Bachelor Degree Project

UNIVERSITY
OF SKÖVDE

1977

The Effects of Probiotics on High Sugar-Induced Type 2 Diabetes Mellitus Symptoms in *Drosophila melanogaster*

Bachelor Degree Project in Biomedicine
30 ECTS
Spring term 2019

Yasser "Al-Garboa" Al-Ghamdi

Supervisor: Katarina Ejeskär
Examiner: Ferenc Szekeres

Abstract

Background: Type 2 diabetes mellitus is a metabolic disorder characterized by the rise of fasting plasma glucose from its normal range (≥125mg/dl). It is marked by insufficient production of insulin from pancreatic β-cells as a result of failed compensation due to insulin resistance. Several treatments are available for the disorder, which mainly focus on improving the sensitivity of insulin in different body tissues. Recently, probiotics were suggested as candidate treatments for type 2 diabetes and for extending lifespan as well. This experiment aims to investigate such claims using *Drosophila melanogaster* as a disease model.

Results: Other than the observed low average weights in treated larva samples, probiotics did not show any other significant results in affecting the length, glucose, glycogen, and trehalose levels (One-Way ANOVA and Kruskal-Wallis, $p > 0.05$). Real-time PCR was only carried out once. Thus, no statistical tests were reliable enough to analyse the data obtained. The longevity study, on the other hand, did show significance (Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test, $p < 0.0001$), as the probiotic *Bifidobacterium lactis* extended the lifespan of adult flies feeding on a high sugar diet significantly when compared to the control ones feeding on only high sugar diet without probiotics.

Conclusion: Except for weight measurements, none of the other results was reliable enough to make a concrete conclusion on whether the treatments indeed worked in reversing type 2 diabetes symptoms or not. Real-time PCR results did show some effects of some of the treatments at different developmental stages. However, unless Real-time PCR is repeated at least once using the same protocol, no deduction can be made. Additionally, the data obtained hint that the dosage used (0.025 g) was too high for larvae and adult flies and might have caused malnutrition by blocking their midgut and decreasing food absorption. Hence, false significant or non-significant results were acquired instead. Still, a promising deduction can be made, as these results could indicate a weight loss effect of probiotics through decreasing food absorption, which can be the topic of future research.

Further studies are required using a much lower probiotic dosage if *Drosophila* is used as a disease model. Although, other models such as mice or rats are recommended in this case, in order to reach a solid conclusion about the effectiveness of probiotics in treating type 2 diabetes mellitus. Baring these thoughts in mind and based on the results of this experiment, the null hypothesis indicating that there is no significant relationship between the use of probiotics and reversing type 2 diabetes mellitus symptoms is therefore accepted.

Popular scientific summary

Diabetes is one of the most prevalent metabolic disorders in the world. As of 2014, the number of affected people worldwide was 422 million, and the number of deaths caused by this disorder was 1.6 million in 2016. It is characterized by rising blood glucose levels due to insufficient insulin production. This inadequate production of insulin is a result of either damaged pancreatic β-cells, which is referred to as type 1 diabetes, or the inability to reach the body tissues demands for insulin due to "failed compensation" and insensitivity to insulin, which is type 2 diabetes in this case. The actual cause of type 1 is still unknown, where genetic factors and lifestyle are the leading causes of type 2.

Treatments for type 1 diabetes usually involve synthetic insulin, reducing the intake of carbohydrates and physical activities. Type 2 diabetes treatments, on the other hand, include drugs that improve the performance and sensitivity of insulin, synthetic insulin in some cases and physical activities as well. Recent studies have suggested that probiotics might be of help improving insulin performance in diabetic patients. In 2017, a scientific article proposed that probiotic supplementation significantly enhanced "fasting insulin in type 2 diabetes patients". Another article suggested that probiotics will also extend longevity in animals.

A project was designed using fruit flies "*Drosophila melanogaster*" as study models to investigate the effects of probiotics in reversing type 2 diabetes symptoms. The main reason behind this model choice was its ability to develop insulin resistance in similar manners as humans. In nature, flies feed on rotten fruits which mainly contain the carbohydrate "fructose". This sugar does not have the same harmful effects as glucose. Thus, in this project, flies were fed a diet high on sucrose, which was considered to be an overdose to some extent and caused addiction. Hence, flies were feeding constantly nonstop in this case. As a result, insulin was continuously produced, causing flies' body tissues to store more and more glucose in different forms, one of which was fat. At this stage, the increased production of insulin and its constant interaction with its receptor resulted in insulin tolerance where insulin became inefficient at making an impact when binding to its receptor. Therefore, initiating "insulin resistance" and type 2 diabetes in the long term as a consequence of failed compensation. Flies in this project were also fed different types of probiotics that were tested and proven to have positive effects on health. Various measurements of weight, length, glucose, glycogen and trehalose (common insects' sugar) levels were taken at different developmental stages of the fruit fly. Measurements of longevity and gene products of specific genes, which are involved in regulating glucose levels, were also taken.

This study aimed to examine the claims made by previous articles that linked probiotics to reversing the symptoms of type 2 diabetes. However, most of the results obtained did not show significant effects of probiotics in reversing the symptoms. Exceptions include results obtained from the weight measurements, where larvae treated with probiotics had significantly lower average weights than the untreated ones. Yet, the analysis of all the results obtained suggest that these low weights were probably due to the accumulation of high doses of probiotics in the midgut of these larvae. Thus, obstructing the absorbance of glucose and other nutrients causing malnutrition. Therefore, the low average weights were the result of developmental delays and not due to the effectiveness of the probiotics themselves. However, further studies need to be made, as some technical issues were encountered, which could have significantly altered the results. Consequently, false significant or insignificant data were obtained instead. Still, a promising deduction can be made, as these results could indicate a weight loss effect of probiotics through decreasing food absorption, which can be the topic of future research.

Contents

Abbreviations

LSD   Low Sugar Diet
HSD   High sugar diet
T2DM   Type 2 diabetes mellitus
PEPCK   Phosphoenolpyruvate carboxykinase 1
Fbp   Fructose biphosphatase
Zw   (Zwischenferment) Glucose-6-phosphate dehydrogenase G-6-PD
Hex-t1   Hexokinase
RPL32   Ribosomal protein L32
CC   Corpora cardiac
AKH   Adipokinetic hormone
ILP   Insulin-like peptide
GLUT1   Glucose transporter type 1
Mio   Mondo
MIX   Bigmax
SIK3   Salt inducible kinase 3
Dimm   Dimmed
Amon   Amontillado
LDVC   Large dense core vesicles
VGCC   Voltage-gated calcium channel
InR   Insulin receptor
Chico   Insulin receptor substrate
PI3K   Phosphatidylinositol 3 kinase
PTEN   Phosphatase and tensin homolog
PDK1   Phosphoinositide-dependent protein kinase-1
AKT   Protein kinase B
TORC1   Target of rapamycin complex 1
S6K1   S6 kinase 1
FOXO   Fox O transcription factor
Inulin+1   *Inulin + L. paracasei*
Inulin+2   *Inulin + L. plantarum*
Inulin+3   *Inulin + L. acidophilus*
Inulin+4   *Inulin + B. animalis*
Inulin+5   *Inulin + B. breve*
Inulin+6   *Inulin + B. lactis*

Introduction

Type 2 diabetes mellitus and disease models

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by the rise of fasting plasma glucose from its normal range (≥125mg/dl) (Janghorbani and Amini 2012; Kharroubi and Darwish 2015). The constant ingestion of sugar, glucose to be exact, leads to constant production of insulin, as the body wants to clear the excess glucose from the blood. This continuous release of insulin leads to ''insulin tolerance", where the hormone fails to make an impact when binding to its receptor, therefore, resulting in insulin resistance. The resistance then causes inadequate production of insulin from pancreatic β-cells in the long term "failed compensation" (Kasuga 2006). Thus, T2DM is devolved at this point. The International Diabetes Federation (IDF) reported in 2013 that 8.3% of adults between the ages (20-79) are affected by this disorder worldwide, which approximately accounts for 382 million people. This percentage could rise to 10.1% by 2035, according to IDF reports (Kharroubi and Darwish 2015).

In order to study T2DM and develop effective treatments, researchers have been designing different animal models to carry out molecular and pharmacological studies concerning T2DM, before engaging in human clinical studies. These models are ranging from rodent models (e.g. mice, rats and gerbils), feline models (e.g. domestic cats), swine models, primate models (e.g. African green monkeys and baboons) (Cefalu 2006) and fly models (e.g. *Drosophila melanogaster*) (Graham and Pick 2016). The latter is more favourable by many researchers, not only for their rapid generation time and low costs (Morris et al. 2012) but also for the many conserved pathways they share with mammals (Appendix D, Figure D1). Besides the ability to use them in research without the need for ethical permits (Trinder, Daisley, Dube and Reid 2017).

Carbohydrates regulation in *Drosophila*

In a healthy *Drosophila*, insulin-like peptide (ILP) and adipokinetic hormone (AKH) control the regulation of carbohydrates circulating the hemolymph, by acting as insulin and glucagon analogous and having similar if not the same signalling pathways and outcomes (Alfa and Kim 2016). These outcomes include glycogenesis, lipogenesis and glucose uptake from circulation for ILP. Lipolysis, gluconeogenesis and trehalose release into the circulation as for AKH. There are eight known variants of ILPs. Seven of them act like insulin and an 8th that has relaxin-like functions coordinating growth (Álvarez-Rendón, Salceda and Riesgo-Escovar 2018). ILP2, ILP3 and ILP5 constitute the majority of adult flies' insulin, wherein larvae, ILP1 also has some contributions in sugar regulation (Alfa and Kim 2016). Insulin-producing cells, which are β-cells analogous, express all the different variants of ILPs. These cells have bodies that are located in the brain of *Drosophila*, where ILPs are expressed, and axon terminals located at different parts of the body, where ILPs are released into the hemolymph (Luo et al. 2014). AKH, on the other hand, is produced by corpora cardiac (CC) cells, which are located in the neuroendocrine ring gland as Figure 1.B shows (Park, Bustamante, Antonova, McLean and Kim 2011). Out of the different variants of ILPs, ILP2 is structurally the closest to vertebrate insulin (Álvarez-Rendón, Salceda and Riesgo-Escovar 2018).

When fed a low sugar diet (LSD), *Drosophila* breaks down and absorbs the carbohydrates via the intestinal enterocytes in the midgut. This process exists in both adult flies and larvae without much differences, yet further stages, when glucose enters the circulation, differ between larvae and adult flies. In adult flies for example, some of the glucose is transferred to the fat body cells where it is converted to trehalose. Trehalose is the most abundant carbohydrate in the hemolymph, where It acts as a negative regulator of CC cells. Thus, the more trehalose produced, the less AKH is produced from CC cells and vice versa. Moreover, trehalose is synthesized from glucose-6-phosphate and

uridine-diphosphate glucose (UDP-glucose) by the enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase. Nevertheless, not all of the glucose is converted to trehalose, as some have essential functions in the release of ILP from insulin-producing cells (Mattila and Hietakangas 2017). When the concentration of glucose in the circulation is high, the insulin-producing cells sense this increased concentration through glucose transporters (GLUT1) located on their membranes (Figure 1.A). Thus, the high levels of glucose lead to an increase in the expression of ILP2 (Graham and Pick 2016). In larvae, however, trehalose acts as a "biphasic" regulator of CC cells. In this case, high and low concentrations of trehalose stimulate the production of AKH. Additionally, glucose is not sensed by insulin-producing cells via GLUT1 in larvae, and hence, glucose concentration does not affect the expression of ILP. (Mattila and Hietakangas 2017).

Insulin-like peptide production and regulation
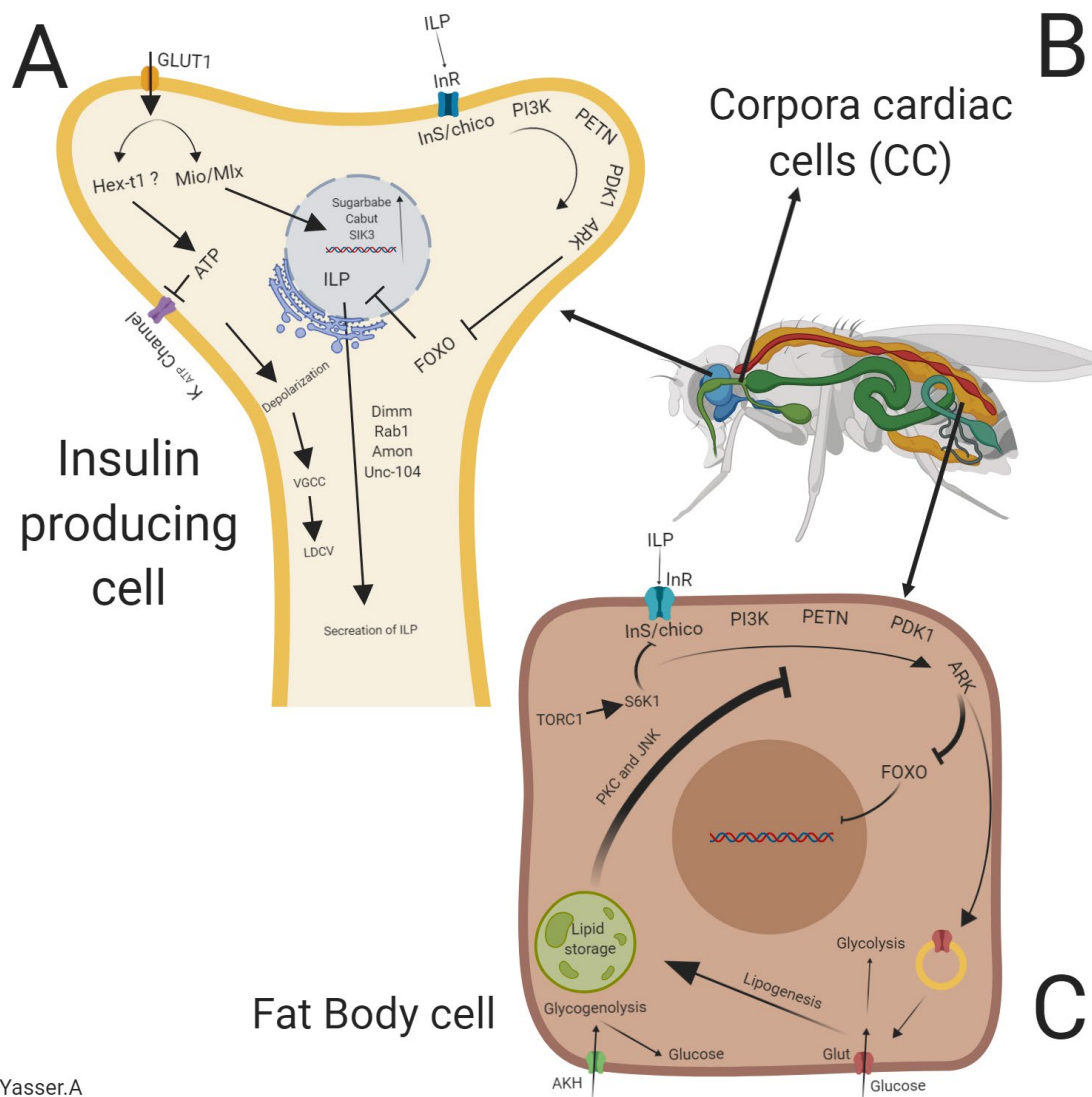
Figure 1. The locations of insulin-producing cells, corpora cardiac (CC) cells and fat body cells in adult *Drosophila melanogaster*. A: Insulin-like peptide production and its signalling pathway in insulin-producing cells. B: Corpora cardiac cells, where the adipokinetic hormone (AKH) is produced. C: Insulin signalling in fat body cells, where most glucose is stored. (ILP) Insulin-like peptide. (AKH) Adipokinetic hormone. (GLUT1) Glucose transporter type 1. (Mio) and (MIX) transcription factors Mondo and Bigmax respectively. (SIK3) Salt inducible kinase 3. (Dimm) Dimmed. (Amon) Amontillado. (LDVC) Large dense core vesicles. (Hex) Hexokinase. (VGCCs) Voltage-gated calcium channels. (InR) Insulin receptor. (chico) Insulin receptor substrate. (PI3K) Phosphatidylinositol 3 kinase. (PTEN) Phosphatase and tensin homolog. (PDK1) Phosphoinositide-dependent protein kinase-1. (AKT) Protein kinase B. (TORC1) Target of rapamycin complex 1. (S6K1) S6 kinase 1. (FOXO) Fox O transcription factor.

When glucose levels are high in the circulation, insulin-producing cells sense this increased concentration by the amount of glucose transported intracellularly via GLUT1. The elevated glucose levels then activate the transcription factors Mondo (Mio) and Bigmax (Mlx). These, in turn, activate the genes responsible for glycogenesis, lipogenesis, glycolysis, pentose phosphate pathway and ILP expression. Those activated genes encode transcriptional regulators such as Sugarbabe, Cabut and salt inducible kinase 3 (SIK3) (Mattila and Hietakangas 2017). Other expressed genes include the transcription factor Dimmed (Dimm), Amontillado (Amon), GTPase, Rab1 and UNC-104 which are essential for the processing and loading ILP into large dense core vesicles (LDVC) for extracellular transport. Simultaneously, glucose entering insulin-producing cells are phosphorylated through an unknown hexokinase (HEX). This glucose influx leads to ATP production from the mitochondria via glycolysis. The ATPs generated inhibit K_{ATP} channels causing the depolarization of insulin-producing cells membrane. This signal ends up activating unknown voltage-gated calcium channels (VGCCs), causing the fusion of ILP expressed and LDVC for axonal transportation to target production site. Insulin-producing cells also have an autocrine feedback signalling pathway that allows them to sense and regulate the amount of ILP produced (Figure 1.A) (Alfa and Kim 2016).

In insulin signalling, the binding of ILP to the insulin receptor (InR) in fat body cells, for instance, stimulates a "phosphorylation cascade". The cascade starts with insulin receptor substrate (chico), going through phosphatidylinositol 3 kinase (PI3K), phosphatase and tensin homolog (PTEN), phosphoinositide-dependent protein kinase-1 (PDK1) and ending up phosphorylating protein kinase B (AKT). These first signalling steps take place at the cell membrane of Insulin-producing cells with the aid of phosphatidylinositol 4,5-biphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate (PIP3), which act as proteins binding sites. PDK1 and AKT can then function in the cytoplasm by phosphorylating tuberous sclerosis proteins 1-2 (Tsc1-2) and Fox O transcription factor (FOXO) respectively. The phosphorylation of these two has vital functions in regulating ILP production, especially FOXO, as it "counteracts" insulin actions, although, it has positive contributions to longevity (Álvarez-Rendón, Salceda and Riesgo-Escovar 2018). Upon phosphorylation, FOXO remains in the cytoplasm instead of entering the nucleus, and thus, its transcription activities decrease. The inhibition of Tsc1-2 activities, on the other hand, leads to the inhibition of the target of rapamycin complex 1 (TORC1). The latter stimulates insulin resistance when activated through its effector ribosomal protein S6 kinase 1 (S6K1) (Figure 1.C). Thus, its inhibition prevents insulin resistance. Furthermore, knockdown of S6K1 has been proven to protect against insulin resistance (Alfa and Kim 2016; Álvarez-Rendón, Salceda and Riesgo-Escovar 2018).

Inducing type 2 diabetes mellitus in *Drosophila*

In nature, flies feed on rotten fruits which mainly contain the carbohydrate "fructose" (Hales, Korey, Larracuente and Roberts 2015). This sugar does not have the same harmful effects as glucose. Thus, feeding flies a diet high on sucrose is considered to be an overdose to some extent and might cause addiction (Pasco and Léopold 2012). Consequently, flies will feed constantly nonstop in this case. As a result, insulin will be produced continuously, causing flies' body tissues to store more and more glucose in different forms, one of which is fat. At this stage, the increased production of insulin and its constant interaction with its receptor will result in insulin tolerance where insulin becomes inefficient at making an impact when binding to its receptor. Therefore, initiating insulin resistance and type 2 diabetes in the long term as a consequence of failed compensation (Alfa and Kim 2016; Álvarez-Rendón, Salceda and Riesgo-Escovar 2018).

On a cellular level, insulin signalling in fat body cells functions in activating lipogenesis, glycogenesis and in the fusion of GLUT to the cell membrane. The latter facilitates the uptake of glucose from the circulation. When feeding on HSD, glucose is absorbed and transferred to the fat body. In fat body cells, excess glucose is converted to triglycerides through (lipogenesis) and stored in lipid droplet within these cells. Lipid accumulation leads to the activation of protein kinase C (PKC), which acts as

an antagonist to insulin signalling, through the inhibition of AKT and PDK1 activities, and hence, stimulation of FOXO and TOR complex activities. The latter two are negative regulators of insulin signalling, as mentioned above. Lipid accumulation can also activate Jun-N-terminal kinase (JNK) signalling pathway, leading to the inhibition of insulin signalling and thus, insulin resistance as illustrated in Figure 1.C (Alfa and Kim 2016; Mattila and Hietakangas 2017). Alfa and Kim (2016) suggest that FOXO has a role in increasing insulin sensitivity by increasing the expression of InR under low glucose conditions. Consequently, this leads to insulin resistance through failed ILP compensation. (Alfa and Kim 2016; Álvarez-Rendón, Salceda and Riesgo-Escovar 2018).

Type 2 diabetes mellitus and probiotics

In order to reverse T2DM, researchers have been studying the effects of probiotics on the microbiota of *Drosophila*. Probiotics have been known for their various benefits on health. These benefits include reduction of serum cholesterol, modification and enhancement of immune responses, treatment of acute diarrheal disease, improvement of lactose metabolism, protection of the intestinal mucosa from pathogenic penetration, anti-inflammatory and anti-oxidation benefits, vitamins synthesis, protection against allergies at early stages in life and reversing T2DM symptoms. However, some of these benefits are not based on sufficient evidence (Kechagia et al. 2013).

In a healthy *Drosophila*, the gut microbiota is mainly comprised of bacteria of the firmicutes phylum (lactobacillus is the dominant genus). Smaller portions of proteobacteria (acetobacter is the dominant genus) also exist in its microbiota composition. Firmicutes are the main "good" bacteria that reside in the microbiota, and their increased numbers provide notable benefits, including protection against chronic inflammations, such as diabetes (Tsai et al. 2019). Proteobacteria, nevertheless, have adverse effects on health when their numbers increase in the gut. The exact mechanism in which probiotics reverse insulin resistance is still unknown and understudy (Trinder et al. 2017). Westfall, Lomis and Prakash (2018) suggest that gut dysbiosis results in excess lipogenesis and oxidative stress leading to elevated FOXO levels and therefore causing insulin resistance. Hence, probiotics can reverse insulin resistance by restoring the microbiota balance in the gut. Another claim proposed that dysbiosis, caused by increased densities of proteobacteria populations, lead to elevated lipopolysaccharides (LPS) levels entering the host system. Additionally, high LPS levels can cause systemic inflammations which might result in insulin resistance through the activation of JNK pathways. Therefore, administrating probiotics will restore the microbiota balance, thus reversing insulin resistance (Tsai et al. 2019). Still, most of the papers available discuss the benefits of probiotics on T2DM from a "dysbiosis" point of view, and not from an obesity or high sugar consumption perspective.

The genes of interest and their predicted expression changes

In this experiment, several genes involved in glucose metabolism will be analysed. These genes include phosphoenolpyruvate carboxykinase 1 (*PEPCK*), fructose biphosphatase (*Fbp*), glucose-6-phosphatase dehydrogenase (*Zw*) and hexokinase type 1 (*Hex-t1*). Generally speaking, when T2DM is developed, the genes responsible for glucose catabolism (e.g. glycolysis and lipogenesis) will be downregulated. The ones responsible for glucose anabolism (e.g. gluconeogenesis), however will be upregulated (Cersosimo et al. 2018). Furthermore, when T2DM is developed, glucose will not enter the cell in sufficient amounts and will remain in the blood instead. Hence, glycolysis frequency will decrease, and cells will start making glucose to obtain more energy (gluconeogenesis). *PEPCK* is involved in gluconeogenesis and is expected to be repressed under LSD and at the initial stages of HSD. This repression is caused by the activated transcription regulator (Cabut). Once T2DM has been induced, *PEPCK* will be expressed by activated FOXO. *Fbp* is also involved in gluconeogenesis and is expected to follow the same expression pattern as *PEPCK*. *Zw* gene, encoding one of the major pentose phosphate pathway (PPP) components glucose-6-phosphate dehydrogenase G-6-PD, is

expected to be expressed normally under LSD. Its expression, however, will be upregulated shortly after feeding on HSD and before T2DM induction, and downregulated once *Drosophila* develops T2DM. The dehydrogenase is phosphorylated and activated by SIK3, and has an essential contribution in generating triglycerides. *Hex-t1*, nevertheless, is involved in phosphorylating hexoses as part of metabolism. It is expected to be expressed under LSD, upregulated instantly after feeding on HSD, and downregulated once T2DM is devolved (Mattila and Hietakangas 2017; Tillmann, Bernhard and Eschrich 2002; Visinoni et al. 2012; Xu, Osborne and Stanton 2005).

Aim of the study

This experiment aims to study the effects of probiotics on high sugar-induced T2DM symptoms in *Drosophila*, using six probiotic strains (*L. paracasei, L. plantarum, L. acidophilus, B. animalis subsp lactis, B. breve, and B. lactis HN019*) and the prebiotic (*Inulin*). Different measurements will be taken to investigate the aim including, weight, length, carbohydrate levels, longevity and gene expression. The HSD is expected to shorten life span, induce cardiac dysfunction, cause arrhythmias, reduce the size, delay the development, cause obesity and insulin resistance leading to T2DM. The null hypothesis indicates that there is no significant relationship between the use of probiotics and reversing T2DM symptoms.

Materials and method

Drosophila melanogaster breeding

Wild type flies were allowed to mate in a fly cage attached to a Petri dish containing specific fly food "apple juice plate" (Table 1). A paste made of yeast and water was also applied to the food surface to attract the flies further toward the food. The cage was incubated at 25°C overnight. This process was repeated five times to supply enough eggs for five trials. The trials were carried out using two different methods.

Method 1 (for trials 1 and 2)

Embryos collection and transfer

Twenty-four hours post-incubation, the apple plate was detached from the cage and covered with a thin layer of 1X PBS solution. The eggs were mobilised using a fine brush, and the PBS solution containing the eggs was then poured into a 50 ml falcon tube. The procedure was repeated several times until most of the eggs in the petri dish were collected. The embryos were then transferred to the desired plate/vial using a 1000 ul pipette with a tip, which was cut at 5 mm from the bottom to allow more eggs transfer.

Embryos growth

The eggs were transferred and allowed to grow in new apple plates. Each plate contained a small paste of one of the following: only HSD (1 M sucrose), only LSD (0.15 M sucrose), HSD mixed with 0.005 g of a single strain of probiotics, HSD mixed with 0.005 g of a single strain of probiotics and 0.005 g of *inulin* or HSD mixed with 0.005 g of multi-probiotics. The plates were then incubated at 25°C. The recipes for the HSD, LSD and the strains of probiotics used are provided in Table 1 and Appendix 1.

Larvae transfer to vials

At third instar stage, larvae were transferred to vials containing HSD, LSD, HSD with 0.005 g of a single strain of probiotics, HSD with 0.005 g of a single strain of probiotics and 0.005 g of *inulin* or HSD with 0.005 g of multi-probiotics.

Sample collection for further measurements

Larvae from treatment and control samples were collected on the same day. Length and weight were measured immediately after collection, without freezing the samples.

Weight and length measurements

Larvae were weighed in grams (g) while alive using a scale. The length was measured by placing larvae on a grid paper and measuring the length covered in millimetre (mm).

Method 2 (for trials 3, 4 and 5)

Embryos collection and transfer

Twenty-four hours post-incubation, the apple plate was detached from the cage and covered with a thin layer of 1X PBS solution. The eggs were mobilised using a fine brush, and the PBS solution containing the eggs was then poured through a paper mesh for embryos collection. The eggs were then transferred to the desired plate/vial using a spatula.

Embryos growth

The eggs were transferred and allowed to grow in Petri dishes containing one of the following: only HSD (1 M sucrose), only LSD (0.15 M sucrose) or HSD with 0.025 g of a single strain of probiotics sprinkled on the surface of the food. The plates were then transferred to beakers which were sealed with Para-films afterwards, to ensure no flies can contaminate the plates with new eggs. The Para-films were punctured to allow aeration and the beakers were incubated at 25°C (Figure 10).

Flies transfer to vials

Larvae were allowed to grow inside the incubated beakers until the adult stage. The flies were then anaesthetised with CO_2, and transferred to vials containing HSD, LSD or HSD with 0.025 g of a single strain of probiotics added to the top of the food. The vials were sealed with Para-films, which were punctured to allow aeration. Appendix E, Figure E1 illiterates how probiotics were used in methods 1 and 2.

Sample collection for further measurements

Treatment and control samples of 3rd instar larvae and adult flies (5 days after developing to adults) were collected and kept in -20°C freezer for further measurements. Third instar larvae from LSD control sample were collected first. Third instar treated larvae and larvae from the HSD control sample were collected 3-4 days later due to developmental delays.

Weight and length measurements

Third instar Larvae and adult flies were weighed in grams (g) after being frozen using a scale. Photos of 3rd instar larvae and adult flies were taken next to a ruler using Sony Cybershot DSC-H300. The photos obtained were then used for length measurements using ImageJ.

Table 1. The composition of apple juice plates, low sugar diet (LSD) and high sugar diets (HSD) for vials and apple plates.

Apple juice plate recipe	Low sugar diet (LSD) recipe for vials	High sugar diet (HSD) recipe for vials	Low sugar diet (LSD) recipe for apple plates	High sugar diet (HSD) recipe for apple plates
- 960 ml of water - 18 g bacto agar-agar - 20 g sucrose - 40 ml apple juice - 12 ml of 10% ethanolic Nipagin	- 200 ml of water - 1.4 g agar - 13 g inactive yeast - 10.3 g sucrose (0.15 M) - 6 g cornflour - 1.5 Nipagin	- 200 ml of water - 1.4 g agar - 13 g inactive yeast - 68.4 g sucrose (1 M) - 6 g cornflour - 1.5 Nipagin	- 40 ml of water - 13 g inactive yeast - 2 g sucrose (0.15 M) - 6 g cornflour	- 40 ml of water - 13 g inactive yeast - 14 g sucrose (1 M) - 6 g cornflour

Carbohydrates measurements

Glucose, glycogen and trehalose were measured according to the protocol (Tennessen et al. 2014) with few adjustments such as four adult flies and 3rd instar larvae were used for each protocol instead of five adult flies and 25 2nd instar larvae. Another adjustment included the way glycogen and trehalose were measured, since their standard curve did not work as expected. Thus, the concentrations of glucose monomers, which used to make-up glycogen and trehalose, were used as estimated measurements of original glycogen and trehalose concentrations. The absorbance was measured using the FLUOstar Omega Microplate Reader at 340 nm.

RNA extraction and purification

Whole RNA extraction was carried out using Rneasy plus mini kit (Qiagen, Cat. #74134) according to manufacturer protocol. RNA purity was measured using the Nanodrop ND-1000 spectrophotometer at absorbance 260 nm and 280 nm. RNA $A_{260/280}$ ratio of around 2 was considered to be pure (Appendix 2).

Reverse transcription

The protocol was carried out using the High-Capacity RNA-to-cDNA^{TM} Kit (Thermo Fisher Scientific, Cat. #4387406). RNA concentrations were normalised to 1000 ng per 20 ul reaction using nuclease-free water or MilliQ water (Appendix 2). The reverse transcription reaction was run according to the following protocol: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C for 20 minutes.

Real-time PCR

TaqMan probes (Appendix 3) and TaqMan Gene Expression Master Mix (Applied Biosystems) were used to perform qPCR according to the protocol recorded in Table 2:

Table 2. Real-time PCR protocol steps.

Step	Description
1	Probes were diluted 1:40 using MilliQ H_2O or ddH_2O.
2	2ul of diluted probes were transferred into the assigned wells in a 96 well RT-qPCR plate.
3	cDNA samples were diluted 1:40 with MilliQ H_2O or ddH_2O
4	The TaqMan Master mix was added to the diluted cDNA samples in 1:1 ratio
5	2ul of cDNA/master mix was transferred to appropriate wells
6	The plate was covered, vortexed and centrifuged at 1500 rpm for 1 minute.
7	The plate was transferred to Thermo Fisher Pikoreal real-time PCR machine, and the reaction was started according to the following protocol:
	Temp.: 50°C; Time 02:00 min
	Temp.: 95°C; Time 10:00 min
	Number of cycles to loop:40; Number of steps inside loop:2
	Temp.: 95°C; Time 00:15 sec
	Temp.: 60°C; Time 01:00 min

Results obtained were further analysed using ΔCt method, to compare the relative gene expression between the different samples. The method was carried out by plotting the logarithmic relative expression of each gene in each sample i.e $\log_2 (2^{-\Delta Ct})$.

Statistical analysis of results

Various statistical tests were performed using SPSS. The Shapiro-Wilk test was used to determine the normality of data. One-Way ANOVA followed by *Post hoc Tukey* test was performed to define significance in case of normally distributed data and Non-parametric Kruskal-Wallis test in case of non-normal data. Two-way ANOVA was performed using Ct values obtained from qPCR. Kaplan-Meier survival analysis and Log-rank (Mantel-Cox) test were performed for the longevity study (using Prism GraphPad). Box-Whisker plots were used to exclude outliers from all results. Levene's test of distribution was also used to check for homogeneity of variances. Two-tailed, t-test was performed assuming equal variances to assess the significance among the normalised data. The significance value considered was $p < 0.05$.

Results

Weight measurements

Trials 1 and 2 using method 1 (0.005 g of probiotics) (individual results)

One of the biological markers associated with insulin resistance is weight gain. Therefore, measuring weight was the first protocol carried out to investigate the aim of this project. When this experiment was designed, the intention was to use 0.005 g of probiotics as treatment. Thus, the first duplicate experiment (1st and 2nd trials) were conducted using this amount. Consequently, both trials gave negative results, that showed no significance ($p > 0.05$) between the weights of larvae of the different samples as Appendix A, Figures A1.A and A1.B illustrate. The data also showed contradictory results, where larvae feeding on HSD had higher average weight values than the ones feeding on LSD in the

1st trial, and vice versa in the 2nd trial. Hence, the samples were disposed of, the experiment was discontinued, and the method was modified to ensure maximum effects of probiotics and diet (refer to materials and method for more details).

Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (individual results)

The data obtained from the triplicate (3rd, 4th and 5th trials) showed some consistency in terms of weight differences between the controls. Except for adult samples from the 3rd trial, larvae and flies feeding on LSD had higher weights than the ones feeding on HSD, as Appendix A, Figures A1.C - A1.H display. However, when statistical tests were conducted, none of these weight differences between the controls gave any significance (p>0.05). When comparing the weights of treated *Drosophila* and controls, the results did show significance (p<0.05) with larvae being more affected than adult flies. All probiotics showed significant results (p<0.05) at different trials. However, only three probiotic strains (*L. plantarum*, *L. acidophilus* and *B. lactis*) remained constant in terms of significance among the triplicate (3rd, 4th and 5th trials) at larval stage (Appendix A, Figures A1.C, A1.E and A1.G). Only *L. plantarum, L. paracasei and B. animalis* were "significantly" lower than both controls at larval stage (Appendix A, Figures A1.C and A1.G), as the other treatments were "significantly" lower than LSD control only.

In trial 3, larvae treated with *B. animalis* were more affected in terms of weight "reduction" out of the three, where the ones treated with *L. acidophilus* were the least affected. In trial 4, larvae treated with *B. lactis* had the least average weight, where the ones treated with *L. plantarum* and *L. acidophilus* had the highest average weights out of the three probiotics. In trial 5, *L. plantarum* was the most effective treatment in "reducing" larvae weights, where *L. acidophilus* and *B. breve* was the least (Appendix A, Figures A1.C, A1.E and A1.G).

At the adult stage, on the other hand, no consistent results can be observed, and only two probiotics (*B. animalis* and *B. breve*) showed significance (p<0.05) in (3rd and 5th trials), each in one trial respectively (Appendix A, Figures A1.D and A1.H). Both treatment samples, in this case, had significantly higher average weights (p<0.05) than HSD samples, and only *B. animalis* treated sample was significantly higher than both, HSD and LSD controls (p<0.05). Interestingly, both treatment samples were also significantly different from the controls when they were at the larval stage.

Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (combined results)

When combining (averaging) the results of the biological triplicate, all treated larva samples exhibited significantly lower average weights (p<0.05) when compared to the LSD sample (Figure 2A). The treatment groups had also ''non-significant'' lower weights (p>0.05) when compared to the HSD sample. *L. paracasei*, *L. plantarum* and *B. lactis* samples had the lowest weight averages, *L. acidophilus* sample, though, had the highest average weights. Adult flies nonetheless, did not show any significant deviations (p>0.05) from both controls (Figure 2B). Nevertheless, one interesting observation came from the adult sample treated with *B. animalis*, which had a higher average weight than all the other samples, including the controls.
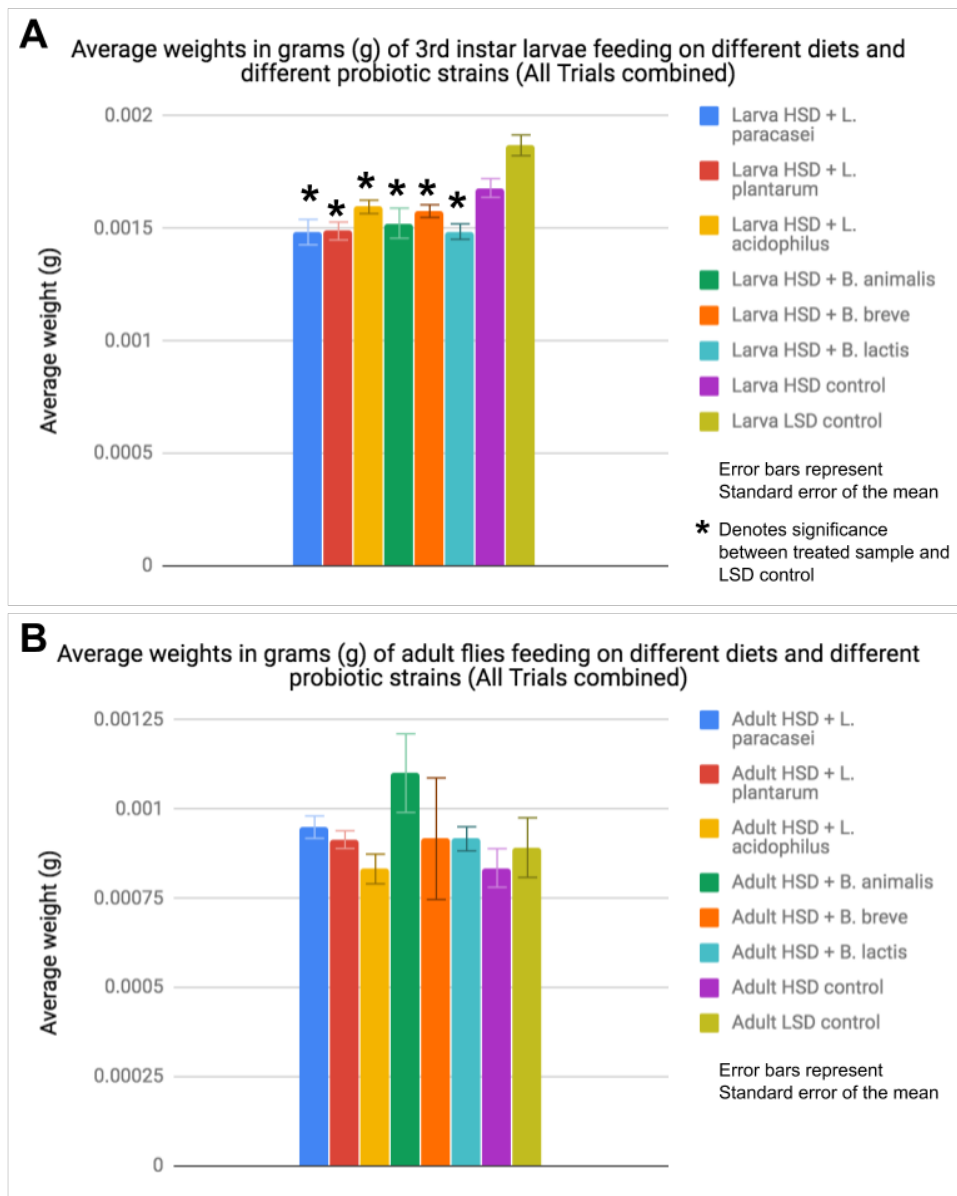
Figure 2. A: Average weight in grams (g) of larvae feeding on HSD, LSD and HSD with treatments. All larvae treatment groups showed significant results (denoted by Asterisk *) when compared to LSD sample (One-Way ANOVA and post hoc Tukey, $p<0.05$). All data were normally distributed (Shapiro-Wilk, $p>0.05$) and variances were equal (Levene's test, $p>0.05$). B: Average weight in grams (g) of adult flies feeding on HSD, LSD and HSD with treatments. No significant differences between controls and adult samples (One-Way ANOVA, $p>0.05$ and Kruskal-Wallis, $p>0.05$). All data were normally distributed (Shapiro-Wilk, $p>0.05$) except for *B. breve* treated sample, due to insufficient data collected. Variances were not equal (Levene's test, $p<0.05$).

Length measurements

Trials 1 and 2 using method 1 (0.005 g of probiotics) (individual results)

Length is another biological marker used to investigate insulin resistance in this experiment. The duplicate (1st and 2nd trials) showed no significant results ($p>0.05$) between the different treatment samples and controls (Appendix A, Figures A2.A and A2.B). The data also showed contradictory results, where larvae on HSD had higher average length values than the ones feeding on LSD in the 1st trial, and vice versa in the 2nd trial. Thus, these trials were discontinued and improved later on.
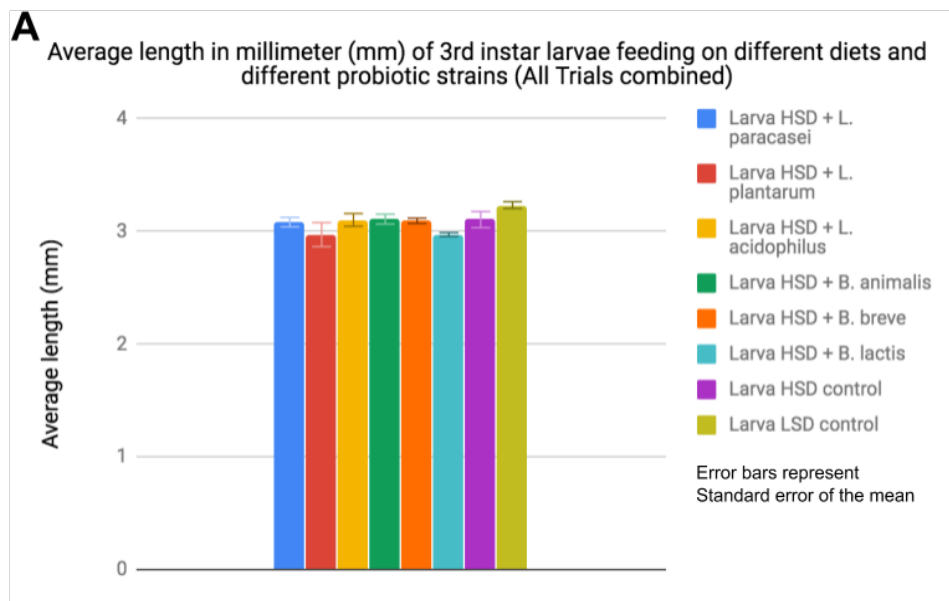
Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (individual results)

Trials 3, 4 and 5 indicate increased lengths in larvae feeding on LSD in comparison with larvae feeding on HSD (Appendix A, Figures A2.C, A2.E and A2.G). In the 5th trial, larvae treated with *B. lactis* and *L. plantarum* exhibited significant decrease lengths ($p < 0.05$) when compared to larvae feeding on LSD, with the latter probiotic having a more significant effect than the former (Appendix A, Figure A2.G). No other significant results were observed among larvae in other trials.

When analysing adult *Drosophila* results, 3rd and 4th trials display higher average lengths of flies feeding on HSD than the ones feeding on LSD (Appendix A, Figures A2.D and A2.F). However, in trial 5 the reverse is shown, as the LSD sample had higher average length than the HSD sample (Appendix A, Figures A2.H). Still, none of these results is considered significant ($p > 0.05$), when statistical tests were performed. Flies treated with *L. plantarum*, *B. animalis* and *L. paracasei* however, exhibited significantly higher average lengths ($p < 0.05$) than the ones feeding on LSD in the 3rd trial, with *L. plantarum* being the most effective and *L. paracasei* being the least effective of the three (Appendix A, Figures A2.D). Flies of trials 4 and 5, did not show any significant difference ($p > 0.05$) in average length between the treated samples and controls (Appendix A, A2.F and A2.H).

Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (combined results)

Combining the results of the triplicate did not produce any significance ($p > 0.05$) as Figure 3A, and 3B illustrate. Moreover, flies and larvae samples feeding on LSD only had higher average lengths than the ones feeding on HSD.

A Average length in millimeter (mm) of 3rd instar larvae feeding on different diets and different probiotic strains (All Trials combined)
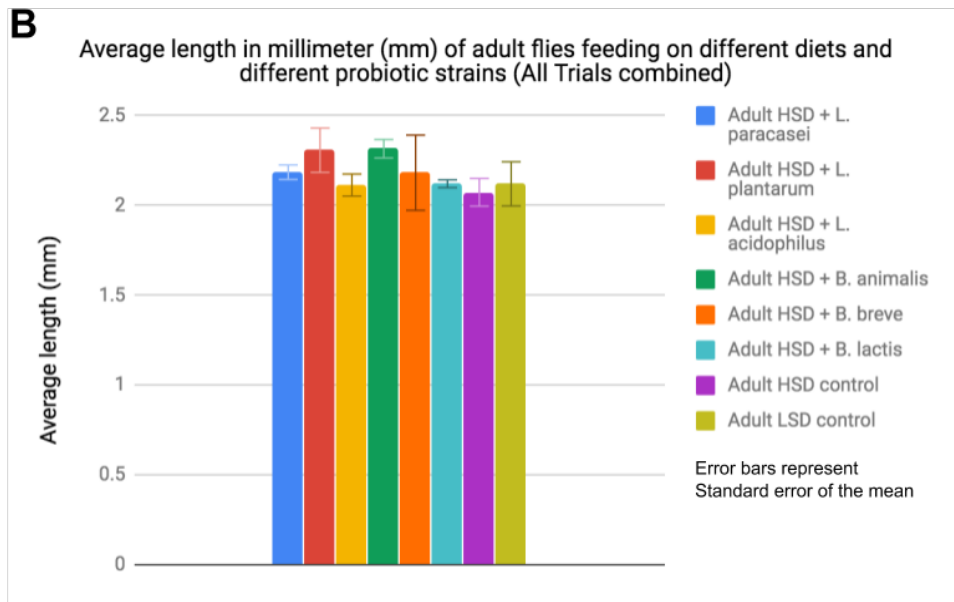
Figure 3. A: Average length in millimetres (mm) of larvae feeding on HSD, LSD and HSD with treatments. No significant differences between controls and larvae samples (One-Way ANOVA, p>0.05 and Kruskal-Wallis, p>0.05). All data were normally distributed (Shapiro-Wilk, p>0.05) except for the HSD sample (Shapiro-Wilk, p<0.05). Variances were equal (Levene's test, p>0.05). B: Average length in millimetres (mm) of adult flies feeding on HSD, LSD and HSD with treatments. No significant differences between controls adult and larvae samples (One-Way ANOVA, p>0.05 and Kruskal-Wallis, p>0.05). All data were normally distributed (Shapiro-Wilk, p>0.05) except for *B. animalis* due to insufficient data collected, and *B. lactis* (Shapiro-Wilk, p<0.05). Variances were not equal (Levene's test, p<0.05).

Carbohydrates measurements

Glucose, glycogen and trehalose levels were measured using whole-body tissue (whole larva/whole fly) in order to assess the viability of the weight and length measurements. The results obtained from each trial of the biological triplicate were insufficient for statistical tests (Appendix A, Figures A3.A - A3.R). Thus, all results were combined, and outliers were eliminated to carry out such tests and to determine if there were actual significant differences (p<0.05) between control and treated samples (Figures 4, 5 and 6). The protocols were not carried out using the 1st and 2nd trials as they were discontinued after the weight and length measurements gave insignificant results (p>0.05).

Glucose protocol: Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (combined results)

When the combined results of the triplicate were plotted, flies and larvae feeding on LSD had slightly higher whole-body glucose concentration than the ones feeding on HSD. Larvae treated with *B. animalis*, *B. breve* and *B. lactis* had higher concentrations of glucose than the rest (Figure 4A). High concentrations of glucose were also observed in adults treated with *L. acidophilus, L. animalis, B. breve* and *B. lactis* (Figure 4B). Nonetheless, none of these values displayed any significance (p>0.05) when statistical tests were performed.
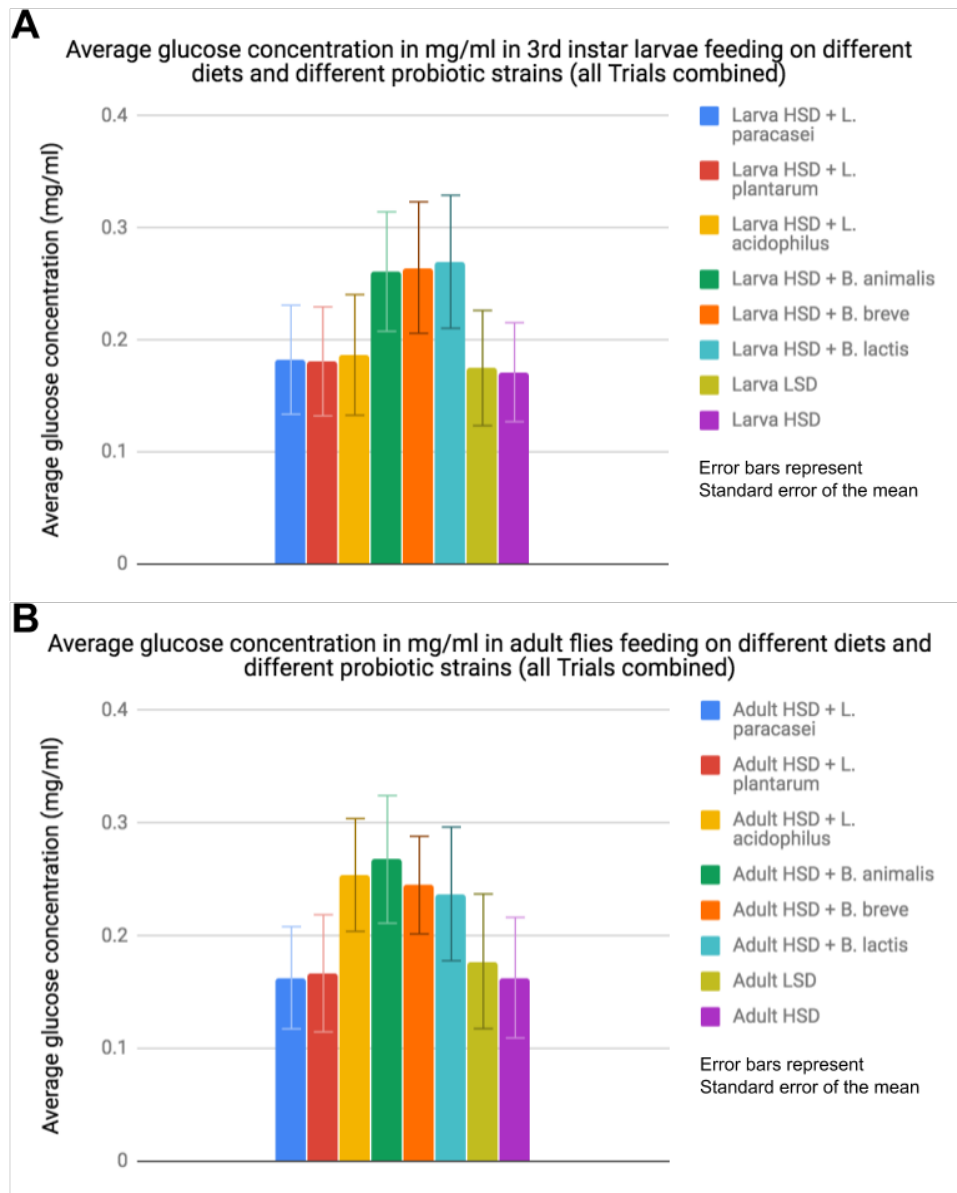
Figure 4. A: Average glucose concentration in mg/ml of 3rd instar larvae feeding on HSD, LSD and HSD with treatments. B: Average glucose concentration in mg/ml of adult flies feeding on HSD, LSD and HSD with treatments. No significant differences between controls and adult/larvae samples (One-Way ANOVA, p>0.05 and Kruskal-Wallis, p>0.05). Most data were normally distributed (Shapiro-Wilk, p>0.05). All variances were equal (Levene's test, p>0.05).

Glycogen protocol: Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (combined results)

Similar measures were taken when assessing the concentration of glycogen in different samples. The combined triplicate results showed that flies and larvae feeding on HSD had higher glycogen concentrations than the ones feeding on LSD (Figures 5A and 5B). Larvae and adult flies treated with *L. paracasei* and *L. plantarum* strains had the highest glycogen content, and *B. breve* had the lowest glycogen content at both developmental stages. Nevertheless, none of these differences was significant (p>0.05) as statistical tests indicate.
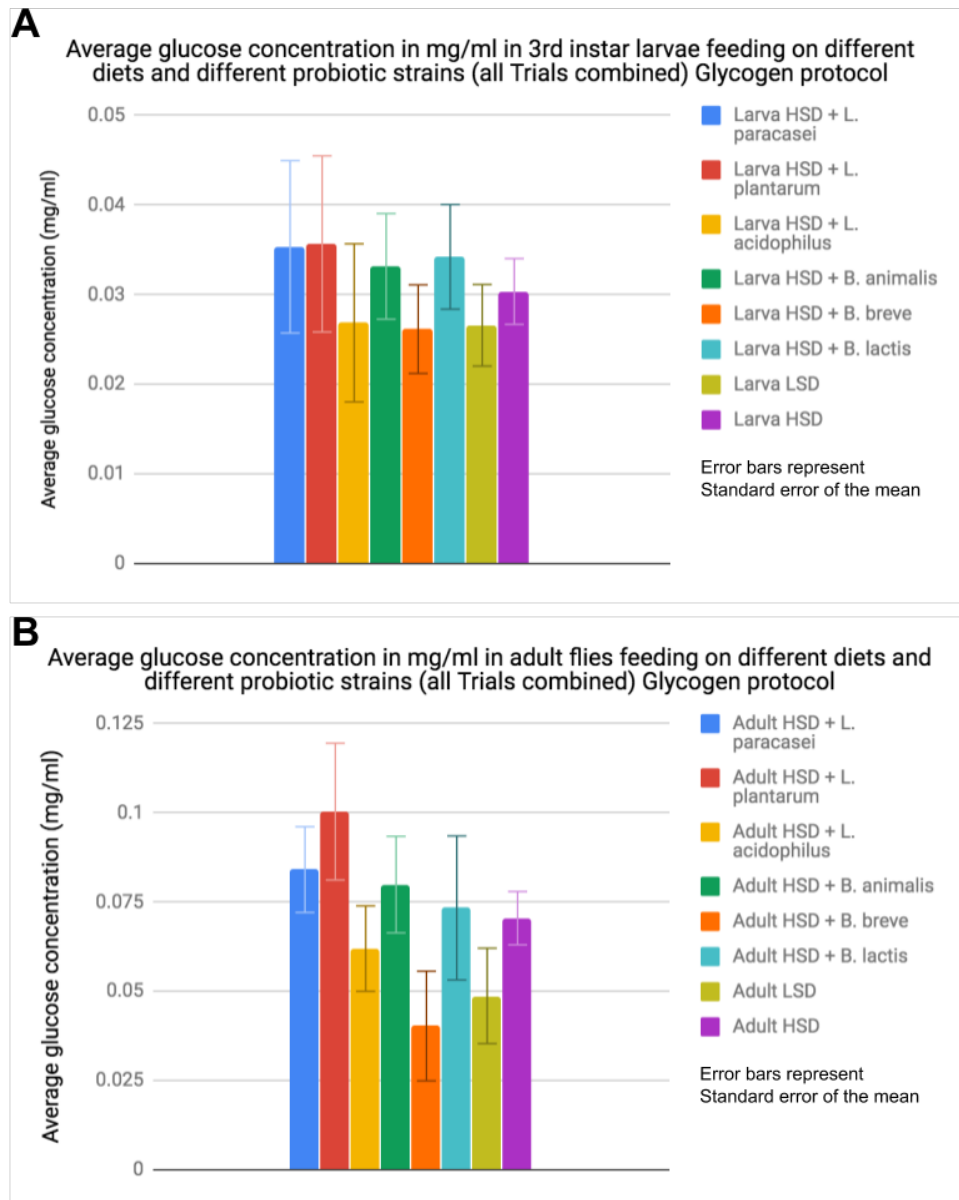
Figure 5. A: Average glucose (glycogen) concentration in mg/ml of 3rd instar larvae feeding on HSD, LSD and HSD with treatments. B: Average glucose (glycogen) concentration in mg/ml of adult flies feeding on HSD, LSD and HSD with treatments. No significant differences between controls and adult/larvae samples (One-Way ANOVA, $p>0.05$ and Kruskal-Wallis, $p>0.05$). Most data were normally distributed (Shapiro-Wilk, $p>0.05$). All variances were equal (Levene's test, $p>0.05$).

Trehalose protocol: Trials 3, 4 and 5 using method 2 (0.025 g of probiotics) (combined results)

Trehalose concentrations in adult flies feeding on both HSD and LSD were the same, wherein larval stage, HSD samples had slightly more of trehalose than LSD samples. Overall, the concentrations of trehalose in treated samples were higher than the ones in controls, except for larvae treated with *L. paracasei* and *B. lactis*. Adults treated with the latter, however, had the highest concentration of whole-body trehalose. Larvae and adult flies treated with *L. plantarum* and *B. breve* also had high levels of trehalose at both stages (Figures 6A and 6B).
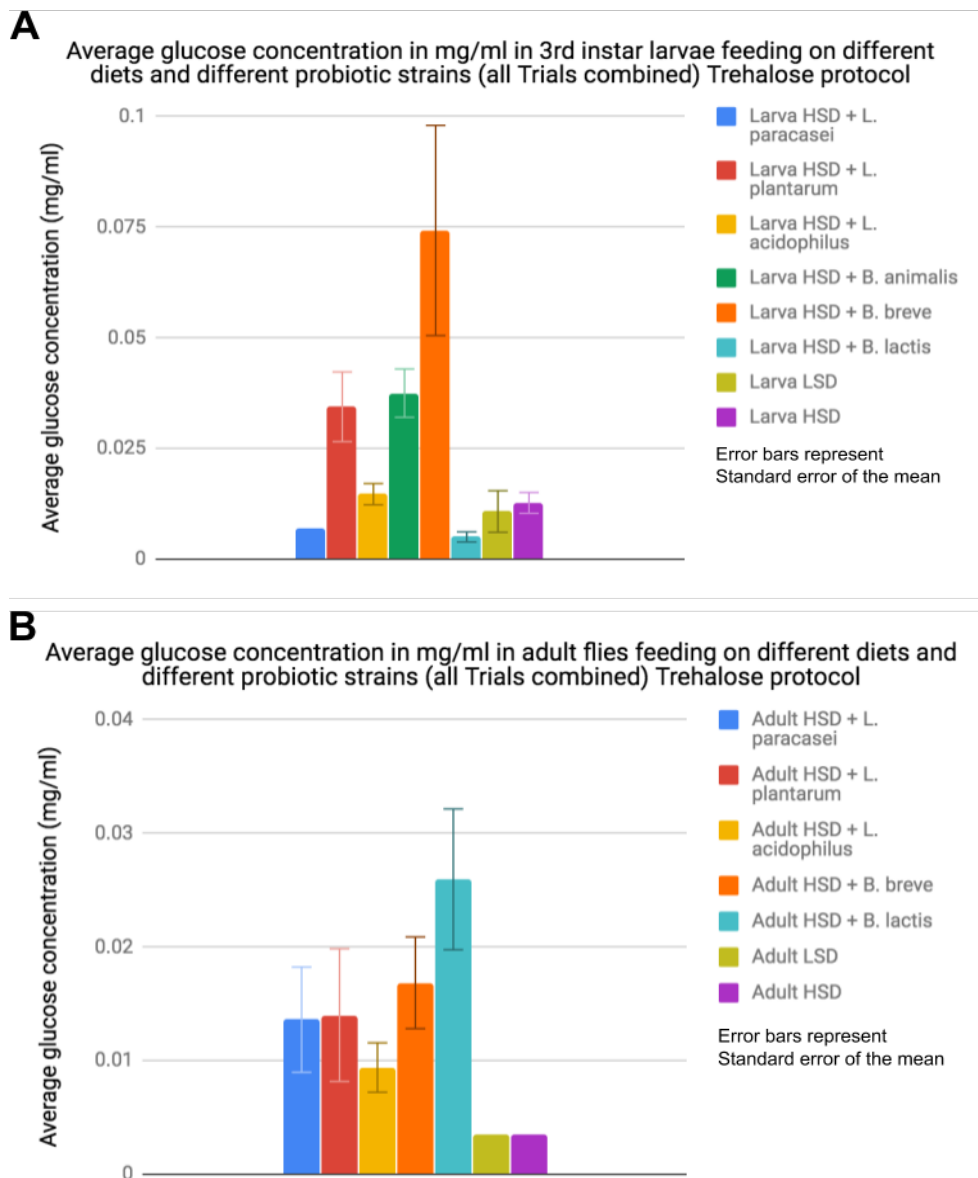
Figure 6. A: Average glucose (trehalose) concentration in mg/ml of 3rd instar larvae feeding on HSD, LSD and HSD with treatments. B: Average glucose (trehalose) concentration in mg/ml of adult flies feeding on HSD, LSD and HSD with treatments (*B. animalis* was excluded from the chart due to insufficient results obtained). No significant differences between controls and adult/larvae samples (One-Way ANOVA, p>0.05 and Kruskal-Wallis, p>0.05). Most of the data were normally distributed (Shapiro-Wilk, p>0.05). Variances were equal (Levene's test, p>0.05) except for glucose concentrations (trehalose protocol) in larvae (Levene's test, p<0.05). No standard deviations were produced for glucose concentrations (trehalose protocol) in adult HSD and LSD samples due to the insufficient number of results collected. Similar case with glucose (trehalose) concentration in larva treated with *L. paracasei*.

Real-time PCR

qPCR was conducted with trial 3 samples using method 2 (0.025 g of probiotics)

PEPCK, *Fbp* and *Hex-t1* had lower expression in larvae feeding on LSD than in the ones feeding on HSD, wherein *Zw*, the expression was lower in HSD sample, when compared to LSD sample. *L.*

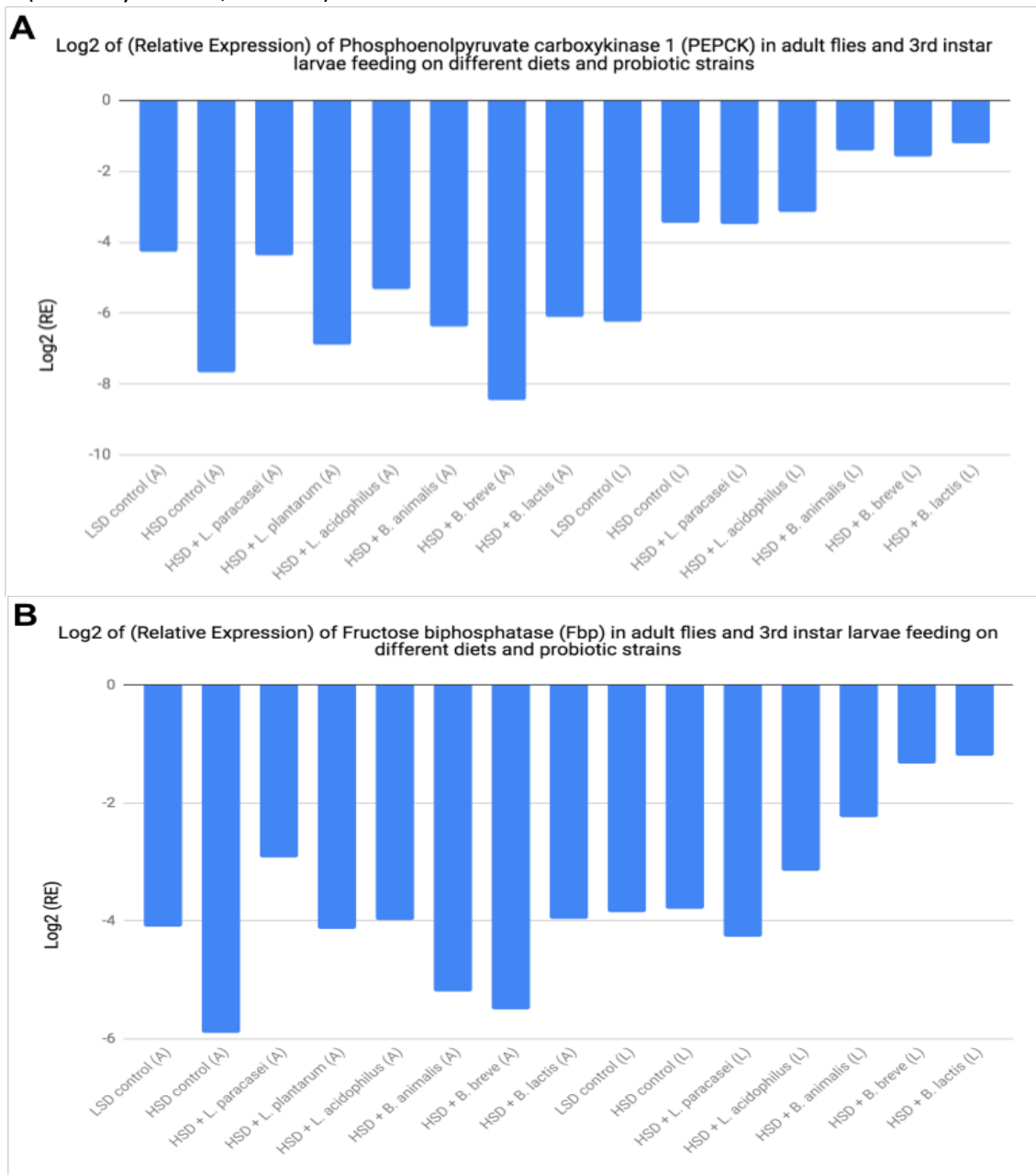*paracasei* treated larva sample was the closest to LSD sample in terms of expression levels in all the genes examined, where *B. lactis* was the furthest (Figures 7A-7D).

In adult flies on the other hand, all four genes had lower expression in HSD control sample when compared to the LSD sample. Treatment samples *L. paracasei*, *L. acidophilus* and *B. lactis* had the closest gene expression levels to LSD sample in all four genes. Interestingly, *B. breve* treated adult sample had the lowest expression values out of all treatment samples in all four genes (Figures 7A-7D).

The relative expression of these genes cannot be validated in terms of significance, as the experiment was only carried out once. Thus, no statistical tests were performed using the ΔCt or ΔΔCq data. Nonetheless, a rough "non-reliable" estimation of significance was carried out using Ct values, to compare the effects of treatments on different genes. The result was significant in this case (Two-way ANOVA, P<0.000).

A Log2 of (Relative Expression) of Phosphoenolpyruvate carboxykinase 1 (PEPCK) in adult flies and 3rd instar larvae feeding on different diets and probiotic strains

B Log2 of (Relative Expression) of Fructose biphosphatase (Fbp) in adult flies and 3rd instar larvae feeding on different diets and probiotic strains

C Log2 of (Relative Expression) of Hexokinase type 1 (Hex-t1) in adult flies and 3rd instar larvae feeding on different diets and probiotic strains

D Log2 of (Relative Expression) of Glucose-6-phosphate dehydrogenase G-6-PD (Zw) in adult flies and 3rd instar larvae feeding on different diets and probiotic strains
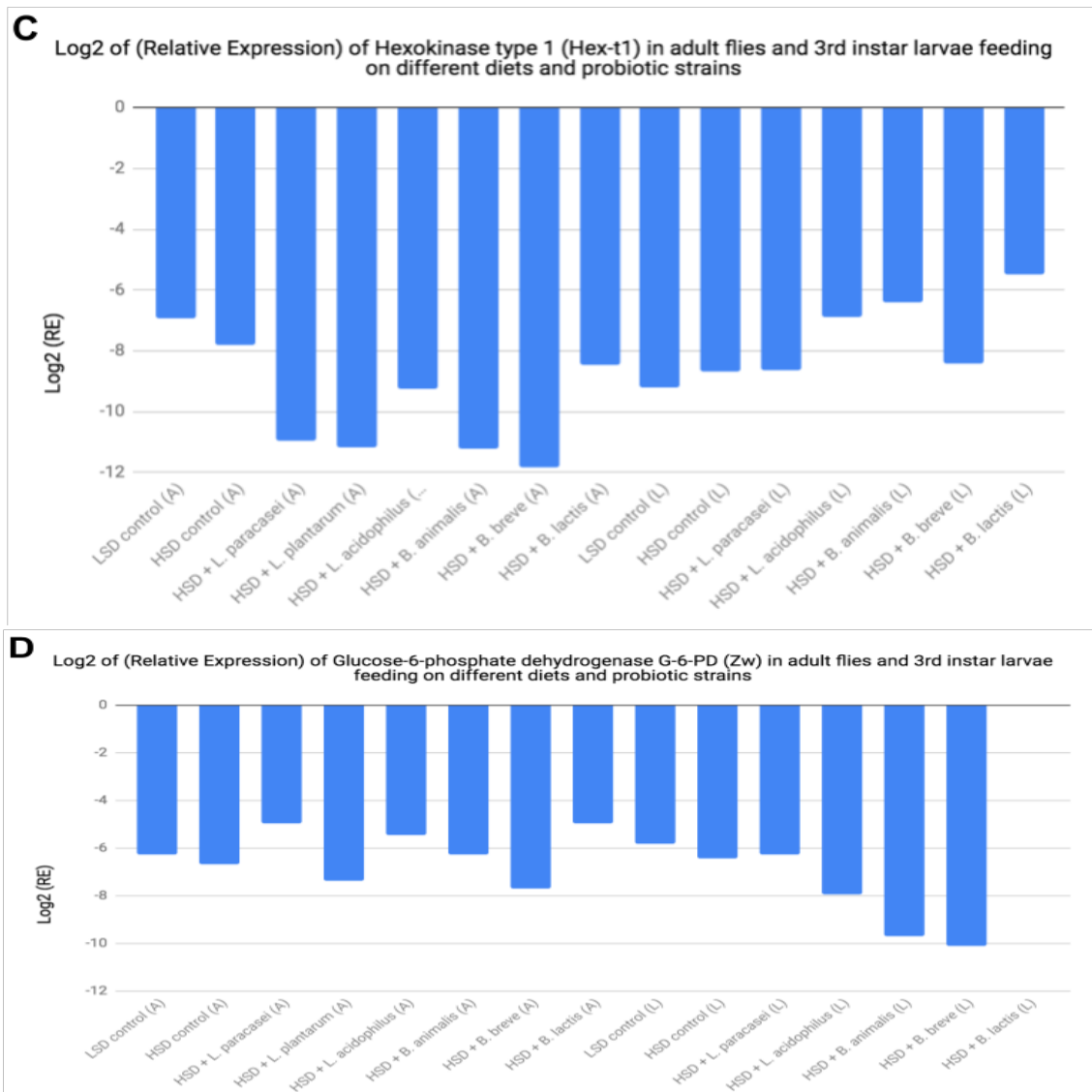
Figure 7. Log$_2$ of (Relative Expression) of A: *PEPCK* expression in adult flies and 3rd instar larvae feeding on HSD, LSD and HSD with probiotic treatments. B: *Fbp* expression in adult flies and 3rd instar larvae feeding on HSD, LSD and HSD with probiotic treatments. C: *Hex-t1* expression in adult flies and 3rd instar larvae feeding on HSD, LSD and HSD with probiotic treatments. D: *Zw* expression in adult flies and 3rd instar larvae feeding on HSD, LSD and HSD with probiotic treatments (*B. lactis* sample expression in larvae was not plotted for this gene, due to the lack of Ct values of *Zw* for this sample*. L. plantarum* expression in larvae was not plotted for all genes, due to the lack of Ct values of the reference gene, thus, normalization was not carried out for this sample in the first place). All expression data were normalized using the values of the reference gene *RPL32*. Statistical test of Ct values (Two-way ANOVA, P<0.000), still, results cannot be statistically validated. (Appendix C for raw data)

Longevity measurements

Drosophila samples using method 1 (0.005 g of probiotics)

One of the issues encountered when analysing the initial version of the Kaplan-Meier survival curve, combining results from all samples, was the overlapping of curves, which make it difficult to interpret (Appendix B). Hence, separate graphs for each sample was made to make the readings clear (Figure 8A-8E). A general statistical test between all the samples under study, showed a

significant difference between the curves (Log-rank (mantel-Cox) test, (p<0.05)). The same test was run to compare between individual samples and controls, which showed that *B. lactis* treated sample had significantly higher survival rate than HSD sample (p<0.05) (Figures 8C and 8D). The sample treated with *L. acidophilus*, on the other hand, had a significantly lower survival rate when compared to LSD control (p<0.05) (Figures 8B and 8E). The results produced were based on around 60 days of observations.
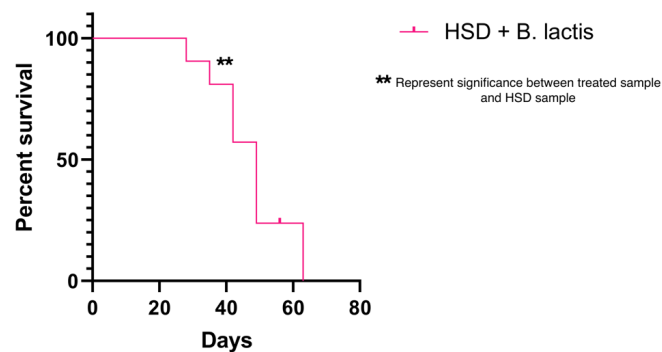
A
Kaplan-Meier survival plot of flies feeding on High sugar diet and Lactobacillus paracasei
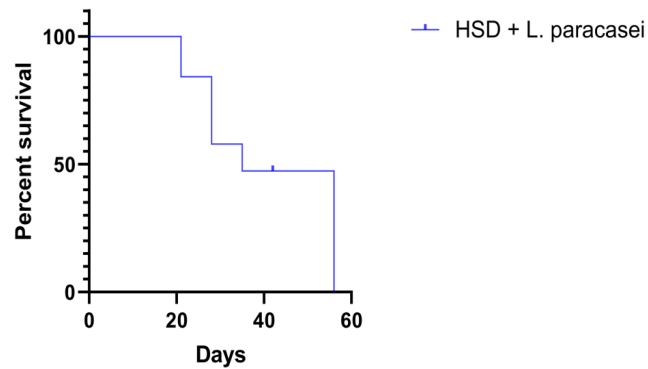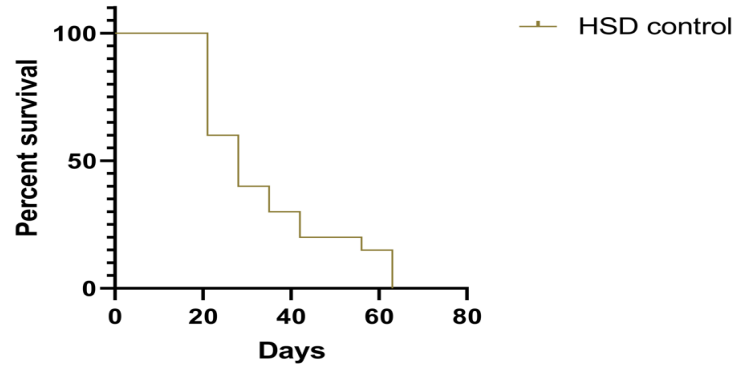

B
Kaplan-Meier survival plot of flies feeding on High sugar diet and Lactobacillus acidophilus
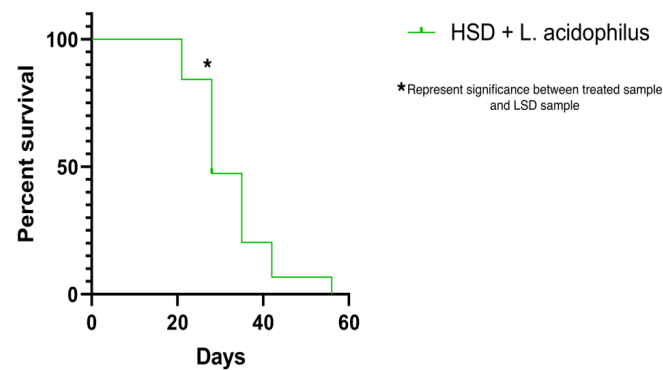
C
Kaplan-Meier survival plot of flies feeding on High sugar diet and Bifidobacterium lactis

D

Kaplan-Meier survival plot of flies feeding on only High sugar diet

E

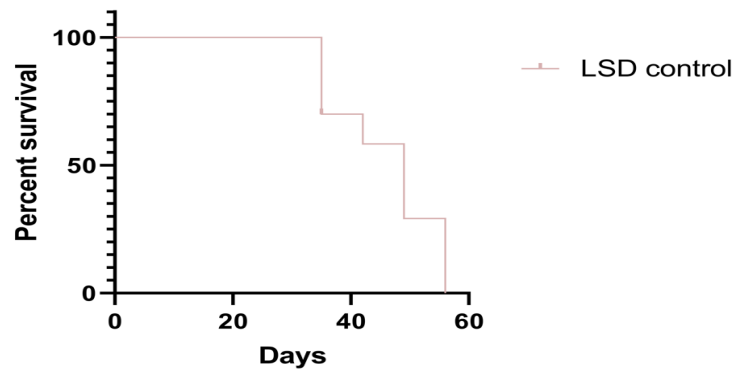**Kaplan-Meier survival plot of flies feeding on only Low sugar diet**

Figure 8. Survival curve (Kaplan-Meier) of adult flies feeding on A: HSD and *L. paracasei*. B: HSD and *L. acidophilus* C: HSD and *B. lactis*. D: HSD only. E: LSD only. * Asterisk denotes significance (p<0.05) and is determined using the Log-rank (mantel-Cox) test.

Normalization of results

Normalization of trials 3, 4 and 5 results, method 2 (0.025 g of probiotics)

The collected data of length and carbohydrate levels were normalized using weight in order to produce comparable ratios between the different samples. HSD samples data were also used in the normalization process, to exclude the effects of the diet itself from each treatment sample and to act as a calibrator (i.e. to compare samples to it). The normalization was carried out for each trial individually and then the results were averaged and plotted.

The normalization was carried out using the equations:

Ratio difference of (length* weight (i.e. size)) between all samples and HSD control sample =

$$\frac{Average\ length\ of\ the\ sample\ (mm) \times Average\ weight\ of\ the\ sample\ (g)}{Average\ length\ of\ the\ HSD\ control\ sample\ (mm) \times Average\ weight\ of\ the\ HSD\ control\ sample\ (g)} \tag{1}$$
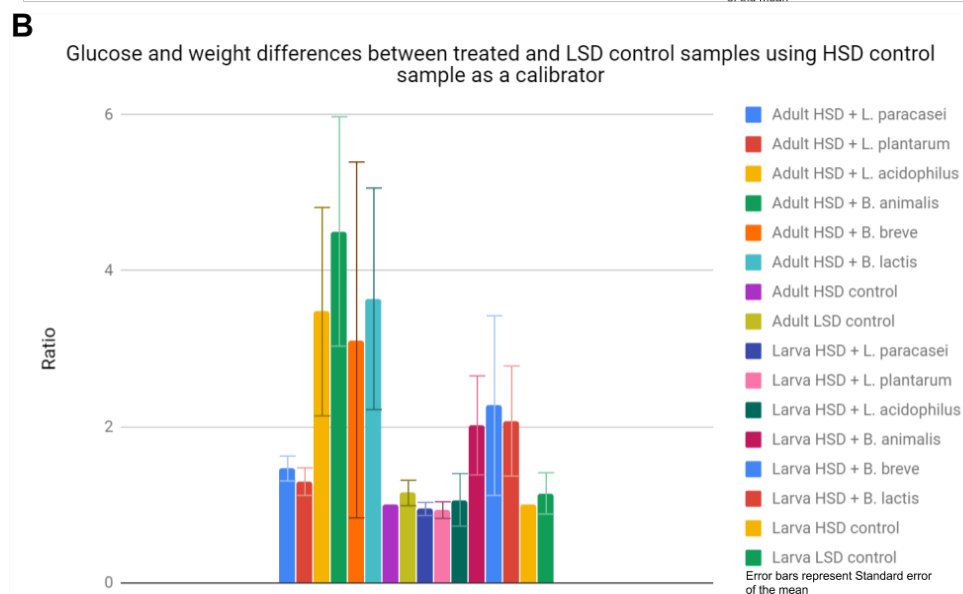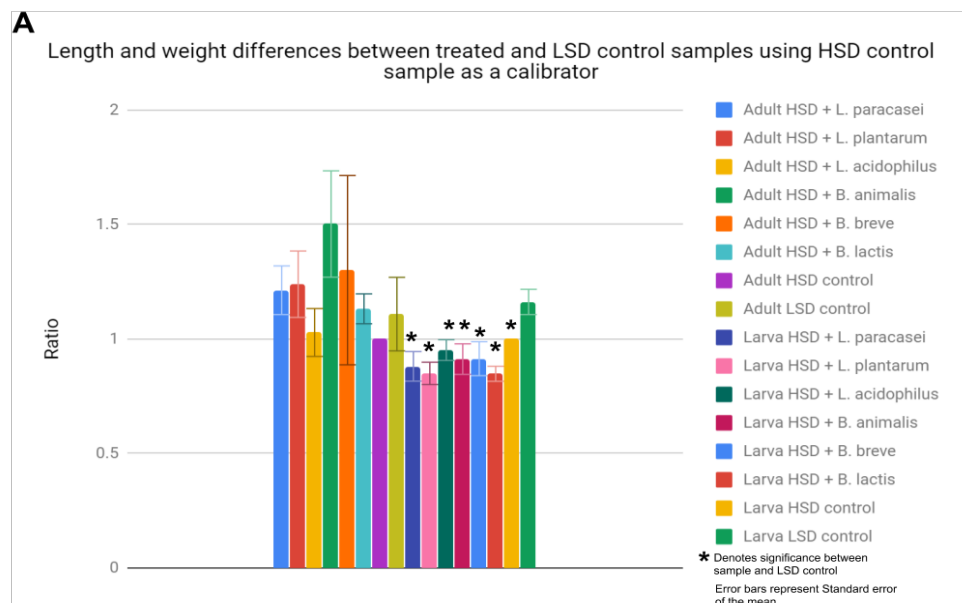
Ratio difference of (glucose* weight (i.e. whole-body glucose levels)) between all samples and HSD control sample =

$$\frac{Average\ glucose\ levels\ of\ the\ sample\ (mg/ml) \times Average\ weight\ of\ the\ sample\ (g)}{Average\ glucose\ levels\ of\ the\ HSD\ control\ sample\ \left(\frac{mg}{ml}\right) \times Average\ weight\ of\ the\ HSD\ control\ sample\ (g)} \tag{2}$$

19

Ratio difference of (glucose* weight for glycogen protocol (i.e. whole-body glycogen levels)) between all samples and HSD control sample =

$$\frac{Average\ glucose\ levels\ of\ the\ sample\ (mg/ml) \times Average\ weight\ of\ the\ sample\ (g)}{Average\ glucose\ levels\ of\ the\ HSD\ control\ sample\left(\frac{mg}{ml}\right) \times Average\ weight\ of\ the\ HSD\ control\ sample\ (g)} \quad (3)$$

Generally speaking, all the results obtained post-normalization (Figure 9A-9C), match the results collected pre-normalization (Figures 2-5), which are presented in other parts of results above. The notable differences were the significance of the length data (p<0.05) of larvae when normalized, which was not the case when length measurements were considered ''individually'', and the significance of the HSD control sample (p<0.05) when compared to the LSD control sample (Figure 9A). None of the other normalized data showed any significant deviations from the pre-normalized results presented above. Trehalose results, however, were less credible, as some of the samples had only one plausible reading to use (Appendix A, Figure A3.M - A3.R). Thus, no statistical tests were run using data obtained from these samples, and no reliable normalization procedures were carried out either.

A Length and weight differences between treated and LSD control samples using HSD control sample as a calibrator

Legend:
- Adult HSD + L. paracasei
- Adult HSD + L. plantarum
- Adult HSD + L. acidophilus
- Adult HSD + B. animalis
- Adult HSD + B. breve
- Adult HSD + B. lactis
- Adult HSD control
- Adult LSD control
- Larva HSD + L. paracasei
- Larva HSD + L. plantarum
- Larva HSD + L. acidophilus
- Larva HSD + B. animalis
- Larva HSD + B. breve
- Larva HSD + B. lactis
- Larva HSD control
- Larva LSD control

* Denotes significance between sample and LSD control
Error bars represent Standard error of the mean

B Glucose and weight differences between treated and LSD control samples using HSD control sample as a calibrator

Legend:
- Adult HSD + L. paracasei
- Adult HSD + L. plantarum
- Adult HSD + L. acidophilus
- Adult HSD + B. animalis
- Adult HSD + B. breve
- Adult HSD + B. lactis
- Adult HSD control
- Adult LSD control
- Larva HSD + L. paracasei
- Larva HSD + L. plantarum
- Larva HSD + L. acidophilus
- Larva HSD + B. animalis
- Larva HSD + B. breve
- Larva HSD + B. lactis
- Larva HSD control
- Larva LSD control

Error bars represent Standard error of the mean

C

Glucose and weight differences between treated and LSD control samples using HSD control sample as a calibrator, (Glycogen protocol)

Legend:
- Adult HSD + L. paracasei
- Adult HSD + L. plantarum
- Adult HSD + L. acidophilus
- Adult HSD + B. animalis
- Adult HSD + B. breve
- Adult HSD + B. lactis
- Adult HSD control
- Adult LSD control
- Larva HSD + L. paracasei
- Larva HSD + L. plantarum
- Larva HSD + L. acidophilus
- Larva HSD + B. animalis
- Larva HSD + B. breve
- Larva HSD + B. lactis
- Larva HSD control
- Larva LSD control

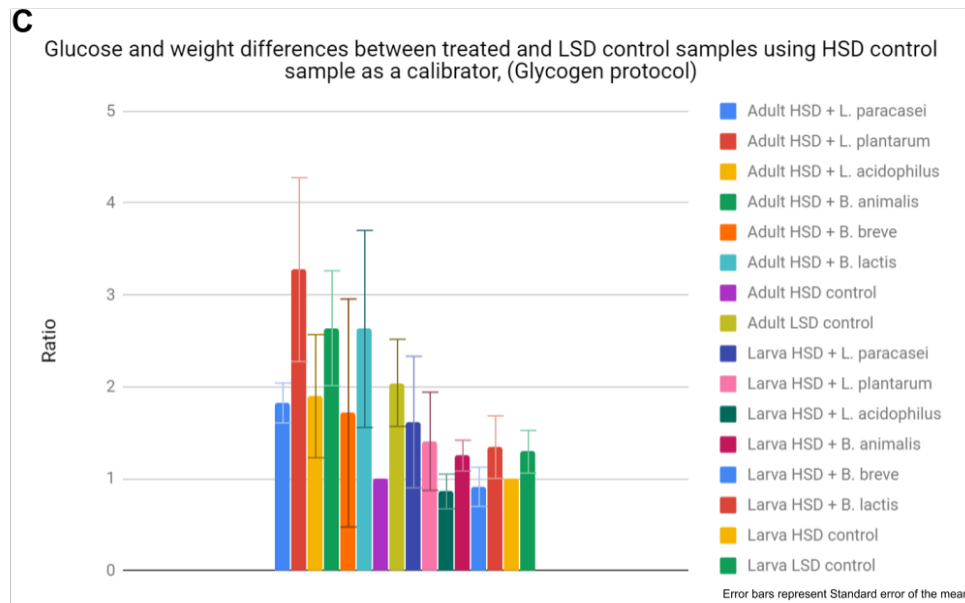Error bars represent Standard error of the mean

Figure 9. Normalized data of A: Length. B: Glucose. C: Glucose for (glycogen protocol). HSD control samples were used as calibrators for comparison between the other samples and are represented in the figures with ratios of 1. Ratios above or below this value, symbolize the magnitude of the increase or decrease from the HSD control sample. * Asterisk denotes significance (p<0.05) between the sample and LSD control sample, and is determined using two-tailed t-test (type 2).

Discussion

Analysis of results

The combination of results obtained from different measurements (weight, length, glucose, glycogen and trehalose) showed no consistent differences between treated samples and controls, besides the fact that most results were insignificant (p<0.05). Nonetheless, minor interesting observations can be seen and discussed. For instance, larvae and adult flies that were feeding on LSD had greater lengths, weights and glucose concentrations than the ones feeding on HSD, although lower glycogen levels at both stages and trehalose levels at larval stage (Figures 2-6). This might have been an outcome of developmental delays, probably through the excessive activation of FOXO (Kramer, Davidge, Lockyer and Stavely 2003), as a consequence of developing insulin resistance. The delay can be seen clearly in Figure 10, as the HSD larvae were still at 1st to 2nd instar, where LSD larvae where already at 3rd instar. Such observations were discussed in multiple articles, including Musselman et al. (2011, 2017), yet none of these papers mentioned higher whole-body glucose levels in LSD samples compared to HSD samples. Logically speaking, these results can be justified, since, in this experiment, whole flies and larvae were used to measure glucose concentrations in different samples. Flies and larvae feeding on LSD were slightly larger than the ones feeding on HSD (Figures 2, 3 and 11). These flies and larvae had more cells and tissues, and therefore, more glucose was required by such organisms. Hence, the high glucose levels in LSD samples compared to HSD samples. Glycogen levels, though, were higher in HSD samples, as the consumption of high quantities of glucose, led to the storage of high quantities of glycogen, thus HSD having more glycogen than LSD samples. Still, these results were not significant to confirm these interpretations (p>0.05).
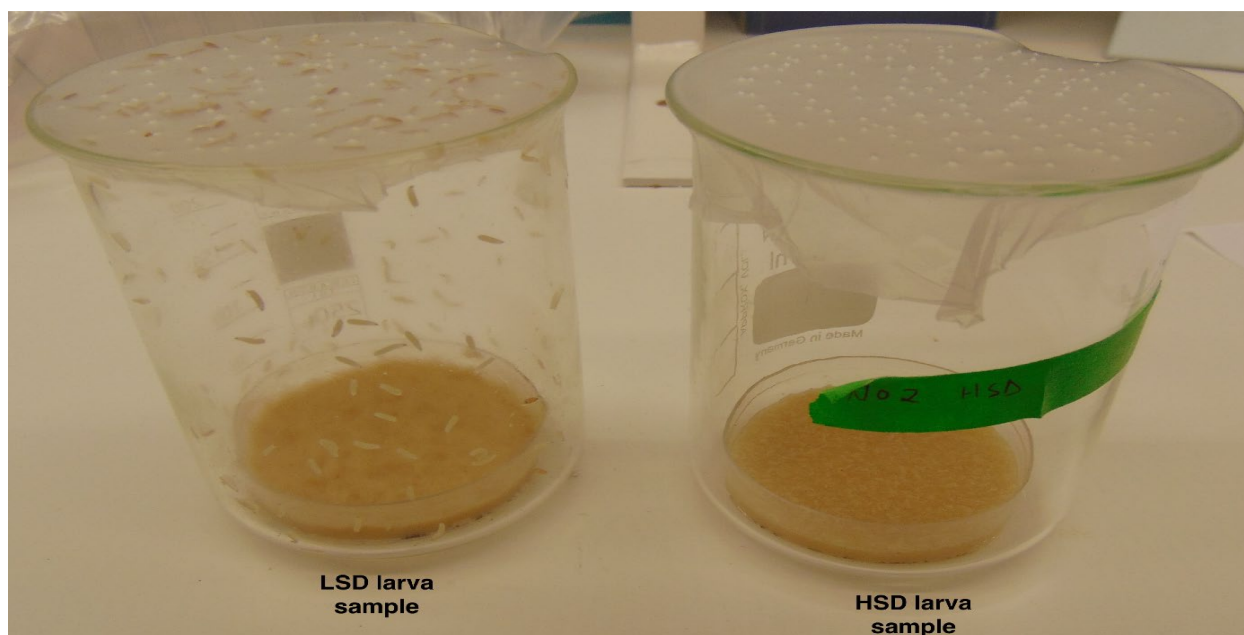
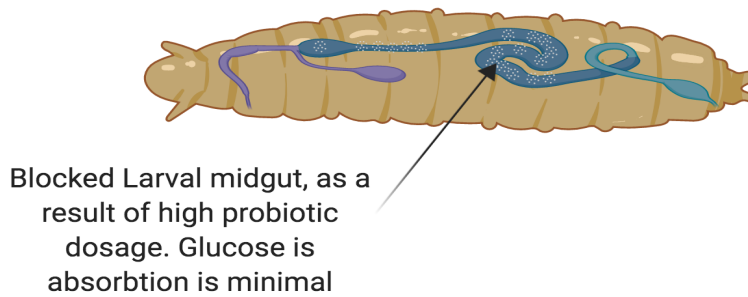Figure 10. LSD and HSD larva samples (4-5 days post eggs hatching).

Figure 11. Male and female flies fed on HSD/LSD, under dissecting microscope (14-16 days post eggs hatching).

Morris et al. (2012) on the other hand, found no significance (p>0.05) in the concentrations of glucose, trehalose and glycogen between female flies feeding on HSD (30% w/V) and LSD (5% w/V), which match the data obtained from this experiment (Figures 4-6). Musselman et al. (2011) however, reported significantly high concentrations of glucose and trehalose in 3rd instar larvae feeding on HSD (1 M) in comparison with the ones feeding on LSD (0.15 M), yet, glycogen concentration was lower in HSD larvae samples than in LSD samples. Nevertheless, these outcomes cannot be compared to this experiment's findings, as Musselman et al. (2011) used hemolymph to carry out these measurements and not whole larvae.
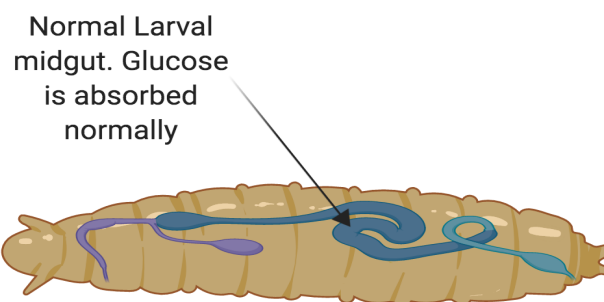
Morris et al. (2012) described an increase in the weight of flies feeding on HSD when compared to the ones feeding on LSD, contradicting Musselman et al. (2011) and this experiment's findings. Moreover, treated larva samples had significantly low average weights (p<0.05) when compared to LSD sample as illustrated in Figure 2, with *L. paracasei* having lower average weight than the other treatments. Such observations might suggest that the treatments did not work, so the HSD caused the insulin resistance, thus delayed the development. Alternatively, it could indicate that the high dosage was blocking the glucose from being absorbed in the midgut, so in a sense, the larvae went through malnutrition which resulted in the significantly low average weights and delayed development (May, Doroszuk and *Zw*aan 2015). The latter explanation could be more logical since the weights of treated samples were even lower than the high sugar control, although not significantly lower (p>0.05). Adult flies, on the other hand, were not significantly affected by the treatments (p>0.05). The fact that adult flies do not feed as much as larvae, and that they were allowed to feed for only 5 days post-hatching from pupa and before collection, could have led to this insignificance. Thus, the treatments did not have the same impact on their physiology. Jones et al. (2018) nonetheless, claimed that the probiotics *B. breve, L. plantarum, L. acidophilus* and *L. paracasei* increased obesity in humans. Such a claim was not found to be true in this project.

When results were normalized, only larva data gave significant results (p<0.05) as indicated in Figure 9A**,** with treated and HSD control samples having significantly lower (length*weight) ratio than LSD sample (p<0.05). These outcomes are mainly due to the significant low weights of the treated samples, when compared to the LSD sample, which decreased the ratio greatly, as the average lengths of the treated samples did not have significant deviations from the LSD or HSD samples. Interestingly, HSD sample did not give significant length or weight results (p>0.05) (Figures 2 and 3), yet when both factors were considered together, a significant outcome was obtained (p<0.05). These results confirm what was discussed above about the treatments not working. The significance, in this case, might be a consequence of either the HSD causing insulin resistance, thus lowering the weights significantly or due to malnutrition, as a consequence of the probiotics obstructing the larval midgut. Hence, less glucose was consumed, as illustrated in Figure 12.

A

Blocked Larval midgut, as a result of high probiotic dosage. Glucose is absorbtion is minimal

B

Normal Larval midgut. Glucose is absorbed normally

Yasser.A

Figure 12. Glucose absorption in larvae with A: Blocked midgut as a consequence of feeding on high dosage of probiotics. B: Normal unblocked midgut. (the larval digestive tract is not illustrated accurately in this Figure)

Interestingly, *B. animalis*, *B. breve* and *B. lactis* treated samples shared common but non-significant high (glucose*weight) ratios at both developmental stages when compared to the other samples, including the controls (Figure 9B). Such observations might indicate that larvae and adult flies in samples containing these specific treatments, did not consume a lot of the probiotics compared to the other samples with other treatments, probably due to the probiotics' taste or even smell. Hence, spending most of the time feeding on the HSD instead, leading to the development of insulin resistance. Hence, the low weights, yet high whole-body glucose. However, this interpretation contradicts with the results obtained from the HSD control sample, since larvae from this sample had not only low weights but also low whole-body glucose. Another plausible explanation is that larvae and adults did not develop insulin resistance, but instead, these probiotics did not obstruct the absorption of glucose as much as the others. In other words, the malnutrition was mild and not as severe as in other samples. Therefore, glucose levels were high, but the average weights were still significantly lower than LSD sample. Moreover, Yadav, Jain and Sinha (2007) suggested that *L. acidophilus* reduces hyperglycaemia significantly. This observation was only found at the larval stage with the sample treated with *L. acidophilus* having lower (glucose*weight) ratio than LSD control sample, although having the same ratio as HSD control sample (Figure 9B). Still, this experiment has failed to prove if such a claim is significant and whether the low ratio was a result of the probiotic effects or the malnutrition.

Glycogen and trehalose data, however, were not good enough to be considered, since their protocols did not work as expected. Thus, the primary protocol was adjusted as described in the materials and method section. Furthermore, not all results produced were plausible as some of the samples gave negative concentration values (excluded from the final analysis), while others gave only one value which was not enough to carry out statistical tests or normalization procedures. Nevertheless, minor observations can still be discussed such as samples treated with *B. animalis* and

B. lactis have higher glycogen and glucose levels than most of the other treated samples, at both larval and adult stages (Figures 9B and 9C). These results although not significant ($p>0.05$), but might hint that the proposed explanation above that the delay in development was because of malnutrition and not insulin resistance might not be accurate for all the probiotics used. Furthermore, if malnutrition was the cause for the significantly reduced size of larvae, then why do larvae treated with *B. animalis* and *B. lactis* have high glucose and glycogen levels in the first place, assuming that the glucose and adjusted glycogen protocols worked properly. The only plausible explanation, in this case, is that larva samples treated with these two probiotics did not develop insulin resistance. In fact, the larvae might have had functional insulin signalling, hence the high whole-body glucose and glycogen levels. The significantly reduced size however, could be a result of other unknown lurking factors. Nevertheless, the insignificance of the carbohydrate measurement data makes it hard to consider these results thoughtfully.

As mentioned before, *PEPCK* is a gene involved in gluconeogenesis. It is typically repressed by Mondo-Mlx target Cabut when *Drosophila* are fed LSD (Mattila and Hietakangas 2017) since glucose can be found in excess intracellularly due to functional insulin signalling. Thus, no need to produce more glucose (no need for gluconeogenesis). Upon insulin resistance, however, *PEPCK* was supposed to be activated and overexpressed by FOXO, which is not always the case in the qPCR results obtained. Figure 7A shows an overall low expression of *PEPCK* when compared to the housekeeping gene, as expected, since this gene is meant to be repressed most of the time. The interesting observation was the differences in the levels of expression between the controls at adult and larval stages. For instance, the gene is less expressed in larvae feeding on LSD than larvae feeding on HSD, which matches (Mattila and Hietakangas 2017) findings. However, the reverse happens at the adult stage, as *PEPCK* is less expressed in flies feeding on HSD than the ones feeding on LSD.

Likewise, *Fbp* is another gene involved in gluconeogenesis (Visinoni et al. 2012). The gene expression was supposed to increase upon the development of insulin resistance, yet Figure 7B shows that the gene is less expressed in HSD feeding flies than in LSD feeding flies, although the reverse takes place at larva stage. Such results were suggesting an increased production of glucose when adult flies were feeding on LSD, contradicting the predicted outcomes and the findings reported by Mattila and Hietakangas (2017) and Visinoni et al. (2012). One explanation for such results could be the difference in the amount of food consumed by *Drosophila* at both developmental stages. Furthermore, larvae consume food constantly, where adult flies spend only seconds feeding. Consequently, larvae are more prone and sensitive to the different diets, since they spent a week feeding on each diet before the different protocols were conducted. Adult flies, on the other hand, had only five days to feed before samples were collected for the protocols. Thus, the diet did not impact them as much as the larvae (Wong, Piper, Wertheim and Partridge 2009).

Zw and *Hex-t1* nonetheless, are both involved in glucose catabolism. Thus, their expression should be increased in LSD feeding flies and deceased in HSD feeding flies if insulin resistance develops (Xu, Osborne and Stanton 2005). Moreover, *Hex-t1* functions in phosphorylating hexoses, including glucose, where *Zw* is involved in pentose phosphate pathway as previously described. The latter also has essential contributions to the production of triglycerides (lipogenesis). Figure 7C shows a lower expression of *Hex-t1* in adult flies feeding on HSD when compared to flies feeding on LSD, where the reverse takes place at larval stage. *Zw* however, exhibits a lower expression in both larvae and adult flies feeding on HSD compared to the ones feeding on LSD (Figure 7D). These findings suggest that more triglycerides were made from glucose in flies and larvae feeding on LSD than the ones feeding on HSD and that more glucose was being phosphorylated in LSD adults' sample than in HSD adults' sample, and vice versa in larvae case.

When evaluating the qPCR data for the treated samples, three probiotics gave relatively close expression values when compared to the LSD control, though the results were not statistically tested

in a sufficient way, as previously mentioned. These include *L. paracasei, L. acidophilus* and *B. lactis* at adult stage and *L. paracasei* at larval stage. The measurement of how effective a treatment is, was based on how close the expression values of the treatment samples to the LSD sample (Figure 7). Park, Yang and Kim (2017) reported that *L. acidophilus* reduces the expression of *PEPCK*. In this experiment, though, the expression was almost same in larvae treated with *L. acidophilus* as the HSD control. In adults, however, the expression was higher in flies treated with *L. acidophilus* when compared to the HSD control sample. No articles were reported discussing the effects of the other probiotics on the other genes, although Plaza-Diaz et al. (2014) did mention that *B. breve* decreased triglyceride levels in rats. Such a claim might indirectly imply that *B. breve* decreases the expression of *Zw*, a gene involved in lipogenesis. Furthermore, the results obtained from the qPCR show that samples treated with this probiotic had the lowest *Zw* expression values among all other samples, at both developmental stages (Figure 7D). Nevertheless, the expression values obtained from the qPCR do not necessarily reflect the effectiveness of the probiotics, as malnutrition might have been a significant factor in controlling gene expression in this experiment, and not the treatments themselves. Additionally, the overall expression of genes responsible for gluconeogenesis (i.e. *PEPCK* and *Fbp*) was high in treated larva samples, where the gene responsible for glucose catabolism (i.e. *Zw*) had an overall low expression values in treated larva sample compared to the controls. This can confirm the malnutrition explanation in a sense, as more glucose is required by these larvae, hence the high expression of *PEPCK* and *Fbs*. On the other hand, less lipids and glucose catabolism were required, thus the low expression of *Zw*.

Gáliková and Klepsatel (2018) and Galenza, Hutchinson, Campbell, Hazes and Foley (2016) observed an increase in lifespan when flies were fed HSD due to the inhibition of insulin signalling. This experiment has failed to display such observation clearly, as some flaws were encountered during the first weeks of the longevity measurements. However, Figures 8C and 8D show that *B. lactis* has significantly extended life when compared to HSD sample, which matches what Matsumoto et al. (2011) reported in mice, although the test models did not follow the same diets like the ones in this experiment (e.g. HSD, LSD …etc). Still, the longevity data obtained during this project are not reliable enough to conclude due to some obstacles encountered during the project.

The issues encountered during the project and their proposed solutions

One of the major issues encountered during this project was the texture of the high sugar diet. During the longevity measurement, many flies were stuck in the food and were dead a few days later as a consequence. These mortalities have affected the data obtained severely since these flies did not die as a result of complication developed from the HSD itself, but rather from the stickiness of the food. A solution to the problem would be achieved by decreasing the mass of sucrose used to the point that insulin resistance is induced, yet the flies do not stick to the diet. A concentration of 0.6-0.7M might be recommended in this case.

The change of method (from method 1 to method 2) was a consequence of obtaining insignificant results (p>0.05), as previously described. This insignificance could have been an outcome of using only a small amount of the diet, which might have been avoided by some larvae. Thus, not all larvae were affected by the diet. In method 2 however, the whole plate had either HSD or LSD, which in a sense, ensured that each larva was defiantly affected by the diet (Appendix E). Furthermore, in method 2, larvae were not collected in the same time from all sample unlike in method 1. Instead, they were allowed to develop to 3rd instar before collection. This is because further protocols used in this project required larvae to be at that stage before carrying them out. If the protocols were carried out for all the samples at the same time ignoring the differences in larval stage, then there would have been significant differences (p<0.05) in all results between the different samples, since all of the treatment samples and the HSD control sample had delayed development (i.e they had

significantly different sizes). Additionally, the delay in development can be seen clearly without the need to carry out other protocols (Figure 10), thus the project was more focused in testing the effects of probiotics in "overcoming" the effects of the HSD when larvae were at the same stage. Hence, all samples collected for further measurements were 3rd instar.

Another issue was the fast-cellular death of larvae, when taken out of the freezer for weight and length measurements, as Figure 13 indicates. The fast tissue decomposition could be minimized if the measurements were taken in a cold room, with pre-chilled equipment. The inaccuracy of the scale used was also a major issue. The fluctuating values obtained from the scale, have probably made a significant difference in weight measurements. This issue could have been avoided if a more sensitive scale was used. Periodic acid Schiff (PAS) staining method could have been used as an alternative for the glycogen protocol, which did not work as expected. Although this method lacks specify (other carbohydrate-containing molecules can be detected and not only glycogen) (Skurat, Segvich, DePaoli-Roach and Roach 2017). Oral inoculation of probiotics grown in media can be a sufficient method to use instead of adding dried bacteria to the diet (Siva-Jothy, Prakash, Vasanthakrishnan, Monteith and Vale 2018). Furthermore, the concentration of bacteria can be set by controlling the colony-forming unit (CFU) using a spectrophotometer. Thus, this method is more accurate than using a dry mass of probiotics, like in this experiment, as the concentration of probiotics, in this case, is unknown.

Figure 13. Example of cellular death of larvae (Black) when photos were taken for length measurements.

Future perspective and conclusion

LPS of gram-negative bacteria is a structure that causes insulin resistance in muscle cells, through the activation of inflammatory responses (release of cytokines) as described by Liang, Hussey, Sanchez-Avila, Tantiwong and Musi (2013). Choi (2011) on the other hand, suggest that exopolysaccharides produced by gram-positive bacteria can reverse T2DM symptoms. These articles specify structural components of bacteria that are likely to be involved in T2DM and thus should be the focus of future research, instead of using whole bacteria. Furthermore, Tsai et al. (2019) proposed that dysbiosis is closely related to systemic inflammation, including diabetes. Hence, it might be wise to study T2DM from an inflammatory point of view, by focusing on signalling pathways involved in systemic inflammations and how to mediate them.

Except for the average weight measurements, none of the other results was reliable enough to make a concrete conclusion on whether the treatments indeed worked in reversing insulin resistance or

not. The qPCR data did show some effects of some of the treatments at different developmental stages. However, unless the qPCR is repeated at least once using the same protocol, no deduction can be made. Furthermore, the data obtained hint that the dosage used in method 2 (0.025 g) was too high for larvae and adult flies. This dose might have caused malnutrition by blocking their midguts and decreasing food absorption, thus giving false significant or non-significant outcomes. Interestingly, this malnutrition can be a topic of future research involving obesity. Probiotics in this case can be used as supplements for weight loss as a result of decreased absorption of food.

Further studies are required using a much lower probiotic dosage if *Drosophila* is to be used as a disease model for T2DM, although, other disease models such as mice or rats are more recommended in this case. Baring these thoughts in mind and based on the results of this project, the null hypothesis indicating that there is no significant relationship between the use of probiotics and reversing T2DM symptoms is therefore accepted.

Ethical considerations and the impact on society

Drosophila models were used in this experiment not only for their rapid generation time and low costs (Jennings 2011; Morris et al. 2012) but also for the many conserved singling pathways they share with mammals. Additionally, the ability to use them in research without the need for ethical considerations makes them even more desirable by researchers (Trinder et al. 2017). They can be easily anaesthetized with CO_2 or ether, and genetically modified as well to use as models for many diseases (75% of genes responsible for diseases in humans, can be found in *Drosophila* as well). They can live for more than 50 days and can be fed only once a month depending on incubation temperature (Linford 2013; Jennings 2011).

This project was designed to help confirm the outcomes of previous studies regarding the relationship between probiotics and T2DM, which will benefit millions of patients as diabetes is considered to be the epidemic of the century according to (Kharroubi and Darwish 2015). The three Rs (replacement, reduction and refinement) will be followed during this project, as Table 3 below, Illustrates. The three Rs aim to avoid the use of animals as much as possible (replacements), minimize the number of animals used (reduction) and to minimize suffering, stress and pain (refinements) (National Center for the Replacement Refinement and Reduction of Animals in Research 2004).

Table 3. The three Rs and the relevant protocols to follow them.

The three Rs	Protocols
Replacement	*Drosophila* will be used as a model, which is considered to be "partial replacement", as *Drosophila* is less capable of pain and stress in comparison to more complex animals (e.g. mice or rats)
Reduction	Variety of tests and techniques will be carried out in this experiment, to get as many useful information as possible using minimal numbers of *Drosophila*. The results will also be published so other groups could benefit from them without the need to redo the experiment and use more models.
Refinement	Vials containing *Drosophila* will be handled gently and placed in an isolated stress-free environment. Anesthetics will also be used (e.g. CO_2).

Acknowledgment

The oxford dictionary defines "glorious" as having, worthy of, or bringing fame or admiration. I define it as Katarina Ejeskär and Ferenc Szekeres. As supervisors, as mentors, and as humble and patient individuals, they are worthy of fame and admiration, they are "glorious".

References

-Alfa R. W. and Kim S. K. (2016) Using *Drosophila* to discover mechanisms underlying type 2 diabetes. *Disease models and mechanisms*, *9* (4), (p. 365-76). DOI:10.1242/dmm.023887

-Ali Z. and Bhaskar S. B. (2016) Basic statistical tools in research and data analysis. *Indian journal of anaesthesia*, *60* (9), (p. 662-669). DOI:10.4103/0019-5049.190623

-Álvarez-Rendón J. P., Salceda R. and Riesgo-Escovar J. R. (2018) *Drosophila melanogaster* as a Model for Diabetes Type 2 Progression. *BioMed research international*, *2018*, (p. 1-16). DOI:10.1155/2018/1417528

-Cefalu W.T. (2006) Animal Models of Type 2 Diabetes: Clinical Presentation and Pathophysiological Relevance to the Human Condition. *ILAR Journal*, 47 (3), (p. 186-198). DOI:10.1093/ilar.47.3.186

-Cersosimo E, Triplitt C, Solis-Herrera C, et al. Pathogenesis of Type 2 Diabetes Mellitus. [Updated 2018 Feb 27]. In: Feingold KR, Anawalt B, Boyce A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc. Available at: https://www.ncbi.nlm.nih.gov/books/NBK279115/ [Accessed 28.05.2019]

-Choi Jang. (2011) Anti-diabetic Effect of the Exopolysaccharides (EPS) Produced from Cordyceps sinensis on ob/ob Mice. *KSBB Journal* 26, (p. 33-40). DOI:10.7841/ksbbj.2011.26.1.033.

-Galenza A., Hutchinson J., Campbell S. D., Hazes B. and Foley E. (2016) Glucose modulates Drosophila longevity and immunity independent of the microbiota. *Biology open*, *5* (2), (p. 165–173). DOI:10.1242/bio.015016

-Gáliková M. and Klepsatel P. (2018) Obesity and Aging in the *Drosophila* Model. *International journal of molecular sciences*, *19* (7). DOI:10.3390/ijms19071896

-Graham P. and Pick L. (2016) *Drosophila* as a Model for Diabetes and Diseases of Insulin Resistance. *Current topics in developmental biology*, *121*, (p. 397-419). DOI:10.1016/bs.ctdb.2016.07.011

-Hales K. G., Korey C. A., Larracuente A. M. and Roberts D. M. (2015) Genetics on the Fly: A Primer on the *Drosophila* Model System. *Genetics*, *201* (3), (p. 815-842). DOI:10.1534/genetics.115.183392

-Janghorbani M. and Amini M. (2012) Normal fasting plasma glucose and risk of prediabetes and type 2 diabetes: the Isfahan Diabetes Prevention Study. *The review of diabetic studies: RDS*, *8* (4), (p. 490-498). DOI:10.1900/RDS.2011.8.490

-Jennings B.H. (2011) *Drosophila* – a versatile model in biology and medicine. *Materials Today, 14* (5), (p. 190-195). DOI:10.1016/S1369-7021(11)70113-4.

-Jones R. B., Alderete T. L., Martin A. A., Geary B. A., Hwang D. H., Palmer S. L. and Goran M. I. (2018) Probiotic supplementation increases obesity with no detectable effects on liver fat or gut microbiota in obese Hispanic adolescents: a 16-week, randomized, placebo-controlled trial. *Pediatric obesity*, *13* (11), (p. 705–714). DOI:10.1111/ijpo.12273

-Kasuga M. (2006) Insulin resistance and pancreatic beta cell failure. *J Clin Invest*. 116 (7), (p. 1756-1760). DOI:10.1172/JCI29189.

-Kechagia M., Basoulis D., Konstantopoulou S., Dimitriadi D., Gyftopoulou K., Skarmoutsou N. and Fakiri E. M. (2013) Health benefits of probiotics: a review. *ISRN nutrition*, *2013*, DOI:10.5402/2013/481651

-Kharroubi A. T. and Darwish H. M. (2015) Diabetes mellitus: The epidemic of the century. *World journal of diabetes*, *6* (6), (p. 850–867). DOI:10.4239/wjd.v6.i6.850

-Kramer J. M., Davidge J. T., Lockyer J. M. and Stavely B. E. (2003) Expressionof Drosophila FOXO regulates growth and can phenocopy starvation. *BMC Developmental Biology*, 3 (5). DOI:10.1186/1471-213X-3-5

-Liang H., Hussey S. E., Sanchez-Avila A., Tantiwong P. and Musi N. (2013) Effect of lipopolysaccharide on inflammation and insulin action in human muscle. *PloS one*, *8* (5). DOI:10.1371/journal.pone.0063983

-Linford N. J., Bilgir C., Ro J. and Pletcher S. D. (2013) Measurement of lifespan in *Drosophila melanogaster*. *Journal of visualized experiments: JoVE*, (71). DOI:10.3791/50068

-Luo J., Lushchak O. V., Goergen P., Williams M. J. and Nässel D. R. (2014) *Drosophila* insulin-producing cells are differentially modulated by serotonin and octopamine receptors and affect social behavior. *PloS one*, *9* (6). DOI:10.1371/journal.pone.0099732

-Matsumoto M., Kurihara S., Kibe R., Ashida H. and Benno Y. (2011) Longevity in mice is promoted by probiotic-induced suppression of colonic senescence dependent on upregulation of gut bacterial polyamine production. *PloS one*, *6* (8). DOI:10.1371/journal.pone.0023652

-Mattila J. and Hietakangas V. (2017) Regulation of Carbohydrate Energy Metabolism in *Drosophila melanogaster*. *Genetics*, *207* (4), (p. 1231-1253). DOI:10.1534/genetics.117.199885

-May C. M., Doroszuk A. and Zwaan B. J. (2015) The effect of developmental nutrition on life span and fecundity depends on the adult reproductive environment in Drosophila melanogaster. *Ecology and evolution*, *5* (6), (p. 1156–1168). DOI:10.1002/ece3.1389

-Morris S. N., Coogan C., Chamseddin K., Fernandez-Kim S. O., Kolli S., Keller J. N. and Bauer J. H. (2012) Development of diet-induced insulin resistance in adult *Drosophila* melanogaster. *Biochimica et biophysica acta*, *1822* (8), (p. 1230-1237). DOI:10.1016/j.bbadis.2012.04.012

-Musselman L.P., Fink J.L., Grant A.R., Gatto J.A. Tuthil II B.F. and Baranski T.J (2017) A Complex Relationship between Immunity and Metabolism in *Drosophila*Diet-Induced Insulin Resistance, *Molecular and Cellular Biology 38 (2).* DOI:10.1128/MCB.00259-17

-Musselman L.P., Fink J.L., Narzinski K., Ramachandran P.V., Hathiramani S.S., Cagan R.L. and Baranski T.J (2011) Disease Models & Mechanisms (4), (p. 842-849). DOI:10.1242/dmm.007948

-National Center for the Replacement Refinement and Reduction of Animals in Research (2004) *The 3Rs.* Available at: https://www.nc3rs.org.uk/the-3rs [Accessed 2019.02.13].

-National Library of Medicine (2019) Type 2 diabetes. Available at: https://ghr.nlm.nih.gov/condition/type-2-diabetes [Accessed 28.05.2019]

-Nayak B. K. and Hazra A. (2011) How to choose the right statistical test?. *Indian journal of ophthalmology*, *59* (2), (p. 85-86). DOI:10.4103/0301-4738.77005

-Pasco M. Y. and Léopold P. (2012) High sugar-induced insulin resistance in Drosophila relies on the lipocalin Neural Lazarillo. *PLoS One.* 7 (5). DOI:10.1371/journal.pone.0036583.

-Park S., Bustamante E. L., Antonova J., McLean G. W. and Kim S. K. (2011) Specification of Drosophila corpora cardiaca neuroendocrine cells from mesoderm is regulated by Notch signaling. *PLoS genetics*, *7* (8). DOI:10.1371/journal.pgen.1002241

-Park S. S., Yang G. and Kim E. (2017) *Lactobacillus acidophilus* NS1 Reduces Phosphoenolpyruvate Carboxylase Expression by Regulating HNF4α Transcriptional Activity. *Korean journal for food science of animal resources*, *37* (4), (p. 529–534). DOI:10.5851/kosfa.2017.37.4.52

-Plaza-Diaz J., Gomez-Llorente C., Abadia-Molina F., Saez-Lara MJ., Campaña-Martin L., Muñoz-Quezada S., Romero F., Gil A. and Fontana L. (2014) Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on hepatic steatosis in Zucker rats. *PLoS One*, 9 (5). DOI:10.1371/journal.pone.0098401

-Skurat A. V., Segvich D. M., DePaoli-Roach A. A. and Roach P. J. (2017) Novel method for detection of glycogen in cells. *Glycobiology*, *27* (5), (p. 416–424). DOI:10.1093/glycob/cwx005

-Siva-Jothy J. A., Prakash A., Vasanthakrishnan R. B., Monteith K. M. and Vale P. F. (2018) Oral Bacterial Infection and Shedding in Drosophila melanogaster. *Journal of visualized experiments : JoVE*, (135). DOI:10.3791/57676

-Tennessen J. M., Barry W. E., Cox J. and Thummel C. S. (2014) Methods for studying metabolism in *Drosophila*. *Methods (San Diego, Calif*.), 68 (1), (p. 105-115). DOI:10.1016/j.ymeth.2014.02.034

-Tillmann T., Bernhard D. and Eschrich K. (2002) Fructose-1,6-bisphosphatase genes in animals. *Gene, 291* (1–2), (p. 57-66). DOI:10.1016/S0378-1119(02)00627-3

-Trinder M., Daisley B. A., Dube J. S. and Reid G. (2017) *Drosophila melanogaster* as a High-Throughput Model for Host-Microbiota Interactions. *Frontiers in microbiology*, *8*, 751. DOI:10.3389/fmicb.2017.00751

-Tsai Y. L., Lin T. L., Chang C. J., Wu T. R., Lai W. F., Lu C. C. and Lai H. C. (2019) Probiotics, prebiotics and amelioration of diseases. *Journal of biomedical science*, *26* (1), 3. DOI:10.1186/s12929-018-0493-6

-Uniport: *Sequence alignments tutorial*. Available at: https://www.uniprot.org/help/sequence-alignments [Accessed 28.05.2019]

-Verna E. C. and Lucak, S. (2010) Use of probiotics in gastrointestinal disorders: what to recommend?. *Therapeutic advances in gastroenterology*, *3* (5), (p. 307–319). DOI:10.1177/1756283X10373814

-Visinoni S., Khalid N. F., Joannides C. N., Shulkes A., Yim,M., Whitehead J., Tiganis T., Lamont B. J., Favaloro J. M., Proietto J., Andrikopoulos S. and Fam B. C. (2012). The role of liver fructose-1,6-bisphosphatase in regulating appetite and adiposity. *Diabetes*, *61*(5), (p. 1122–1132). DOI:10.2337/db11-1511

-Wong R., Piper M. D., Wertheim B. and Partridge L. (2009) Quantification of food intake in Drosophila. *PloS one*, *4* (6). DOI:10.1371/journal.pone.0006063

-Westfall S., Lomis N. and Prakash S. (2018) Longevity extension in *Drosophila* through gut-brain communication. *Scientific reports*, *8* (1). DOI:10.1038/s41598-018-25382-z

-World Health Organization (2018) Diabetes. Available at: https://www.who.int/news-room/fact-sheets/detail/diabetes [Accessed 28.05.2019]

-Xu Y., Osborne B. W. and Stanton R.C (2005) *activation of PKA, which contributes to oxidative stress in rat kidney cortex.* American Journal of Physiology-Renal Physiology, 289 (5), (p. F1040-F1047). DOI:10.1152/ajprenal.00076.2005

-*Yadav H., Jain S. and Sinha PR.* (2007) Antidiabetic effect of probiotic dahi containing Lactobacillus acidophilus and Lactobacillus casei in high fructose fed rats. *Nutrition, 23 (1), (p. 62-68).* DOI:10.1016/j.nut.2006.09.002

-Yao K., Zeng L., He Q., Wang W., Lei J. and Zou X. (2017) Effect of Probiotics on Glucose and Lipid Metabolism in Type 2 Diabetes Mellitus: A Meta-Analysis of 12 Randomized Controlled Trials. *Medical science monitor: international medical journal of experimental and clinical research*, *23*, (p. 3044–3053). DOI:10.12659/MSM.902600

Protocols

-RNeasy plus Mini Kit (Cat #74137), QIAGEN, Hilden, Germany. Available at: https://www.qiagen.com/se/products/discovery-and-translational-research/dna-rna-purification/rna-purification/cell-free-rna/rneasy-plus-mini-kit/#orderinginformation [Accessed 28.05.2019]

-Applied biosystems[TM] High-Capacity RNA-to-cDNA (Cat #4387406), ThermoFisher Scientific, Massachusetts, United States. Available at: https://www.thermofisher.com/order/catalog/product/4387406 [Accessed 28.05.2019]