Bachelor Degree Project

UNIVERSITY OF SKÖVDE

# THE EFFECT OF PROBIOTIC BACTERIA ON HIGH SUGAR DIET INDUCED DIABETES TYPE-2 SYMPTOMS IN FRUIT FLY: WITH FOCUS ON LIPID METABOLISM

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# Abstract

As the prevalence of type 2 diabetes has increased globally, so has the need to further investigate the disorder's underlying features and a potential target for treatment. *Drosophila melanogaster* has proven to be an excellent model organism to study type 2 diabetes (T2D). To see whether it can serve as a model organism to identify the treatment of T2D, a type 2 diabetes like model were created in *Drosophila t*hrough high sugar diet (HSD). The aim of the study was to investigate the therapeutic effects of six different probiotic bacteria strains on T2D characteristics markers.

Longevity, size, and weight measurement were performed. Followed by verification of HSD effects on these phenotypes. It was demonstrated that probiotics could improve weight and lifespan. Treatment using probiotics showed statistically increased body weight in both 3$^{rd}$ instar larvae and adult flies (p-value <0.05). There was no statistically significant difference in length between any of the groups including controls (p-value 0.25). However, the triglyceride assay showed a slightly significant difference between control low sugar diet and few of the treatment groups (*L. paracasei* with p-value 0.037, and *L. acidophilus* with p-value 0.025) fed larvae/adult flies, and there was no statistically significant difference between controls (high and low sugar diets), and probiotics treatment groups (*L. plantarum, B. animalis and B. breve)* fed larvae/adult flies with a p-value >0.05.

To investigate the effect on gene expression of four genes (FASN$^{CG3523}$, *FASN$^{CG3524}$*, *FASN$^{CG17374}$* & *dsREBP)* important in lipid metabolism, qPCR was performed using the Taqman method. All probiotic treatment groups had significantly decreased gene expression of *FASN* compared to the control groups. Findings of this study suggest that *Drosophila melanogaster* can be utilized as a model organism to study T2D and that further studies concerning the effects of probiotic treatment in Drosophila are required to fully understand the interactions and mechanism of action.

# Popular scientific summary

Type 2 diabetes (T2D) is a metabolic disorder characterized by insulin resistance and hyperglycemia. During the initial stage of T2D development, the body experiences high levels of glucose in the blood, beta cells in the pancreas produce sufficient insulin to maintain euglycemia. The production of insulin becomes ineffective for adequate glucose metabolism when part of the insulin-dependent glucose transport is dysfunctional (GLUT-4), therefore causing insulin resistance to glycogenesis in muscle cells. The dysfunction of GLUT-4 may be due to either been inherently present or persistent hyperglycemia (Vargas & Carrillo Sepulveda, 2019). Impairment of β-cell function and insulin resistance must occur simultaneously for type 2 diabetes to develop. An overweight/obese individual usually has some sort of insulin resistance, but diabetes can only develop in individuals who lack an adequate amount of insulin secretion to match the level of insulin resistance. Although, insulin in that individual may be high, but yet, it is not enough to normalize the glycemic level. Treatment of type 2 diabetes currently consists of insulin injection and oral hypoglycemic agents, and more studies are required to uncover the safety and side effects of these agents.

Obesity is often linked to a lot of medical, social and psychological conditions, and the most prevalent of which may be type 2 diabetes. The prevalence of this devastating disease is currently increasing; until recently, T2D diabetes was traditionally only seen in adults, the disease has now begun to appear in children. Type 2 diabetic patients normally represent patients that have had a long progression, which is initially suffering from metabolic syndrome and being obese/overweight for many years. The human gastrointestinal (GI) tract host trillions of microorganisms, this includes thousands of bacterial species, which affects a large number of biological functions as well as metabolism in humans.

Type 2 diabetes can be manageable and therefore, understanding the mechanism behind the development of any metabolic disease may help to decrease the number of death caused by it. The whole body and glucose metabolism is regulated by a series of interaction between organs and tissues. The human brain relies on glucose as its main source of energy. Glucose metabolism provides the fuel for the physiological function of the brain via the production of ATP and generation of neurotransmitters. Therefore complications in glucose metabolism are at the core of many metabolic disorders, including obesity and type 2 diabetes.

There are few studies showing the metabolic function of probiotics in humans and high sugar diet in animal models such as Drosophila and rodents, but this research is the first one focusing on the effect of probiotics in fruit-fly. The method used in this research is raising *Drosophila melanogaster* on high sugar diet after addition of bacterial probiotic strain and then conducting different experiments. This enables the comparisons between normal healthy individuals with those with T2D or loss of function of intestinal microbiota. The experiments conducted were tailored to investigate if the diet and treatment will reverse the symptoms of type 2 diabetes. The main findings are that an HSD diet decreases the weight and that probiotic bacteria improve the weight loss caused by hyperglycemia by managing insulin resistance and energy regulatory pathways. Suggesting that probiotic bacteria may have the potential effect in alleviating metabolic disorders. Another finding was that probiotics decrease the expression of *FASN* involved in the fatty acid synthesis. Therefore the effects of probiotics on high sugar diet is of interest as an improvement of glucose uptake by peripheral tissues and fatty acid clearance from the circulation would be beneficial in the context of many metabolic diseases.

# Abbreviations

| | |
|---|---|
| Sterol regulatory element binding protein | SREBP |
| Fatty acid synthase | FASN |
| Acetyl-coenzyme A carboxylase | ACC |
| Glucose transporter 4 | GLUT4 |
| Pathogen-associated molecular patterns | PAMPs |
| Pattern recognition receptors | PRRs |
| Toll-like receptors | TLR |
| Real-time quantitative PCR | RT-qPCR |
| Complementary DNA | cDNA |
| Gastrointestinal tract | GI |
| High sugar diet | HSD |
| Low sugar diet | LSD |
| Triacylglycerol | TAG |
| Beta-cells | B-cells |
| Lipopolysaccharides | LPS |

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# Introduction

Diabetes has become one of the most prevalent chronic diseases globally that affects about 8.5% of the adult population, which is nearly 422 in 2014. It's a condition in which either the pancreas is unable to produce enough insulin (type 1) or the body becomes insensitive to the insulin it produces (WHO: Global report on diabetes, 2016). In most cases, metabolic disease arises due to impaired cellular glucose uptake. Glucose is essential for the production of energy in most tissues and its homeostasis is controlled by a highly regulated system that involves different organs such as skeletal muscle, liver, and pancreas (Galgani et al., 2008). After a meal β-cells in the pancreas release insulin, which stimulates the uptake of glucose by peripheral tissues for the production of energy and also decreases the endogenous hepatic glucose production (Karlsson, et al., 2007). Excess glucose is stored primarily in the liver and skeletal muscles as glycogen, free fatty acids are stored as triglycerides mainly in adipose tissue but also in the liver (Graham & Pick, 2017).

At the onset of type 2 diabetes tissues become resistant to the hormone insulin and the circulatory levels of glucose increase. Insulin resistance is a situation whereby insulin induced-glucose uptake is malfunctioning in insulin-sensitive tissues (such as skeletal muscle, adipose tissues, and liver), this is as a result of inhibition of insulin signaling pathways. It has been shown that insulin resistance leads to hyperinsulinemia when pancreatic beta cells increase the production of insulin in an attempt to control the level of glucose in the blood (Ye, 2013). It has been reported that the onset of type 2 diabetes is associated with both genetics and environmental factors like physical inactivity and diet (Karlsson, et al., 2007). It has been shown that an elevated level of glucose leads to hyperglycemia. The chronic hyperglycemia resulting from insulin resistance gives rise to serious life-threatening complications such as stroke and heart attack, retinopathy, neuropathy and kidney failure (Karlsson et al., 2007).

Fat reserves are strictly regulated to meet the required energy without exceeding a maximum adiposity threshold. Obesity is an increased accumulation of adipose fat and presents a high risk of developing many serious health problems. Patients with genetic or environmentally induced excess fat storage also exhibit insulin resistance, cardiovascular disease, and hyperglycemia, all hallmarks of metabolic syndrome. Non-alcoholic fatty liver, neuropathy, and retinopathy are also symptoms. In Drosophila, most of the assimilated nutrients are transferred into the fat body to be metabolized and stored as triglycerides. Triacylglycerols (TAG) are the main fat storage form in the fly, as in humans. Therefore, quantification of TAG content is mainly used to define obesity in flies (Musselman & Kühnlein, 2018). Triacylglycerols (TAG) are the main fat storage form in the fly, as in humans. Therefore, quantification of TAG content is mainly used to define obesity in flies (Musselman & Kühnlein, 2018).

Type 2 diabetes represents about 90% of all cases of diabetes, physical inactivity and obesity all have been shown to be the major risk factors of type 2 diabetes, therefore, diet changes, modifications of lifestyle and exercise are the most common and effective treatment for type 2 diabetes (Stolar, 2010). However, the risk of developing type 2 diabetes may also involve environmental factors, particularly the collection of microorganisms which habitat the intestine (Delzenne et al., 2015).

The human gastrointestinal (GI) tract is one of the largest interfaces between the environmental factors and the host cells in the human body. The gut microbiota is the collections of archaea, bacteria, and eukaryotes that colonize the GI tract, the gut microbiota has co-evolved with the host over a decade to form an intricate and mutually beneficial relationship. The number of microorganisms that inhibits the GI tract is more than $10^4$, that is 100 times higher than the number of genomic content

(microbiome) as the human (Thursby & Juge, 2017). The gut microbiota plays a key role in maintaining the intestinal homeostasis, this includes nutrients metabolism, the synthesis of vitamin B12 & vitamin K, xenobiotics metabolism and normal commensal bacteria that inhibit pathobiotic invasion and maintain barrier function. The intestinal microbial composition changes significantly from birth to adulthood. Changes in the microbiota are found to be co-related to metabolic syndrome and systemic inflammation, such as non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, children's dietary behavior and colitis (Tsai et al., 2019).

Gut microbiota has the ability to communicate with the host through specific cell membranes or related molecules that can activate pattern recognition receptors (PPRs). PPRs are mainly involved in the recognition of molecular patterns, called pathogen-associated molecular patterns (PAMPs) which are specific to bacteria and other microorganisms. The most common PPRs are the TLRs. It was believed that the stimulation of TLR-4 by bacterial lipopolysaccharides (LPS) causes an inflammatory response, production of cytokine and chemokine-mediated recruitment of acute inflammatory cells. It has been revealed that the microbiota also contributes to the onset of type 2 diabetes and insulin resistance through mechanisms associated with an increased level in plasma LPS, termed as metabolic endotoxaemia. In previous studies, it has been revealed that in mice model, type 2 diabetes and obesity, metabolic endotoxaemia was associated with alteration in the composition of the gut microbiota and with increased intestinal permeability (Delzenne et al., 2015).

Previous studies conducted using mice model show that a high-fat diet causes an increase of certain gut bacterial species which generate a higher amount of lipopolysaccharides, therefore leads to the progression of insulin resistance. It has also been shown that gut microbiota contributes to glucose homeostasis through bacterial metabolites (Yao et al., 2017). It has been demonstrated in the previous study that administration of prebiotic (*A. muciniphila)* in genetically and diet-induced obese mice, provides beneficial effects on glucose/lipid metabolism by abolishing metabolic endotxaemia and also reduced body weight and improved body composition (Everard et al., 2013).

The probiotics have gained increasing interest for its health benefits. Probiotics refer to a live organism that has a beneficial role in health and disease (Zhang et al., 2016). International Scientific Association for Probiotics and Prebiotics (ISAPP) revealed that the spectrum of products that can be classified as probiotics comprises both beneficial bacteria, drugs and enteral feeding for the amelioration of a disease, a supplement of food for the promotion of health benefit, infant formula such as powdered milk as well as animal feedings. Currently, probiotic definition shows a specific bacterial strain that can effectively enhance the health of humans. (Tsai et al., 2019). Many studies have shown the effect of probiotic bacteria in terms of lowering the blood glucose level, delaying the onset of hyperglycemia as well as diminishing the insulin resistance in diabetic rat. The effect of probiotic bacteria on glucose metabolism is probably due to their immune modulating properties. Some probiotic bacteria strains can enhance the composition and function of the intestinal microflora. This effect inhibits the bacterial endotoxins transport into the bloodstream and reduces the circulation of lipopolysaccharide and pro-inflammatory cytokines, this decrease inflammation. Therefore, probiotic bacteria decrease insulin resistance and preserve β-cells more efficiently (Rezaei et al., 2017).

*Drosophila melanogaster* is particularly an ideal model for obesity and metabolic disease because flies contain organ, tissues, and system that are analogous to all those found to be involved in human obesity and associated metabolic diseases. Beside that *Drosophila* also develop obesity and its

complications during the high caloric diet. Furthermore, most genes and gene families involved in metabolic disease are conserved between humans and flies (Musselman & Kühnlein, 2018). However, in comparison to mammals, *Drosophila* can also be used to study the governing principles of the host's metabolic interaction with its microbiota. Humans are exposed to the diverse microbiotas that populate a wide range of plant and animal food sources, whereas *Drosophila* has a more limited natural food source of rotten fruits, vegetables, fungi, and decomposing plant. The intestinal microbiota of the fruit fly consists of five to 30 taxa in comparison to 500 taxa in the human intestinal microbiota (Wong et al., 2016).

Studies revealed that most mouse, worm or fly models of type 2 diabetes depend on the manipulation of a single gene such as those encoding leptin, the insulin receptor or other genes that provides insights into specific pathways of insulin resistance. Flies provides the ability to dissect the relative contribution of both gene and environment on metabolism. As stated before, Drosophila contains organs that are analogous to most of those associated with both human energy metabolism as well as the targets of diabetic complications, such as kidneys, heart, brain, liver and adipose tissue, GI tract and blood (hemolymph). However, in humans, the monogenic form of type 2 diabetes is rare. In rodents, high calorie-diet are frequently been used to enhance insulin resistance in mutant models, (Musselman et al., 2011).

*Drosophila melanogaster* can serve as an ideal model organism for the pathology of type 2 diabetes as well as a model to analyze the therapeutic effects of different types of probiotic strains. Based on the evidence that probiotics enhance health when consumed by improving gut flora, the aim of the study was to investigate the effect of probiotic bacteria on high sugar metabolism in the fruit fly. The effect of the high sugar diet and probiotics was examined considering the hypothesis that probiotic bacteria have a positive effect on glucose homeostasis in humans with type 2 diabetes and in animal models. The probiotics used in this research include *L.paracasei, L. plantarum, L. acidophilus, B. animalis, B. breve and B. lactis.*

Quite a number of trials has been performed in humans and probiotics was found to be potential benefits in patients with type 2 diabetes. However, based on the hypothesis that a high-sugar diet increases fat storage in *Drosophila,* one of the goals was to investigate and measure the triglycerides in the fat body.

Studies have shown that insulin is an important regulatory hormone in de novo lipogenesis. It stimulates the uptake of free fatty acid primarily by adipose tissue and liver, as well as the conversion of free fatty acid into triacylglycerol. The plasma level of free fatty acid is usually elevated in fasting condition and decreased in physiological condition after a meal. High level of free fatty acid is usually associated with hyperlipidemia, which is one of the risk factors of insulin resistance. Hyperlipidemia includes an elevated level of free fatty acid, cholesterol, and triacylglycerol in the plasma, which is normally downregulated by insulin. Loss of response to insulin by adipose tissues leads to an elevated level of free fatty acid and triacylglycerol in the plasma (Ye, 2013).

Fatty acid synthase *(FASN)* involved in the fatty acid synthesis. In coordination with Acetyl-CoA carboxylase (ACC), *FASN* determines the lipogenic flux from malonyl-CoA into palmitate. The *FASN* gene expression is primarily regulated by hormonal and nutritional signals, and insulin increases the rate of *FASN* gene transcription in murine cell lines as well as in primary human adipocytes. Insulin also increases human *FASN* enzymatic activity and *FASN* gene expression, therefore enabling the increase

of energy storage as fat (Fernandez-Real et al., 2010). It has been shown that in larvae, ACC is required for the synthesis and storage of TAG in the fat body, an insect organ with adipose and hepatic function (Garrido et al., 2015). Conversely, *FASN* is markedly downregulated under the condition of insulin resistance. Therefore, we hypothesized that the expression of *FASN* genes will be highly expressed in the control group affected with T2D symptoms and downregulated in the treatment group that had an improvement in insulin sensitivity.

To assess this finding, Quantitative real-time PCR (RT-qPCR) was conducted to analyze the different gene expression level of *FASN*. These genes include CG3523, CG3524, CG17374 (correspond to human *FASN,* fatty acid synthase), and *dsREBP* (correspond to human *SREBF1*, stearoyl-CoA desaturase-1) *sREBPs* are membrane-bound transcription factors that are found in all animals, and is one of the proteolytic signaling pathways that play a key in the regulation of lipid metabolism (Amarneh et al., 2009).

*RPL32* was used as a reference gene. Earlier studies demonstrated that in larvae, *FASN*$^{CG3523}$ (*FASN1*) is expressed in all tissues, and is induced by sugar diet, and also animals with *FASN*$^{CG3523}$ (*FASN1*) deficiency are extremely sensitive to a moderate increase in sugar diet. While *FASN*$^{CG3524}$ (*FASN*2) and *FASN*$^{CG17374}$ (*FASN3*) are essentially expressed in the carcass, which includes epidermal cells, skeletal muscle, and oenocytes (Garrido et al., 2015). Studies also show that insulin-induced SREBP-1 activation and the glucose-induced *ChREBP* activation act synergistically to promote *FAS* expression (Jensen-Urstad & Semenkovich, (2011).

# Aims

The main objectives of this study were to investigate the therapeutic effects of a high sugar diet and six different probiotic bacteria strains on type 2 diabetes characteristics markers using *Drosophila melanogaster* as a model organism.

# Materials and Methods

Below describe the type of fly strains used, how eggs were collected and incubated on different types of diet for further development.

## *Fly strain and feeding*

The fly strain used in this study was White w1118 (Bloomington stock center). Wild type *Drosophila melanogaster* (both males and females) were caged and incubated at 25℃ for 24 hours (to mate and lay eggs), using egg collection cage containing apple juice plate, yeast and water were also mixed and paste on to the surface of the apple juice plate. Eggs were collected and incubated at 25℃ on food containing different types of diet (low sugar diet, high sugar diet, and high sugar diet + probiotics) for development. Low sugar diet (LSD) contained 0.15 M of sucrose, high sugar diet contained 1 M of sucrose with all other ingredients kept constant (Appendix 1). Treatment includes 0.005g, and 0.025g of freeze-dried probiotics respectively, which was spread on top of the food before adding the eggs.

## *Probiotic Strains*

Six different freeze-dried probiotic strains, which include *L. paracasei, L. plantarum, L. acidophilus, B. animlais subsp lactis, B. breve, and B. lactis HN019* were used in the study.

## *Longevity study*

To test the lifespan, flies were transferred to the vial tubes containing different types of diet (LSD, HSD and HSD + probiotics) and incubated at 25℃. The number of dead flies was recorded every 2 days until all flies were dead. The food/diet and probiotics were changed once a week. Each vial contained 20 flies, and each lifespan assay was repeated twice.

## *Whole body and lipid measurement*

Both 3$^{rd}$ instar larvae and adult flies were collected and weighed on the scale (25 larvae, 30 adult flies, 15 males, and 15 females from each sample). To measure the triglyceride content, 5 3$^{rd}$ instar larvae or 5 adult flies were homogenized in a 1.5 ml Eppendorf tube (first tube larvae and the second tube adult flies) containing 100µl of cold PBS + 0.05% tween 20 (PBST). Homogenate was further diluted to two-fold dilution (50µl homogenate and 50µl of PBST). Then glycerol standard solution of 1.0mg/ml, 0.5mg/ml, 0.25mg/ml and 0.125mg/ml were prepared. To obtain 1.0 mg/ml triolein equivalent standard, 40µl of the glycerol standard solution (Sigma 2.5 mg/ml triolein equivalent glycerol standard; G7793); were diluted with 60µl of PBST to a final volume of 100µl. Then 2-fold dilutions were carried out into PBST to obtain 0.5mg/ml, (50 µl of 1.0mg/ml + 50 µl of PBST), 0.25mg/ml (50 µl of 0.5mg/ml + 50 µl of PBST) and 0.125mg/ml (50 µl of 0.25mg/ml + 50 µl of PBST) respectively.

Thereafter, 20µl of the prepared glycerol standard, fly sample, and a PBST blank were added to the 1.5 ml microfuge tube. Then 20µl of triglyceride re-agent (sigma; T2449) were added to one tube to free the glycerol backbone and then 20µl of PBST was added to the other tube to measure free glycerol. The tubes were then incubated at 37℃ for about 30-60 minutes, followed by centrifugation for three minutes at full speed. Next, 30µl of each sample were transferred to a 96-well plate. One row of the plate had PBST, whereas the second row had triglycerides.

Each sample was then mixed by the addition of 100µl of free glycerol reagent (Sigma; F6428). Wells were sealed with parafilm to prevent evaporation and then incubated for five minutes at 37℃, then followed by centrifugation in a swing bucket-rotor to clear condensate and remove air bubbles that are present in the samples. Then absorbance was measured at 540 nm (Tsai at al., 2019).

Finally, to determine the Triacylglycerol (TAG) concentration for each sample, absorbance for the free glycerol in the untreated samples were subtracted from the total glycerol concentration in samples that have been incubated with triglycerides reagent.

## *RNA Isolation and purification*

The total RNA was extracted from the whole larvae and adult flies from two separate experiments using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol was carried out. The quality, purity, and concentration of the extracted RNA were measured using the Nanodrop spectrophotometer, this was done by measuring the absorbance (A) of the samples at a wavelength of 230nm, 260nm, and 280nm. Next, the sample was assessed using the A260/A280 ratio, where a ratio of ∼2 was considered pure, which is true for all samples (Table 1). The contamination of the samples was also assessed using the ratio of A260/A230 if the ratio is significantly lower than expected (2.0-2.2), it indicates that contamination might have been present in the sample (Nanodrop Technologies, 2007). However, there was no contamination present in the extracted RNA samples.

## *Reverse transcription PCR (RT-PCR)*

In this step, the isolated RNA was converted to Complimentary DNA (cDNA) using the High Capacity RNA to cDNA reverse transcription Kit (Thermo Fisher Scientific), by the process of reverse transcription. The reverse transcription was performed using a PCR machine according to the following temperature settings: 25℃ for 10 minutes, 37℃ for 2 hours and 85℃ for 5 minutes. 1000ng/µl of RNA sample was used for each reaction, and the amount used can be seen in Table 1 & Table 2.

Table 1. RNA concentration of adult flies, A260/30, A260/80 ratio and amount of RNA used for cDNA transcription

| Samples | RNA ng/l | A260/230 | A260/280 | Vol. of RNA | Vol. M-mix | H2O |
|---|---|---|---|---|---|---|
| **LSD** | 231.09 | 1.90 | 2.16 | 4.33µl | 5.8µl | 9.87µl |
| **HSD** | 164.66 | 0.57 | 2.17 | 6.07µl | 5.8µl | 8.13µl |
| **L. paracasei** | 280.06 | 1.19 | 2.02 | 3.57µl | 5.8µl | 10.63µl |
| **L. plantarum** | 299.84 | 1.71 | 2.16 | 3.34µl | 5.8µl | 10.86µl |
| **L.acidophilus** | 157.34 | 1.35 | 2.10 | 6.36µl | 5.8µl | 7.84µl |
| **B. animalis** | 138.43 | 1.03 | 2.12 | 7.22µl | 5.8µl | 6.98µl |

Table 2. RNA concentration of larvae, A260/30, A260/80 ratio and amount of RNA, master mix & water used for cDNA transcription

| Samples | RNA ng/l | A260/230 | A260/280 | Vol. of RNA | Vol. M-mix | Vol. H2O |
|---|---|---|---|---|---|---|
| **HSD** | 439.64 | 2.25 | 2.28 | 2.27µl | 5.8µl | 11.93µl |
| **LSD** | 637.20 | 2.02 | 2.30 | 1.57µl | 5.8µl | 12.63µl |
| **L. paracasei** | 580.22 | 1.85 | 2.22 | 1.72µl | 5.8µl | 12.48µl |
| **L. plantarum** | 280.08 | 1.32 | 2.24 | 3.57µl | 5.8µl | 10.63µl |
| **L.acidophilus** | 696.80 | 2.17 | 2.33 | 1.44µl | 5.8µl | 12.76µl |
| **B. animalis** | 501.23 | 2.35 | 2.28 | 1.10µl | 5.8µl | 13.1µl |
| **B. lactis** | 370.56 | 2.30 | 2.27 | 2.70µl | 5.8µl | 11.5µl |
| **B. breve** | 186.13 | 1.72 | 2.14 | 5.37µl | 5.8µl | 8.83µl |

## *Quantitative PCR*

qPCR was conducted by using Taqman® probe gene expression assay (Thermo Fisher Scientific) according to the following protocol: 1:40 Taqman probe dilutions were made using Milli-Q water. 2µl of the diluted probe were pipetted into 96-well qPCR plate and left to air dry or heated on the heat block for 1 hour at 50°C. The idea was to decrease the volume so we end up with 2µl of the samples mixed with Master Mix. cDNA dilutions were made according to Table 3. 2µl of the diluted cDNA mixed (1:1) with Taqman® Universal PCR Master mix (Thermo Fisher Scientific) was pipetted into the 96-well plate and finally, qPCR was run on a Pikoreal qPCR System (ThermoScientific). Each reaction was run in triplicate. The list of the specific gene probes used can be seen in Table 3. Δcq values were calculated by subtracting the Ct-value for the reference gene *RPL32*, and used for further statistical analysis.

Table 3. cDNA & Taq-probe dilution

| Taq-probe dilution | cDNA dilution for each sample | Master Mix + diluted cDNA |
|---|---|---|
| 3µl of each gene diluted 117µl of Milli-Q H2O | 1µl of cDNA diluted 19µl of H2O | 20µl of diluted cDNA mixed with Master mix |

Table 4. TaqMan probes used for qPCR, RPL32 was used as a reference gene.

| Fly gene name | Probe ID | Human gene name |
|---|---|---|
| ***CG3523*** | DM01801117_g1 | *FASN1* |
| ***CG3524*** | DM01834801_m1 | *FASN2* |
| ***CG17374*** | DM03420571_g1 | *FASN3* |
| ***dsREBP*** | DM01793857_g1 | *SREBF1* |
| ***RPL32*** | DM02151827_g1 | |

## *Statistical analysis*

Statistical analysis was done using Student t-test and one-way ANOVA using *Tukey* test for normal distribution to determine statistically significant differences as well as comparisons between the treatment groups and controls. A p-value <0.05 was considered statistically significant. Kaplan-Meier was used to determine statistically significant differences in the survival distribution between controls and treatment groups, Kaplan-Meier has been chosen because the test (non-parametric) measures the fraction of subjects survival time after treatment. All statistical analysis was conducted in SPSS version 25 and excel.

# Results

The effects of probiotic bacteria on high sugar diet was analyzed, and the result of the lifespan, weight, length, content of triglycerides, and gene expression level of four lipid metabolism genes can be seen below.

## Lifespan

To determine whether an HSD is associated with a decreased survival rate in adult flies. Flies were fed an LSD and HSD alone and HSD supplemented with different probiotic strains. The mean survival time value of adult flies showed a significant difference between low sugar diet (LSD) (46.0)) and high sugar diet (HSD) (33.1) and also between the treatment groups with the exception of *B. animalis* (28.0), HSD+*L. paracasei and L. plantarum* all had (43.3), *L. acidophilus* (32.6), *B. animalis* (28.0), *B. breve* (40.8), and *B. lactis* (47.3) respectively, with a p-value .000. The results showed 50% (*L. paracasei)* survival rate and maximum lifespan of the adult flies were decreased in the HSD fed groups (0.0% survival rate) compared with those of the treatment groups. This indicated that probiotics can limit the adverse effects of the HSD and can increase the lifespan of flies (Table 5)

Table 5. The lifespan of adult flies fed LSD and HSD/HSD + different probiotic strains.

| Diet/Treatment | Mean lifespan (days ± SE) | Median lifespan(days ± SE) |
|---|---|---|
| **LSD** | 46.0±1.1 | 49.0±2.6 |
| **HSD** | 33.1±3.5 | 28.0±3.8 |
| ***HSD+L. paracasei*** | 43.3±3.1 | 56.0±.000 |
| ***HSD+L.plantarum*** | 43.5±2.9 | 42.0±4.6 |
| ***HSD+L.acidophilus*** | 32.6±2.2 | 28.0±2.5 |
| ***HSD+B. animalis*** | 28.0±.000 | 28.0 |
| ***HSD+B. breve*** | 40.8±2.9 | 42.0±3.5 |
| ***HSD+B. lactis*** | 47.3±2.4 | 49.0±1.1 |

The vial tubes containing different diet/treatment were observed every two days to determine the number of deceased *Drosophila* adult flies. Data were analyzed with Kaplan-Meier Log-rank test. The above result showed that *Drosophila* feeding on LSD had an average lifespan of approximately 46 days, flies feeding on HSD had an average lifespan of approximately 33 days. Whereas flies feeding on HSD+probiotics had an increase average lifespan compared to flies feeding on HSD. This result suggests that the probiotics particularly *L. paracasei* and *L. plantarum* with an average mean lifespan of 43 days*, B. breve* with an average mean lifespan of 40 days, and *B. lactis* with an average mean lifespan of approximately 47 days can significantly reduce the adverse effects of the HSD and that can increase the lifespan of *Drosophila* adult flies with p-value 0.000.

## Weight Measurement

To determine whether an HSD increased and or decreased the size and weight of 3rd instar larvae or adult flies, male and female adult flies were combined (14 males and 14 females) under $CO_2$ anesthesia. We measured the weight of 25 larvae and 28 adult flies using a scale. The mean weight of adult flies showed a significant difference between low sugar diet (LSD) and high sugar diet (HSD) (p-value 0.022) (Figure 1). There was no statistically significant difference between LSD controls and the treatment groups that were fed both HSD and probiotics, showing that all these different probiotics treatments did increase the weight (Figure 1). All statistical values can be seen in (Table 6). The weight measurement for the 3rd instar larvae from LSD was first carried out, this is because the treatment groups and the HSD Larvae exhibited a delay in development, and therefore, their measurement was carried out two days later to be in the same developmental stage with LSD sample. Data are shown in Table 7 and figure 2 reveals a significant mean difference in weight between low sugar diet (LSD) fed larvae and high sugar diet (HSD) fed larvae (p-value 0.032). In general, the mean body weight of both larvae and adult flies raised on LSD were statistically higher than the larvae and adult flies raised on HSD. However, two experiments conducted for the adult flies and larvae showed no significant difference in mean weight between the control groups, with a p-value >0.05 (p-value0.21 adult flies and p-value 0.9 larvae) (Data not shown).

Both larvae and adult flies were photographed and the size/length was measured using Image-J software. The results obtained indicate that there was no statistically significant difference in size between all the groups including controls, the average length was in the same range (3mm) (data not shown).

Table 6. Mean, Std. deviation and p-value of adult fly-weight measurement between different groups

| Samples | Mean | Std. deviation | P-value btw LSD | P-value btw HSD |
|---|---|---|---|---|
| LSD | .000921 | .00028 | | 0.022 |
| HSD | .000757 | .00021 | 0.022 | |
| *L. paracasei+HSD* | .000896 | .00033 | 1.000 | .382 |
| L.plantarum+*HSD* | .000841 | .00030 | .940 | .22 |
| *L.acidophilus+HSD* | .000871 | .00028 | .995 | .588 |
| *B. animalis+HSD* | .000961 | .00031 | .999 | .067 |
| *B. lactis+HSD* | .000882 | .00029 | .999 | .497 |

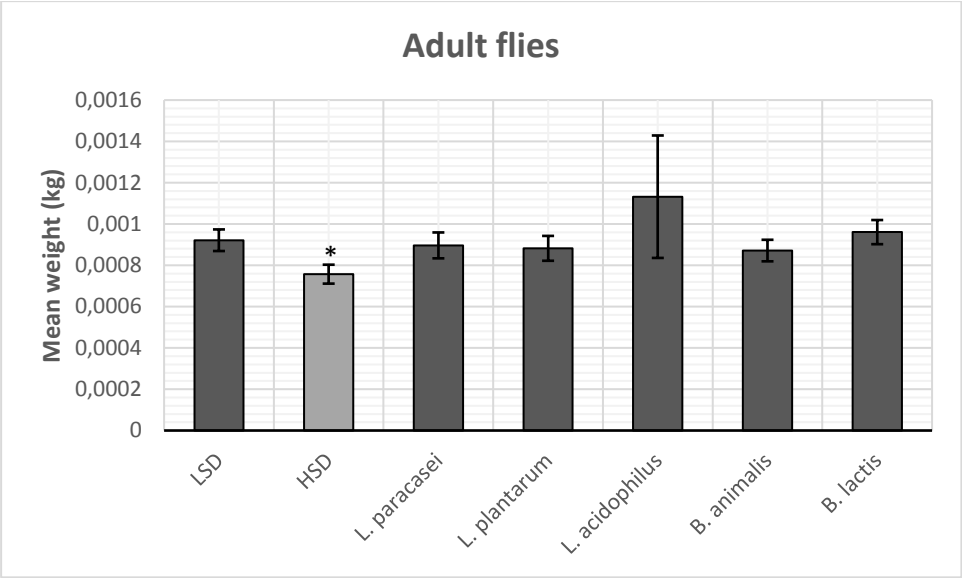


Figure 1. The effects of the HSD and the probiotics on *Drosophila* weight. Adult flies (n=28 for each group). The bar graph represents mean value with error bars of SEM. Statistical significance was determined by one-way ANOVA (p-value 0.022), followed by *post hoc* Tukey test to perform multiple comparisons. Asterisks denote significant differences from the control (* denotes p-value <0.05).

Table 7. Mean, Std. deviation, and p-value of larvae weight measurement between different groups

| Samples | Mean | Std. deviation | P-value btw LSD | P-value btw HSD |
|---|---|---|---|---|
| LSD | .001960 | .0002255 | | 0.032 |
| HSD | .001700 | .0002901 | 0.032 | |
| *L. paracasei +HSD* | .001468 | .0003363 | .000 | .759 |
| *L.plantarum+HSD* | .001428 | .0001948 | .000 | .020 |
| *L. acidophilus* | .001604 | .0002685 | .004 | .998 |
| *B. animalis* | .001572 | .0002590 | .000 | .084 |
| *B. lactis* | .001628 | .0002865 | .000 | .497 |
| *B. breve* | .001540 | .0001893 | .001 | .987 |

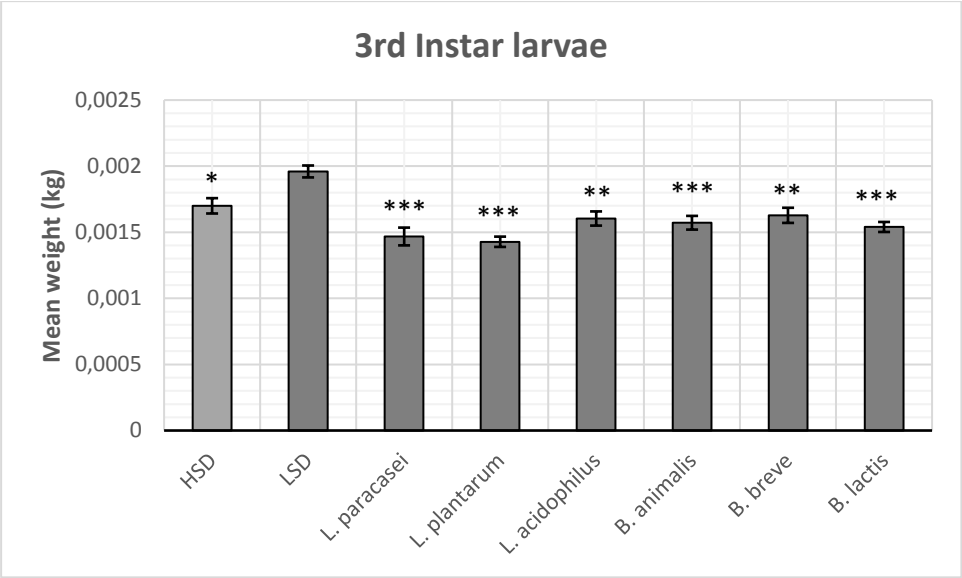


Figure 2. The effects of the HSD and the probiotics on *Drosophila* weight. 3rd instar larvae (n=25 for each group). The bar graph represents mean value with error bars of SEM. Statistical significance was determined by one-way ANOVA (p-value 0.032), followed by *post hoc* Tukey test to perform multiple comparisons. Asterisks denotes significant differences from the control (* denotes p-value <0.05, ** denotes p-value <0.01 & ***denotes p-value <0.001.)

## Coupled colorimetric assay for triglyceride

The initial stage in T2D is often accompanied by obesity. To analyze whether an HSD changed fat storage in *Drosophila,* the content of TAG were measured, which is the major form of fat storage in *Drosophila*. The first experiment using 3rd instar larvae, the results showed that there was no statistically significant difference between LSD control and the treatment groups that were fed both HSD and probiotics with the exception of *L. paracasei* with p-value 0.037 (Figure 3). The mean concentration of the respective groups were HSD (0.13mg/ml), LSD (0.08mg/ml), HSD+*L. paracasei* (0.8mg/ml), HSD+*L. plantarum* (0.7mg.ml), HSD+*L.acidophilus* (0.7mg/ml), HSD+*B. animalis* (0.3mg/ml), HSD+*B. breve* (0.5mg/ml), and HSD+B. lactis (0.6mg/ml) respectively. Adult flies also showed no significant difference between LSD control and the treatment groups that were fed with both HSD and probiotics with the exception of *L. acidophilus* p-value 0.025 (Figure 4). The mean concentration of each group were HSD (0.8mg/ml), LSD (0.5mg/ml), HSD+*L. paracasei* (0.9mg/ml), HSD+*L. plantarum* (0.6mg.ml), HSD+*L.acidophilus* (1.5mg/ml), HSD+*B. animalis* 0.6mg/ml), and HSD+B. lactis (0.9mg/ml) respectively.

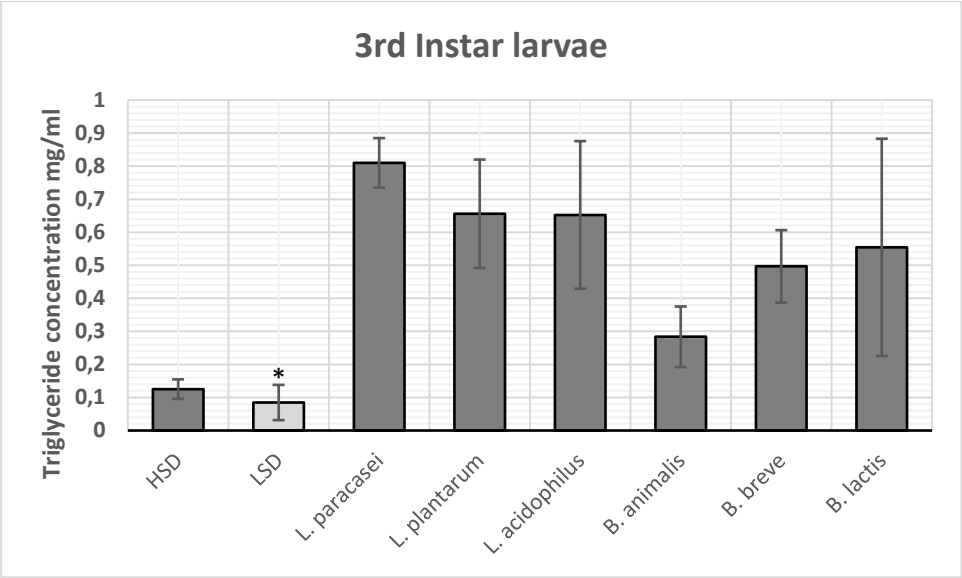


Figure 3. The effects of the HSD and the probiotics on *Drosophila* circulating TAG concentration were assayed using five larvae from each sample (8 Samples in triplicate). The absorbance values were measured at 540nm. The bar graph represents mean value with error bars of SEM. Statistical significance was determined by one-way ANOVA (p-value 0.037), followed by *post hoc* Tukey test to perform multiple comparisons. Asterisks denote significant differences from the control (* denotes p-value <0.05).

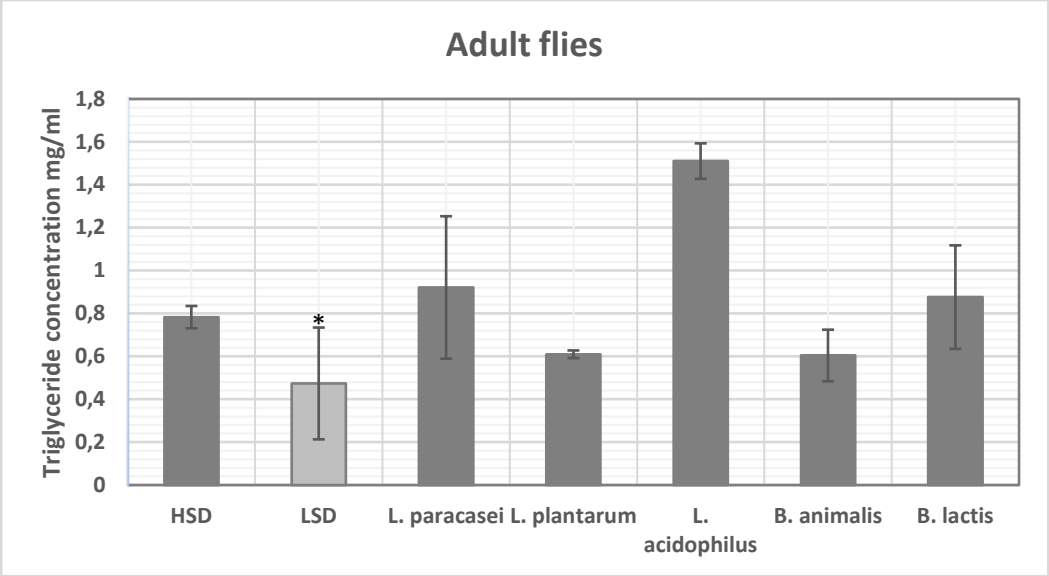


Figure 4. The effects of the HSD and the probiotics on *Drosophila* circulating TAG concentration were assayed using five adult flies from each sample (7 Samples in triplicate). The absorbance values were measured at 540nm. The bar graph represents mean value with error bars of SEM. Statistical significance was determined by one-way ANOVA (p-value 0.025), followed by *post hoc* Tukey test to perform multiple comparisons. Asterisks denote significant differences from the control (* denotes p-value <0.05).

## The relative gene expression level of lipid metabolism genes (*FASN1*, *FASN2*, FASN3 & dsREBP)

To determine the use of *Drosophila* with respect to lipid metabolism, the gene expression level of lipid metabolism genes were analyzed. Total RNA of 4 larvae and 4 adult flies (one experiment) from each sample was isolated and gene expression was measured by qPCR. The expression of four genes, all of which are involved in fatty acids synthesis were analyzed, and the housekeeping gene *(RPL32*) was used to normalize the data.

LSD fed *Drosophila* Larvae had a significantly higher expression level of *FASN1* and *dsREBP*, while the slightly lower expression level of *FASN2* and *FASN3* compared to the HSD fed *Drosophila* larvae. However, HSD fed *Drosophila* larvae had increased expression levels of *FASN2* and *FASN3* compared to all treatment groups, while *dsREBP* were downregulated in HSD fed larvae compared to the treatment groups Figure 5. For adult flies, *FASN2* and *dsREBP* were slightly upregulated in HSD fed adult flies and slightly downregulated in LSD fed adult flies, whereas the expression level of all genes was significantly downregulated in the treatment groups compared to the LSD and HSD fed adult flies (p-value 0.003), with the exception of *L. paracasei* that had increased expression level of *FASN1* compared to other groups. This can be seen in Figure 6. The combined relative gene expression level suggests that the probiotic bacteria had a bigger effect on the gene expression of these lipid metabolism genes than a high sugar level in the diet. The Δcq value was used to plot the graph.

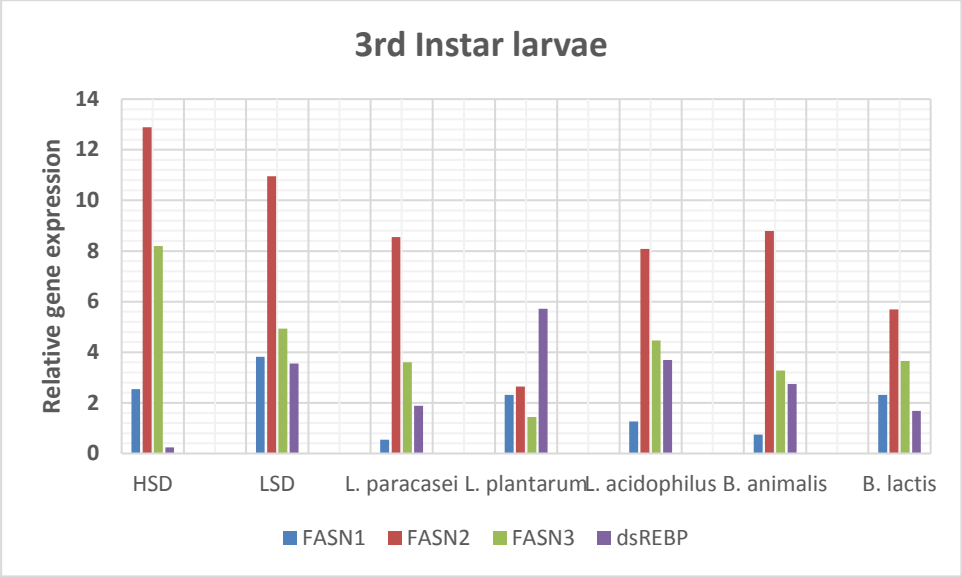


Figure 5. The effects of the HSD and LSD on the expression of *Drosophila* (3rd instar larvae) genes involved in lipid metabolism. Gene expression was quantified by the qPCR and normalized to the housekeeping gene (*RPL32*). Data were analyzed using Excel. *FASN1*, *FASN2* & *FASN3* genes in the treatment groups were slightly downregulated compared control groups.

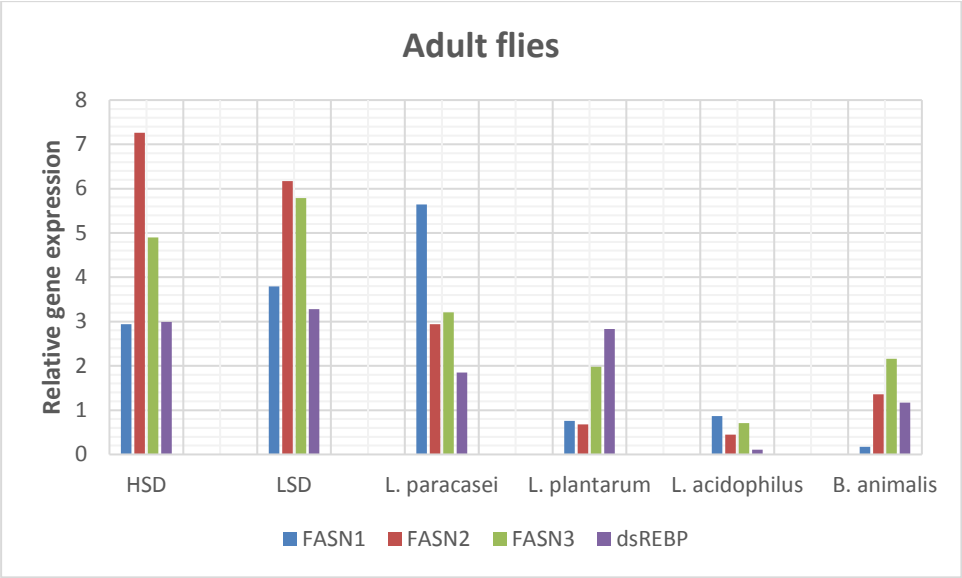


Figure 9. The effects of the HSD and LSD on the expression of *Drosophila* (Adult flies) genes involved in lipid metabolism. Gene expression was quantified by the qPCR and normalized to the housekeeping gene (RPL32). Data were analyzed using Excel. Both genes in the treatment groups were markedly downregulated compared control groups.

# Discussion

In this study, we observed that an HSD significantly reduces the growth rate and development relative to the LSD, causing weight loss. In addition to metabolism, the *Drosophila* insulin signaling pathway has also been found to modulate growth, suggesting that any growth disruption in *Drosophila* may be linked to defects in the insulin signaling pathway (Dhar et al., 2018). We initially hypothesized that *Drosophila* feeding on HSD would become obese compared to those feeding on LSD. Our findings revealed that *Drosophila* raised on HSD had significantly lower weight relative to controls (LSD). This result is similar to the research conducted by Musselman et al., (2013). However, the addition of probiotics significantly improved HSD-induced weight loss (figure 1) and lifespan (Table 5). This decreased weight probably reflects the physiological changes that result in mortality and morbidity increase associated with pro-long high sugar diet consumption. It has been shown that flies fed on HSD for 3 weeks had increased cardiac arrhythmia and that HSD fed flies had an overall reduced lifespan (Buescher et al., 2013). Therefore, the decreased weight in the flies most likely reflects early morbidity associated with the diet.

In mammals, the production of insulin by pancreatic beta cells regulates glucose and lipid metabolism, in similar way corpora cardiaca cells located in the larval ring gland release Adipokinetic hormone (AKH) which acts like pancreatic α-cells (Liu and Huang, 2012). It has been reported that AKH signaling and insulin signaling pathways control energy metabolism in *Drosophila*. Lipids metabolism in *Drosophila* is similar to that of mammals. Many studies revealed that the activation of insulin signaling pathways in non-fat tissue causes an increase in fat storage, and that fat body regulates the release of *Drosophila* insulin-like peptide (DILPs) in the brain by sensing changes in glucose concentration in the diet. Suggesting that fat storage and insulin metabolism are inter-related (Bai et al., 2018). It has been known that patients with T2D have long term obesity followed by the gradual onset of fat metabolism abnormalities. In this study, similar symptoms were observed in HSD fed *Drosophila*, where they had a slight increase in body fat storage compared to LSD (Figure 4). However, some HSD+probiotic fed *Drosophila* experience excessive fat storage compared to control groups. It was revealed that obesity in flies is triggered by an evolutionarily conserved mechanism, which acts through the cellular energy sensor 5' adenosine monophosphate (AMP)-activated protein kinase. In addition, the acidic pH of the GI tract may be essential for fly obesity given that both global vacuolar-type H' adenosinetriphosphatase (ATPase) mutants and flies treated with inhibitors of alimentary acidity accumulate excess fat (Musselman & Kühnlein, 2018). Despite the fact that probiotic supplement may significantly decrease the total LDL-C level. However, the relationship between probiotics and levels of TAG was still not clear (Yao et al., 2017).

In this study, the level of circulating triglyceride was significantly increased in the probiotic treatment compared to *Drosophila* feeding on a high sugar diet, this was contrary to expectation. The result of this study is not consistent with the result of some humans and animal studies. For example, a study conducted by Anderson & Gilliland (1999) on humans showed that five weeks of a probiotic diet lower the plasma level of triglycerides and cholesterol. Another study conducted by Nabavi et al. (2014) also showed that daily intake of 300g of probiotic yogurt containing *B. lactis* and *L. acidophilus* for eight weeks resulted in decreased LDL-C and cholesterol level. This discrepancy between our results and the previous studies could probably be due to the intervention period, probiotic doses, feeding time or different study design. Therefore, further study may be required to elucidate the effect of probiotics on lipid metabolism in *Drosophila.*

Fatty acids are the main energy stores in animals and also important precursors of membrane components and signaling molecules. Metabolism of fatty acids is linked to human diseases. Accumulation of fatty acids in adipose tissue constitutes a critical step in the development of obesity and type 2 diabetes (Parvy et al., 2012). It was reported that Dysregulation of fatty acid synthesis may be postulated to be a feature of massive obesity. Acetyl-CoA carboxylase is considered to be the regulatory enzyme determining the rate of fatty acid synthesis. Long term adaption of the rate of de novo lipogenesis to the hormonal and nutritional status of the animal is correlated to the level of fatty acid synthase and the rate of synthesis of this enzyme (Shilabeer et al., 2009). Two-kind of *FASN* are classically recognized based on the intracellular localization of the enzyme; *FASN*1 is cytosolic and is primarily responsible for de novo fatty acids biogenesis. Whereas the mitochondrial *FASN* provides an octanoyl precursor that is needed for the essential lipoylation pathway. Prototypical *FASN*1 is mainly found in mammals, the enzyme consists of a single gene that produces polypeptide containing all of the reaction centers that are needed to produce a fatty acid. (Menedez et al., 2009).

The role of fatty acid synthesis in the regulation of homeostasis in response to the dietary sugar and expression of lipid metabolism genes was analyzed. To maintain tolerable circulating glucose levels, organisms synthesize and store macromolecules in different organs. Previous research in insects revealed that most of the TAG stored in the fat-body originate from the diet (Garrido et al, 2015). The previous studies (using human adipose tissues) conducted by Berndt et al. (2007) show that an obese individual and patients with type 2 diabetes had higher gene expression level of adipose FASN, suggesting that high expression level of fatty acid synthase (*FASN*) has been shown to be primarily related to impaired insulin sensitivity. A study conducted by Dhar et al. (2018) show that *Drosophila* feeding on HSD had increased gene expression level of fatty acid synthase (*FASN*). This is consistent with our result, it was observed in this study that *Drosophila* adult flies raised on HSD had a higher expression level of fatty acid synthase (*FASN2)*, and *dsREBP*, whereas *Drosophila* larvae feeding on HSD exhibited high expression levels of *FASN2* and *FASN3*. However, *Drosophila* larvae feeding on HSD had a lower expression level of *dsREBP* compared to the other groups. HSD+probiotics fed *Drosophila* larvae had a lower expression level of *FASN1, FASN2,* and *FASN3* (Figure 5). However, *Drosophila* larvae feeding on HSD+L. paracasei, HSD+B.animalis, and HSD+B. lactis also had lower expression level of *dsREBP* compared to the other treatment groups. Whereas HSD+probiotics fed flies had a lower expression level of *FASN1, FASN2, FASN3, and dsREBP* relative to the control groups (Figure 6), this result indicates that probiotic bacteria had a greater effect in the expression of lipid metabolism genes. This trend was similar to that observed in weight (figure 1) and TAG measurement, but in this case, the treatment group had increased circulating triglyceride compared to the HSD & LSD fed larvae (3-4).

As discussed before, fatty acid synthase genes are important in lipid metabolism. in a condition of insulin resistance, high level of free fatty acids may leave the cells within adipocytes and be taken up by the organs that are unable to store an excess amount of fat safely. Insulin acts as an anti-lipolytic hormone in adipose tissue that reduces the release of free fatty acids, insulin resistance by fat cells leads to an elevated level of circulating free fatty acids because the release of free fatty acids is no longer repressed by insulin (Dhar et al., 2018). It has been postulated that sterol regulatory element binding protein *(sREBP*) is one of the proteolytic signaling pathways that play a key role in the regulation of lipid metabolism. *sREBPs* are membrane-bound transcription factor found in all animals (Amarneh et al., 2009). These transcription factors bind to the sterol regulatory elements in DNA, and they are found at the promoters of genes involved in cholesterol and lipid synthesis, thereby promoting gene expression (Lee et al., 2017). Members of the *sREBP* consist of *sREBP-1a*, *sREBP-1c*, and *sREBP-2*,

many studies have shown that in mammals, insulin positively regulates *sREBP-1c* and promotes lipogenesis in liver cells (Liu and Huang, 2012). Giving the role of free fatty acids in mediating insulin resistance, the high expression level of fatty acid synthase genes in *Drosophila* feeding on HSD was observed. We initially hypothesized that an HSD fed *Drosophila* would have higher gene expression level compared to control (LSD) and that the expression level of *FASN* genes would be downregulated in the treatment groups. Few of the results obtained validate our hypothesis, Although, *FASN1 & dsREBP* in LSD fed Drosophila larvae had increased gene expression level compared to the HSD and few of the treatment groups (Figure 5), and therefore further study involving more genes and/or higher sample size may be required in order to fully understand and determine specific roles of *FASN1, FASN2, FASN3* and *dsREBP* with respect to inflammation and insulin signaling in *Drosophila*.

Altogether, our findings confirm the use of *Drosophila* as a novel model organism for studying type 2 diabetes as well as insulin resistance. In conclusion, further elucidation is required to comprehensively understand the function of probiotics on HSD in Drosophila in order to determine the exact utility of glucose and fatty acids entering the cells, as well as the storage of triglycerides

## Ethical aspects & Importance of the research

No ethical approval was required for research using *Drosophila* as a model. The wellbeing of these animals is highly important, therefore the three Rs were strictly followed throughout the research.

Type 2 diabetes has become one of the major public health issues, and therefore contribute to high morbidity and mortality rate worldwide. In 2017, the prevalence of diabetes in Sweden was 6.9%. Diabetes type 1&2 are the major risks factors for quite a number of chronic disease including kidney disease and cardiovascular disease. The major event behind T2D is that the amount of glucose in the blood is higher than needed. As β-cells being the organ that releases insulin, it is necessary to focus on that organ (β-cell) in order to understand more about the molecular or mechanism contributing to the pathogenesis of several metabolic diseases/disorders. Since insulin regulates the blood glucose homeostasis, by stimulating the glucose uptake and glycogen storage in the cell. Therefore decreased blood glucose level. Research on the development of effective treatment might help patients with type 2 diabetes to enhance glucose homeostasis. Deepen our knowledge about how unhealthy lifestyle can pose a risk of serious life-threatening conditions will not only save lives but also decrease the strain on public healthcare, and therefore leads to a better healthier population.

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# Appendix 1- Food preparations

High sugar and low sugar diet were prepared by mixing 1.4g agar with 200ml water and brought to a boil. 13g inactive yeast, 6g corn flour and 68.4g sugar for high sugar and 10.3g for low sugar diet were added and boiled while mixing. The mixture was to 60℃ to 55℃ and 1.5ml Nipagen solution was added while mixing. The mixture was poured into vial tubes and apple plates approximately 3-4 cm thick layer.

# Appendix 2- Information about the RT-PCR master mix quantities used in the PCR experiment

**Table 1. Reverse Transcription PCR master mix**

| Reagent | Volume (µl) | Used µl |
|---|---|---|
| 10× RT Buffer | 2.0µl | 40µl |
| 25× dNTP mix (100mM) | 0.8µl | 16µl |
| 10× RT Random Primers | 2.0µl | 40µl |
| Multi-scribe Reverse Transcriptase | 1.0µl | 20µl |