (more efficient). Its absorption maxima/ emission maxima are 351nm/461nm. The working dilution is 1µg/ml.
- **Phalloidin:** It binds and stabilizes F-actin (filamentous actin) and prevents depolymerization of actin fibers.

Red: Excitation/Emission (nm): 540/565

Green: Excitation/Emission (nm): 495/518

1.4 TUNEL Assay

Entire guts dissected from the *Drosophila* adult flies in 1X PBS followed by overnight fixation in 5% paraformaldehyde at 4°C. After removing the fixative, five times 0.4% PBT washes on a shaker (60 rpm) of 15 minutes each was given. Incubated the tissues in 100mM Sodium citrate solution for 50 minutes at 75°C. Kept the samples at RT for 20 minutes and given 3 PBS washes of 10 minutes each. Added total volume (20 µl) of Enzyme Solution to the remaining 180 µl Label Solution to obtain 200 µl TUNEL reaction mixture. Tissues were incubated (37°C) for one and a half hours in this mixture on Nunc multiwell plates. Then samples were washed three times for 10 minutes each

using PBS at room temperature (60 rpm shaker) and incubated with DAPI for 1 hour. Samples washed in 1X PBS for two times of 5 minutes each and mounted (Bridge mounting) in vectashield. [39]

Positive control: GMR-Gal4-UAS-hid/Cyo (third instar larval eye disc).

1.5 SMURF Assay

SMURF Assay is a *Drosophila* feeding assay to access food intake by co-ingestion of dye. Leakage of food from gut epithelium also can be detected. 400 mg of food dye dissolved in 1 ml of sterile water and mixed well by shaking and vortexing to prepare the stock. Sedimentary dye removed using filtration. The stock was added to the melted Normal Diet to make 7.8% concentration of dye (78µl in 1 ml) in food. The dyed food (melt) later equally poured to vials and kept for drying with mouth closed. Places these vials at -22°C for 15- 20 minutes and then kept upside down at RT to remove moisture. Cleaned the surface of the vials and transferred eight females and four males each from ND & HSD 15day bottles to different vials and labeled them. Images were taken after 12-18 hours.[40][41]

Imaging

Images of the etherized flies were taken by using stereomicroscope (AxioCam ICc 1) (AxioVision LE software).

1.6 Bacterial culture & plating

Adult flies were given two times 70% ethanol washes of 2 minutes each followed by two sterile water washes. After this sterilizing fly body surface, fly's entire guts dissected without crop and malpighian tubules from foregut-hindgut in autoclaved sterile 1X PBS and collected into 300µl PBS in sterilized MCT. Inside Laminar hood homogenize gut using autoclaved pestles and added 700µl PBS. Spread 100µl on LB agar bacterial plates and kept at 25^0 C for incubation after sealing plates with parafilm for 3-5 days.[42]

Imaging

Images of the bacterial plates were taken using Gel doc (UVP- BioSpectrum 310 imaging system).

Statistical Analyses

Statistical analyses for gut width quantification and Bacterial plating and culturing were done using Microsoft Excel 2010, and the significance was determined using students t-test (two- tailed distribution of unequal variance).

1.7 Preparation of LB Agar plates

In 500 ml autoclaved reagent bottle 2.5% of LB and 1.5% Agar powder mixed in distilled water and mixed well. Put for autoclaving after placing autoclave tape. Take out the hot mixture after autoclaving inside the laminar hood and make LB pour plates using sterile plates. Keep the moisture-free plates upside down at 4^0C after covering them with Aluminium foil.

1.8 Buffers and Reagents

10X PBS: For a volume of 500ml, 40g NaCl, 1g KCl, 7.2g Na_2HPO_4, and 1.2g K_2HPO_4 were weighed. 490 ml dH_2O was added. pH was adjusted to 7.2. The total volume scaled to 500ml.

0.3% (or 0.4%) PBT: For a volume of 40ml, 120µl (or 160µl) of 100% TritonX added to 40ml 1X PBS

Sodium Citrate Solution 100mM: For a volume of 20ml, 588mg of tri-sodium citrate anhydrous in 0.1% PBT

10% PFA: For a volume of 2ml, 0.2g of PFA weighed and 2ml volume made with 1X PBS

Chapter 3-Results and Discussions

1. Results and Discussions

1.1 Morphological variations in the gut of adult flies fed on High Sugar Diet

We chose the adult fly gut for studying the effects first because the primary encounter with diet happens there. To nalyse the effect of HSD on the intestine, the entire gut was dissected out without break and stained with Phalloidin and DAPI at Day 5, Day 10 (hyperglycemic, pre-diabetic) and Day 15(diabetic). (**Figure 8**)

Reduction in whole gut size (except crop size) was observed in HSD guts. We observed a negligible reduction in length on Day 5 and severe phenotype on Day 15. However, a significant decrease in the gut width was observed in all the dissection time points (Day 5, Day 10, and Day 15). Quantified date of width is given in (**Figure 9**).

1.2 High Sugar Diet does not cause permeability and feeding defects until Day 15

A *Drosophila* feeding assay- Smurf Assay (SA) was used to test the feeding, aging, and permeability or leakage-functional defects.

We found that HSD does not cause any such defects until Day 15. There was no leakage of dye and the reduction of food in the midgut area due to the reduction in size (wholly or partially- we need to quantify food intake using other Assays) compensated by increased crop size. These results suggest that in diabetic flies till day 15 there is no leakage even though the size diminished significantly and flies are feeding on the HSD. (**Figure 10**)

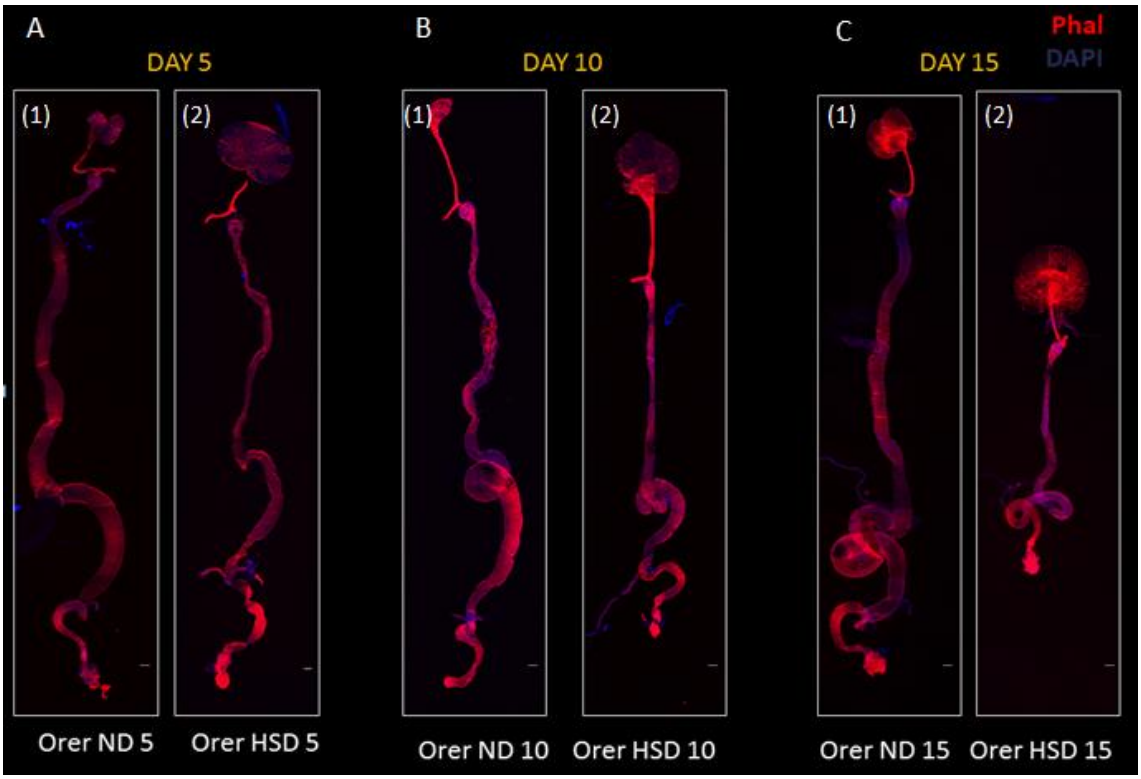

Figure 8: ***Gradual reduction in the size of gut on feeding flies on High Sugar Diet***

Scale bar-20µm. Gut of adult flies fed either on (1) ND- 0.15M sucrose or (2) HSD- 1M sucrose stained with **Phalloidin** and **DAPI** at time points (**A**) Day 5 (**B**) Day 10, and (**C**) Day 15 respectively. Gradual reduction in the overall size (all regions other than crop) of gut observed.

1.3 High Sugar Diet enhances the membrane Integrity of the gut epithelial cells

For identifying the cause(s) of gut size reduction, we checked the status of individual cells using cell membrane (junction) specific markers (Dlg, Fas3)**(Figure 11)**. From our analyses of gut size it was evident that there was significant reduction in the width of the gut **(Figure 9)**.

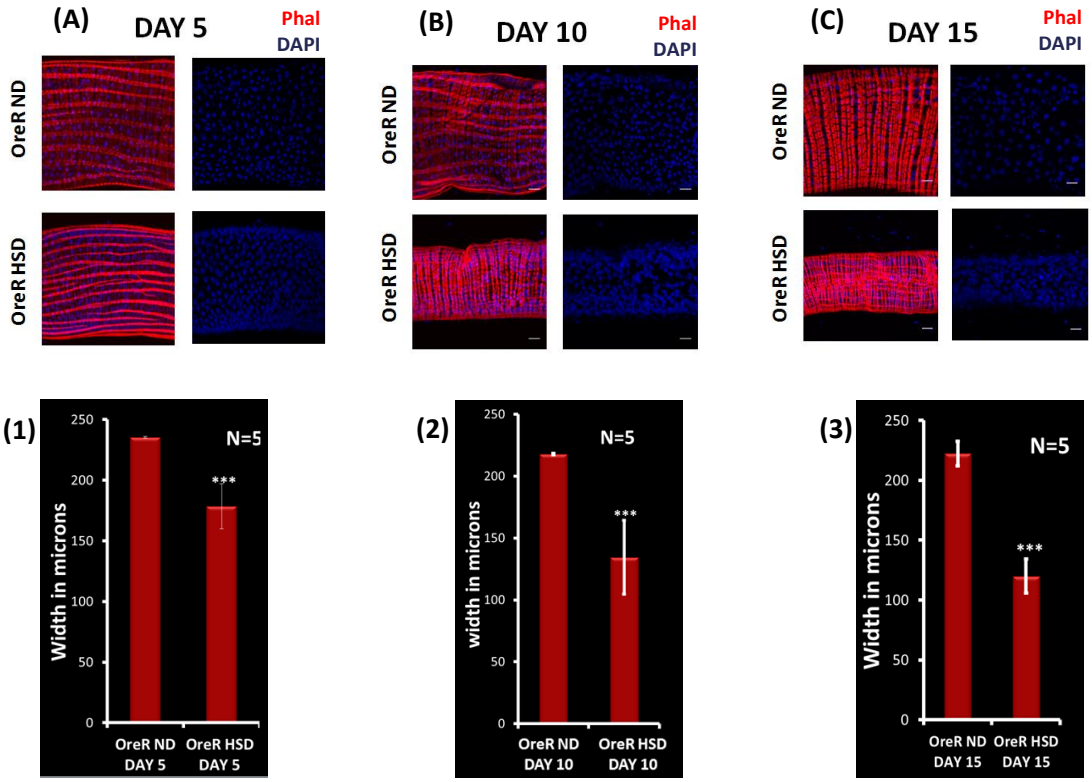

Figure 9: Quantification of reduction in the width of the midgut on feeding flies on High Sugar Diet

Feeding on HSD causes gradual decrease in width in ND: HSD ratio of (**A**) **~1: 0.77** on Day 5 (graph (1)), (**B**) **~1: 0.62** on Day 10 (graph (2)), and (C) **~1: 0.55** on Day 15 (graph (3)). Scale bars: 20 µm

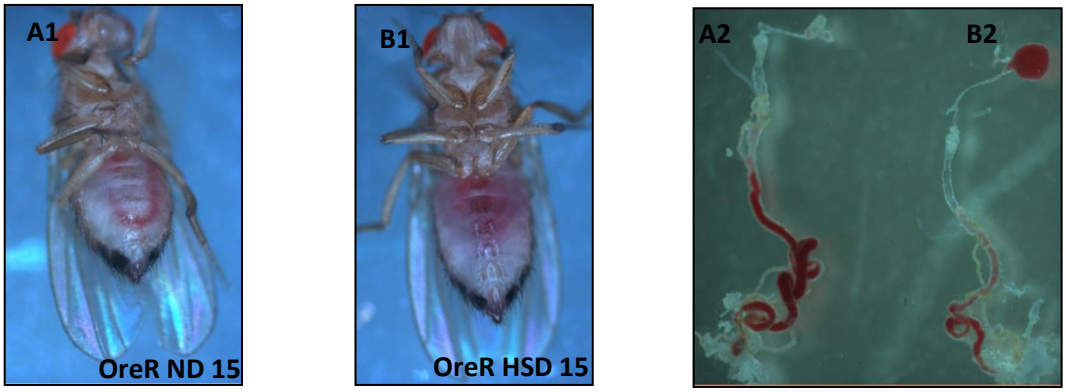

Figure 10: SMURF ASSAY- ND and HSD flies are SMURF negative until Day 15

Both (A) ND and (B) HSD guts are SMURF negatives. (**A1, B1**) There is no dye coloration on abdomen or other body parts. (**A2, B2**) dissected gut images show, in ND food is more in midgut due to increased width and in HSD food is less at midgut, but more at crop. Feeding needs to be quantified.

Staining of the gut with antbodies against Dlg revealed that the reduction in width was achieved due to a significant shrinkage in cell size.

The cell tight junction marker had a more intense expression in the gut of HSD fed flies. This could be due to compaction of cells and cell matrices or increased expression of these protein products or both. This result implies that HSD enhances cell membrane integrity and diminishes the gut epithelial cell size (**Figure 11 A'-B', C'-D'**).

1.4 High Sugar Diet results in increased Cell death in midgut – TUNEL Assay

Through cell membrane markers we found that one possible reason for depletion in gut size might be the reduction in cell size. Next, we wanted to check the survivability of the cells under HSD. For this purpose, we analyzed cell death in the midgut with TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) Assay. As expected, the number of cells undergoing apoptosis furthered in HSD fed fly guts. (**Figure 12**).

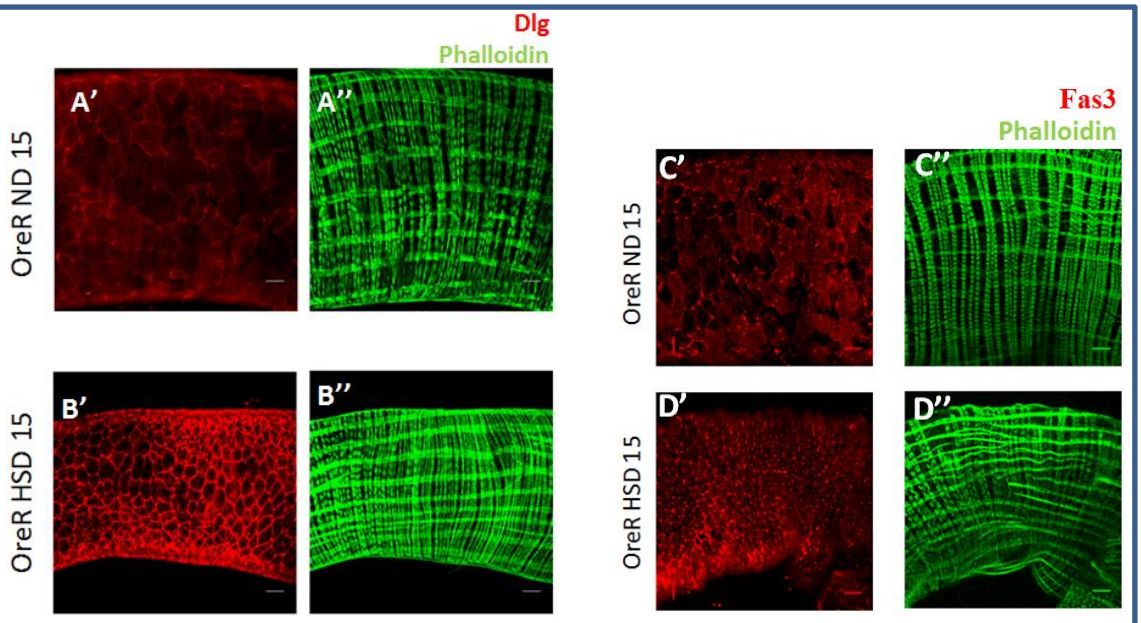

Figure 11: High Sugar Diet enhances cell membrane integrity and diminishes cell size in the midgut. Scale bar-20µm

Expression of cell tight junction marker **Dlg** in (**A'**) ND (**B'**) HSD midgut. HSD caused reduction in cell size and the increased intensity of **Dlg** marker indicates enhancement of cell membrane integrity. Another cell membrane marker **fas3** used to verify the results (**C'**, **D'** corresponds to ND and HSD midgut respectively). (**A'', B''.C'', D''**) **phalloidin** staining

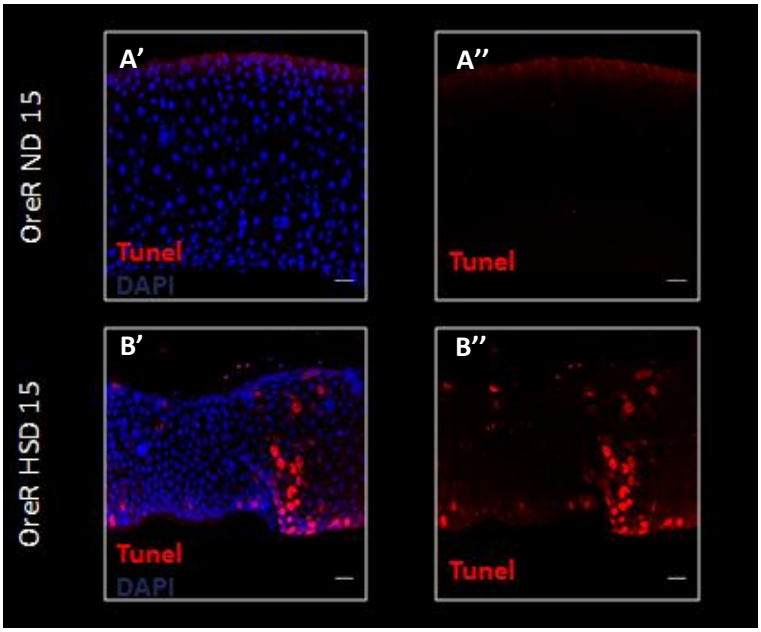

Figure 12: High Sugar Diet promotes apoptosis in the midgut.

Scale bar-20µm.Cell death was detected using TUNEL assay. (A'') ND midgut cells undergo rare and isolated cell deaths whereas (B'') HSD midgut undergo large number of cell deaths.

(A'& B' ND & HSD) stained with both **DAPI** and **Tunel**

1.5 High Sugar Diet affects gut homeostasis by upregulating Notch and downregulating JAK/STAT signaling activity in the midgut.

There are several signaling pathways involved in the maintenance and differentiation of Intestinal Stem Cells (ISCs). Wnt, Notch, JAK/STAT, JNK, EGFR, etc. are some well-studied pathways among them. For understanding the reason for altered structure in organ and cellular levels, we used different transgenic lines to analyze Notch and JAK/STAT signaling. Here we show the effect of HSD on these two pathways using expression status of NRE-GFP, *upd3*-Gal4>UAS-GFP/Cyo and *upd3*-LacZ/Cyo; Δr/TM6B, Tb (supporting data).

Using Notch pathway reporter NRE-GFP, we observed that the number of GFP+ cells increased in the midgut of 1M sugar batch compared to control batch. (**Figure 13**)

Using the *upd3*-Gal4>UAS-GFP/Cyo genetic construct, which reports the expression of cytokine ligands activating JAK/STAT pathway, we found a decrease in their expression, and thus JAK/STAT signaling (**Figure 14 (1)**). Downregulation of JAK/STAT activation was further confirmed using *upd3*-LacZ/Cyo; Δr/TM6B, Tb (**Figure 14 (2)**).

Also, we also checked the expression of *upd1*-LacZ in the gut. Bur fluorescence was faintly detected in both ND and HSD gut.

1.6 High Sugar Diet causes a drastic reduction in the quantity of gut commensal bacteria

Diet is known to affect the gut microbiome population and diversity. Observing other characteristic changes due to HSD, we hypothesized that it might have impacts on the gut commensal bacteria as well. We cultured commensal bacteria from the whole gut on LB agar plates. Steps followed for culturing is provided in the methods section. Under given conditions, after 3-5 days of culturing, we found a significant decrease in the bacterial population on HSD-LB agar plates (**Figure 15**). We quantified this result by calculating CFU (Colony Forming Units), and there is almost ninety percent reduction in colonies. This observation indicates that HSD decreases the total quantity of gut commensal bacteria.

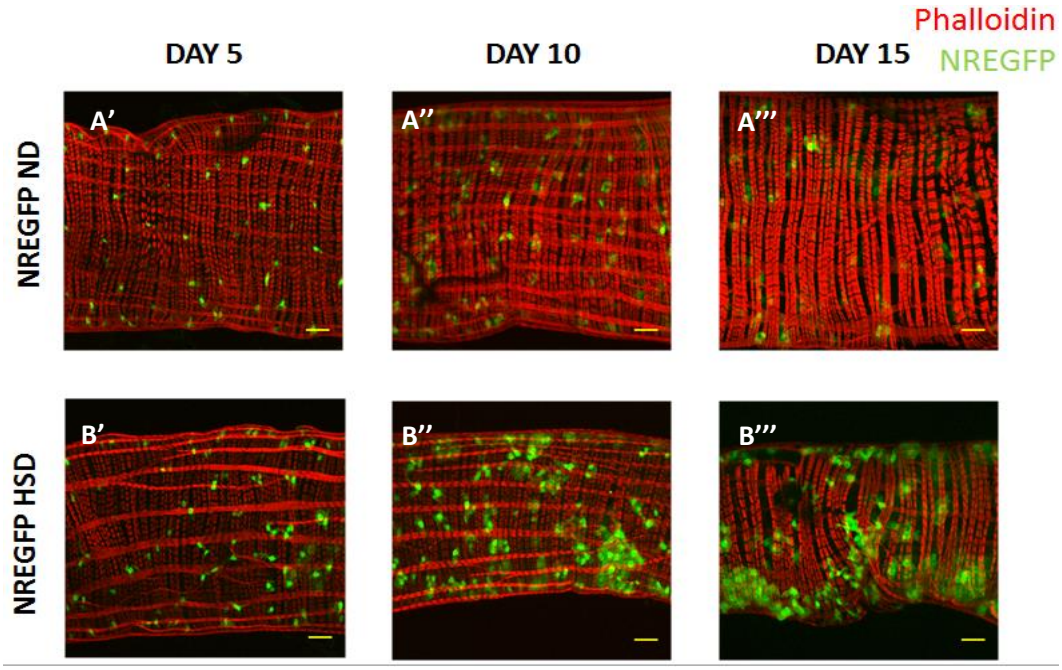

Figure 13: High Sugar Diet gradually induces the Notch pathway.

Scale bar-20µm. Expression pattern of **NREGFP** reporter line in ND (A) and HSD (B) posterior midgut on (A',B') Day 5, (A'',B'')Day10, and (A''',B''') Day 15. HSD midgut shows gradual increase in the expression while expression in ND gut remains unchanged.

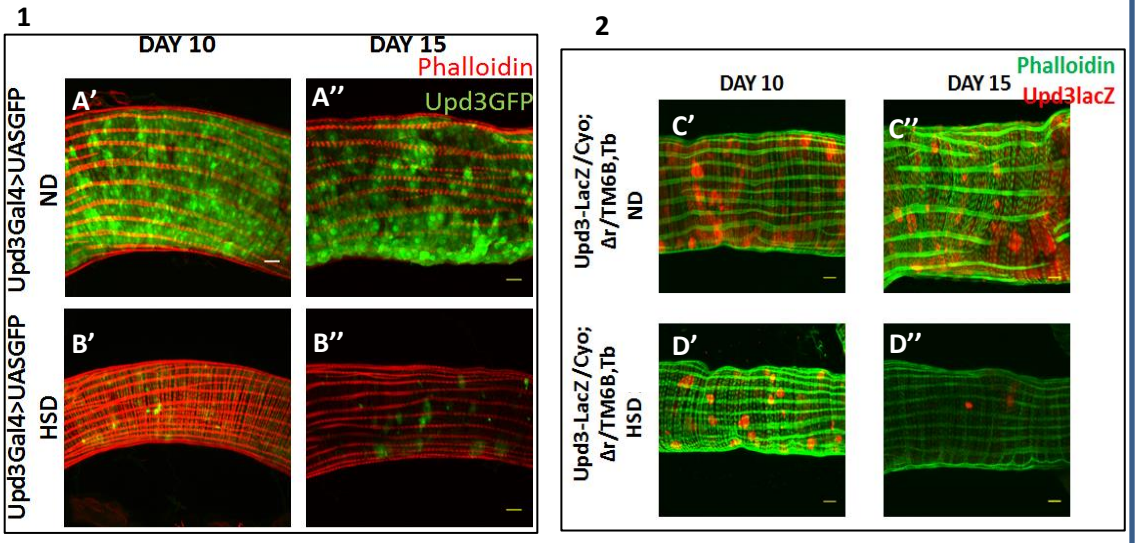

Figure 14: High Sugar Diet causes downregulation of upstream ligands of JAK/STAT signalling.

Scale bar-20µm. JAK/STAT pathway activation can be deduced by studying the expression of upstream Upd ligands. Upd3 levels in (A) ND (B) HSD midgut analysed using ***Upd3*Gal>4UAS-GFP** (along with **phalloidin** staining) on (**A',B'**) Day 10 and (**A'',B''**) Day 15 and observed decreased expression. This findings verified using another reporter- ***Upd3*-LacZ** (along with **phalloidin** staining) on (**C',D'**) Day 10 and (**C'', D''**) Day 15, (where C'.C'' ND and D'.D'' HSD)

1.7 High Sugar Diet lessen the expression levels of anti-microbial peptides (AMPs) in the midgut

According to our previous results, the gut commensal bacterial population was affected by HSD feeding. Host's microbiome and immunity are correlated. So we wanted to extend our studies into immune pathways further. IMD and Toll pathways are two crucial immune pathways in *Drosophila*, respectively *Diptericin* and *Drosomycin* are the anti-microbial peptide end products of these pathways.

We analyzed the status of these pathways using the Dro-GFP-Dpt-lacZ reporter. As expected the expression was affected in HSD midgut(**Figure 16**). This result implies that the High Sugar Diet decreased the expression level of anti-microbial peptides-*Drosomycin* and *Diptericin*. It suggests that immune pathways got affected by feeding flies onHigh Sugar Diet.

1.8 Alterations in gut structure get rescued by shifting the flies back to ND from HSD

After finding above mentioned variations in the gut due to High Sugar Diet, we next wanted to carry out rescue experiments. Feeding the flies back on Normal diet after maintaining them on HSD for 10 days was able to partially rescue the defects with gut size within 5 days (**Figure 17**).

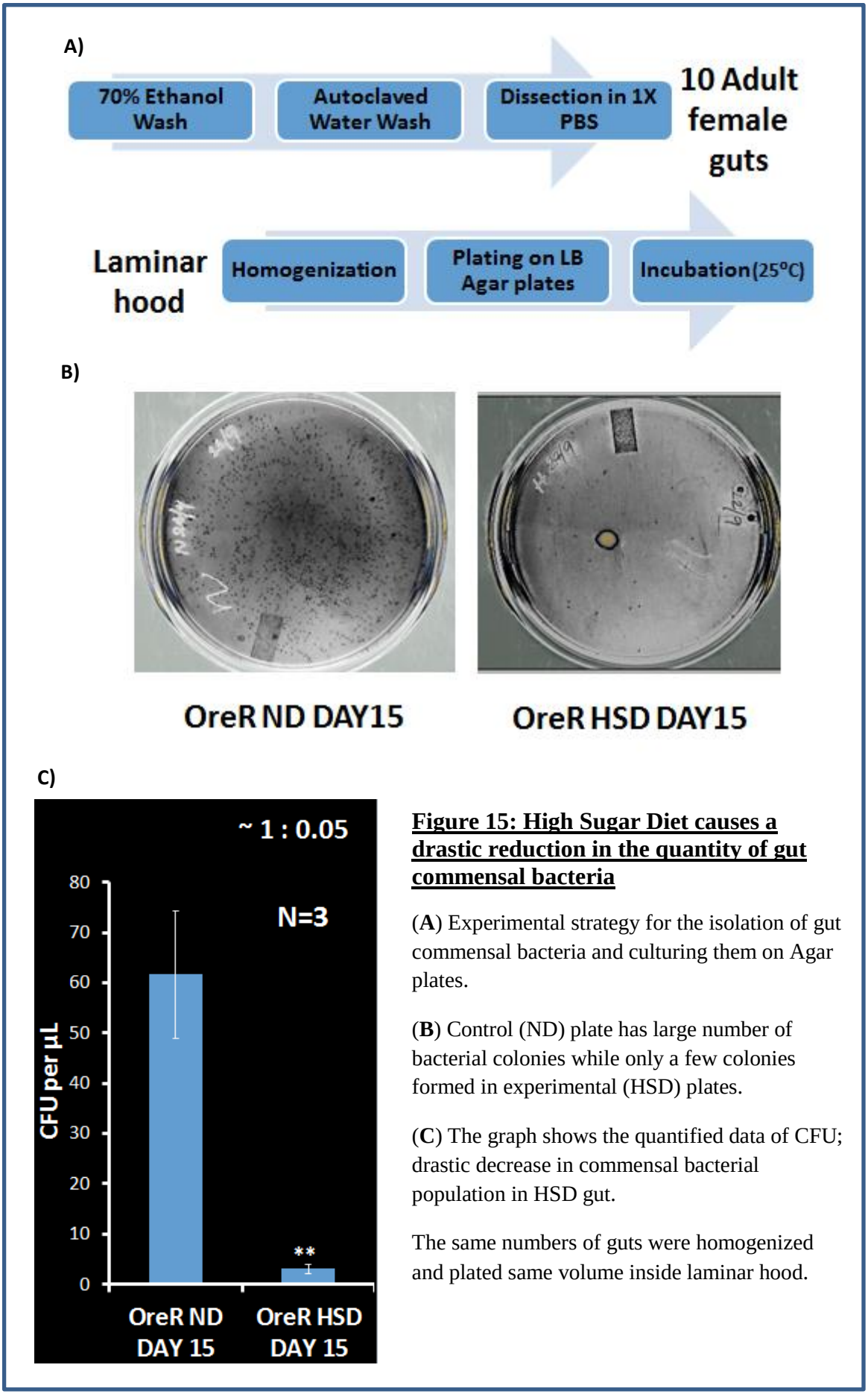

Figure 15: High Sugar Diet causes a drastic reduction in the quantity of gut commensal bacteria

(**A**) Experimental strategy for the isolation of gut commensal bacteria and culturing them on Agar plates.

(**B**) Control (ND) plate has large number of bacterial colonies while only a few colonies formed in experimental (HSD) plates.

(**C**) The graph shows the quantified data of CFU; drastic decrease in commensal bacterial population in HSD gut.

The same numbers of guts were homogenized and plated same volume inside laminar hood.

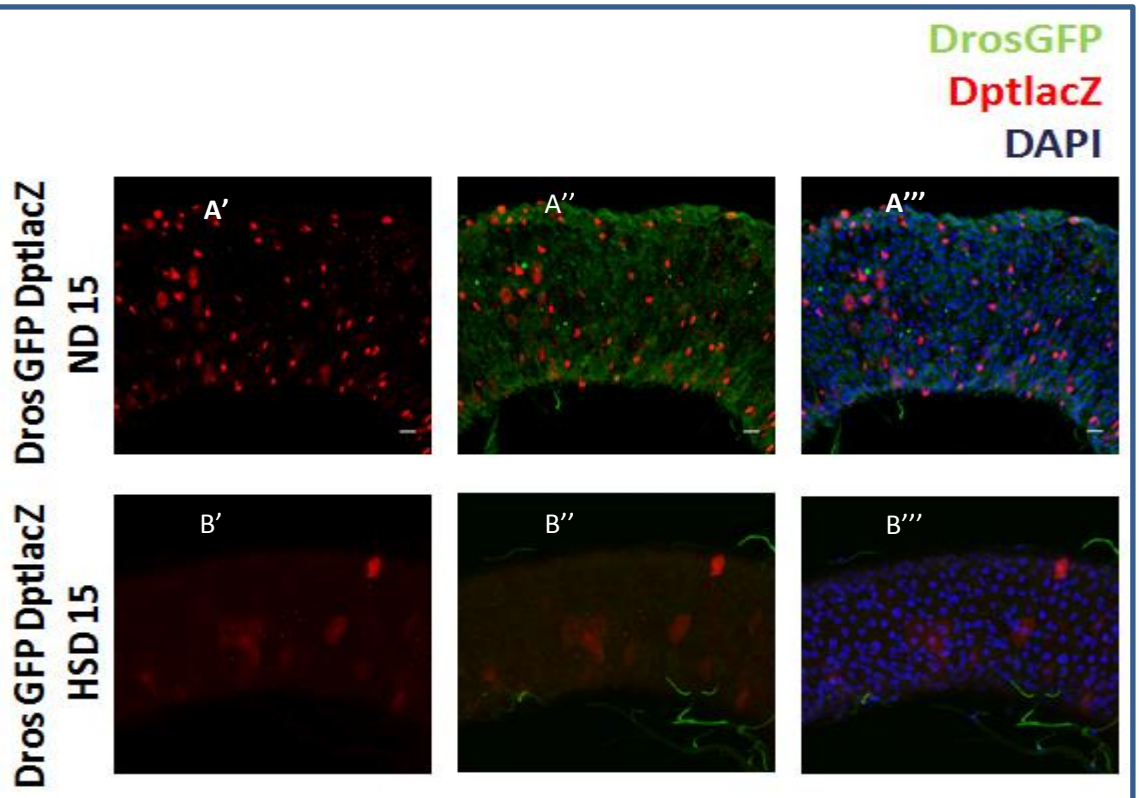

Figure 16: High Sugar Diet causes reduction in the expression levels of anti-microbial peptides (AMPs) in the midgut.

Scale bar-20µm. Expression pattern of AMPs (**A', B'**) **DptLacZ**, (**A'', B''**) **DrosGFP** and **DptLacZ** and (**A''', B'''**) **DAPI**, **DrosGFP** and **DptLacZ** in the midgut (ND, HSD). Their expression got affected and reduced in HSD midguts.

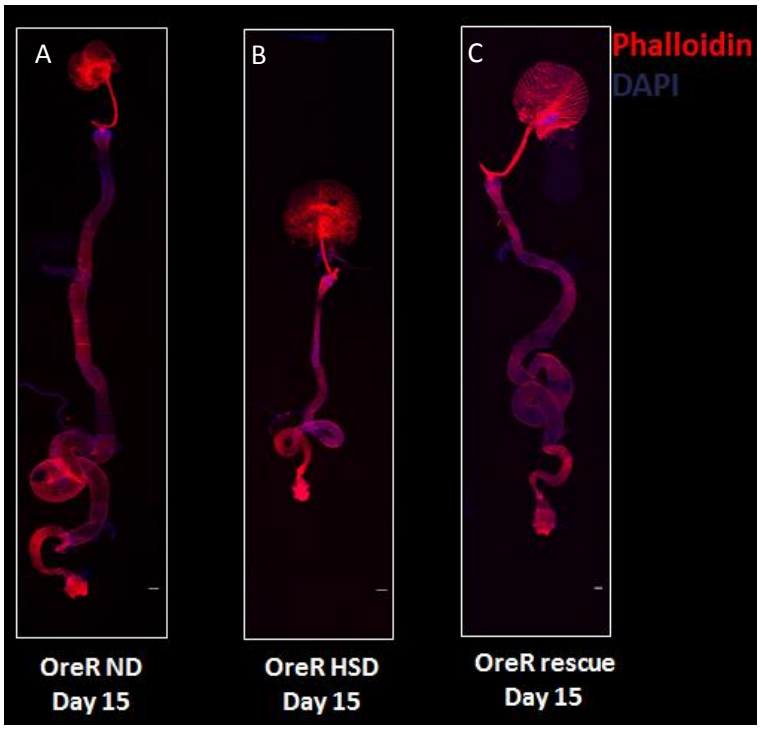

Figure 17: Alterations in gut structure get rescued by shifting the flies back to ND from HSD

Scale bar-20µm. Structural analyses with **DAPI** and **Phalloidin** staining of (**C**) Day 15 rescue gut, which fed on HSD for10 days and later transferred to ND for 5 days before dissection. Images (**A**) and (**B**) are Day 15 ND and HSD guts respectively. (**C**) Rescue is similar to (**A**) ND with exception of crop size similarity with (**B**) HSD gut

Discussions

Nutrition and health are correlated. Unhealthy diets can generate diseases. A high sugar diet can cause a variety of abnormalities including Type 2 diabetes (T2D) and obesity. The first contact of food happens in the gut. Other than being a tube just for digestion, the gut is becoming a main regulator of multiple biological processes. Through our study, we are trying to demonstrate the impacts of High Sugar Diet on gut and gut homeostasis.

To start with, we observed that the guts of the HSD fed flies are short and thin. There might be two possible reasons for this effect- (a) reduced cell size and (b) increased cell death. Our results demonstrate that there is increased cell death and the gut cells also shrink in size. However, it's also possible that these cells do not undergo normal proliferation. This aspect need to be addressed. Existing literature also indicate that HSD has variable effect on different cells types of the midgut [42] that needs to be studied in detail.

We have checked the functional effects related to gut permeability of high sugar diet in addition to cell tight junction staining using *Dlg* on gut, we performed smurf assay and all the control and experimental flies were smurf negative until day 15. Both these results demonstrate absence of functional defects due to leakage and enhanced cell membrane integrity in flies fed on high sugar diet [40].

The JAK/STAT pathway promotes ISC proliferation while the notch pathway often dictates ISC differentiation [28][1]. Our results of reduced JAK/STAT signaling and enhanced Notch signalling indicates that HSD might disrupt the intestine homeostasis by promoting ISC differentiation and depleting ISCs. To check this hypothesis different cell type markers are to be used to identify the status of each cell types. Also studying the other signaling pathways such as Wnt, EGFR, JNK, etc. involved in maintaining gut homeostasis would benefit to get an overall estimation of HSD effects on the gut.

Major defense mechanisms reported in the gut are via expression of anti-microbial peptides (AMPs) and reactive oxygen species (ROS). They have complementary duties- AMPs encounter ROS resistant bacterial (pathogen) species. The resident gut microbiome is also known to fight with external pathogen's entry.

Through our results demonstrate a sharp decline in the number of gut commensal bacteria and subsequent reduction in the expression of genes coding for AMP, to understand the effect of HSD on immunity, it would be intriguing to check their levels even under infection conditions.

The role of the gut microbiome in host physiology, development and immunity is a very relevant topic among researchers for a long time. But still many molecular mechanisms behind host and microbiome crosstalk are unknown. Since *Drosophila* has relatively simple microbiome consisting of only 1-30 taxa, there is growing interest to use the fly model to study and elucidate such mechanisms [38].Fruitfly lacks vertical microbiome transfer like humans. The (gut) microbial populations solely depend on the diet for its establishment and maintenance. Many studies are showing the importance of microbiome maintenance and some tools for replenishing them into a healthy equilibrium state [47][48]. For example, manipulating the microbial populations at different life stages for rescuing cancer or immune deficient phenotypes [49][50][51][52][53]. Gut microbiota is also known to affect fly's germline [54]. Our results with affected gut commensal bacteria and connected immune pathways also provide possibilities of rescuing the HSD phenotype by manipulation of the gut microbiome.

Even though our experimental model resembles T2D only after 13 days (threshold period) on HSD, the hyperglycemic (sugar levels in the blood remains higher than usual) condition is affecting the intestine from a very early period. Interference at this prediabetic state could help to rescue the phenotype. One rescue experiment with the strategy of shifting back to a normal healthy diet showed desired results with partial phenotypic recovery. In this case, the rescue of signaling pathways and cell death needs to be done. The rescue experiments before and after diabetic condition will provide more estimate about the period of intervention.

The High Sugar Diet fed flies laid less number of eggs compared to Normal Diet fed flies. 2-3(data not quantified) days of lagging (in HSD batches) observed in different developmental stages also. The entire body size also got reduced progenies. These observations open up the potential of this model to study hereditary consequences. We further intend to extend our studies to other organs (ovary, eye, brain, etc.) and relate them with diabetic phenotype.

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