



**MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES**



**INVESTIGATING THE EFFECTS OF *HALOMONAS
SMYRNENSIS* LEVAN AS SOLUBLE FIBER ON FAT
DEPOSITION AND LIFESPAN USING
CAENORHABDITIS ELEGANS AS MODEL**

HALİL ÖNDER ÖZBAŞAK

MASTER THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Dr. Ebru TOKSOY ÖNER

Thesis CO- Supervisor

Assoc. Prof. Pınar OBAKAN YERLİKAYA

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Halil Önder ÖZBAŞAK, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended his thesis entitled “**INVESTIGATING THE EFFECTS OF *HALOMONAS SMYRNENSIS* LEVAN AS SOLUBLE FIBER ON FAT DEPOSITION AND LIFESPAN USING *CAENORHABDITIS ELEGANS* AS MODEL**”, on September 30, 2019 and has been found to be satisfactory by the jury members.

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ÖZET

Obezite, insülin direnci ve tip 2 diyabet, 1980'lerden itibaren sürekli olarak dünya genelinde yaygınlaşan hastalıklardandır. Her ne kadar araştırmalar bu hastalıkların yaygınlaşmasını hakkında kesin bir yargıya varamasa da; bir olguda bilimsel fikir birliği vardır: Vücut yağ yüzdesinin artması, insülin direncinin gelişmesi riskini, obezitenin belirleyici özelliklerindne birisi olan tip 2 diyabet riskini artırır. *Caenorhabditis elegans*, nispeten kısa bir ömre sahip ve insan hastalıklarına ilişkin genlerin % 40'ının ortologlarına sahip olan serbest yaşayan bir nematottur. Levan, çeşitli bakteri, arke, fungi ve bazı bitki türleri tarafından üretilebilen 2,6 beta glikosidik bağlantıya bağlı fruktoz homopolimeridir. Çalışmamızda *Halomonas smyrnensis* halofilik bakterilerden elde edilen *Halomonas levan*'ın ve hidrolize türevinin *C. elegans* üzerindeki yağ azaltıcı etkisini gösterilmiştir. Buna ek olarak, levan, nematodlar, yüksek glukozlu diyet için model için kullanılan % 2 glukoz ile aynı anda uygulandığında, nematodların vücutlarında yağ birikimini kayda değer ölçüde azalttığı tespit edilmiştir. Ayrıca, levanın nematodların yaşam süresi üzerindeki olumlu etkisinin dair ilk bulgular elde edilmiştir. *Halomonas levan*'ın *C. elegans* üzerindeki yağ azaltıcı etkisinin de insan popülasyonu için de geçerli olabileceğini düşünürsek, bu bulgular obeziteye karşı *Halomonas levan*'ın gıda katkısı olarak kullanılmasının önünü açması bakımından önemlidir.

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ABSTRACT

Obesity, insulin resistance and type 2 diabetes mellitus are closely related diseases that persistently have risen in prevalence consistently worldwide starting from 1980s. Although research is non-conclusive about the root of these diseases; there is scientific consensus over one fact: increased body fat percentage increases the risk developing of insulin resistance, type 2 diabetes mellitus which are the hallmarks of obesity. *Caenorhabditis elegans* is a free-living nematode which has a relatively short life span and has orthologues of 40% of genes related to the human diseases. Levan is fructose homopolymer connected with 2,6 beta glycosidic linkages which can be produced by various types of bacteria, archaea, fungi and some plants. In our study, we have shown the fat reducing effect of *Halomonas* levan and its hydrolyzed derivative on *C. elegans*. Moreover, levan also significantly reduced intestinal fat deposition when nematodes were treated with in combination with 2% glucose which is used for model for high glucose diet. Furthermore, preliminary observations of levans positive effect on nematodes' health span implicated that *Halomonas* levan's fat reducing effect on *C. elegans* may also positively affect humans and can be used as food additive for weight loss.

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HALİL ÖNDER ÖZBAŞAK

SYMBOLS

°C	: Celsius
μl	: Microliter
d	: Day
dH₂O	: Demineralized water
kg	: Kilogram
m²	: Meter square
mL	: Milliliter
mm	: Millimeter
rcf	: Relative centrifugal force
w	: Weight

ABBREVIATIONS

ANOVA	: Analysis of Variance
BMI	: Body Mass Index
CGC	: <i>Caenorhabditis</i> Genome Center
FOXO	: Fork Head Transcription Factor
FUDR	: 5-Fluorodeoxyuridine
GI	: Glycemic Index
HDL	: High-Density Lipoprotein
HL	: Hydrolyzed Levan
IFD	: Intestinal Fat Deposition
IGF-I	: Insulin Like Growth Factor I
INSR	: Insulin Receptor
LB	: Lysogeny Broth
LDL	: Low-Density Lipoprotein
LPS	: Lipopolysaccharide
MSG	: Monosodium Glutamate
NCD	: Non-Communicable Diseases
NGM	: Nematode Growth Media
ORO	: Oil Red O
PPR	: Pharyngeal Pumping Rate
SE	: Standard Error
SEM	: Standard Error Mean
T2DM	: Type 2 Diabetes Mellitus
WHO	: World Health Organization

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1. INTRODUCTION

Obesity is a disease that comes with wide array of comorbidities ranging from type 2 diabetes mellitus (T2DM), hypertension and heart disease. Even though T2DM and obesity is strongly associated and highly intertwined it is still not clear that which one of them causes other one (Kahn, Hull et al. 2006). While research on these diseases' mechanisms are still unclear statistically it is clear that prevalence of these diseases increasing and turned into full-blown global epidemic. For obesity projections for year 2030 shows that, if the if same trend rooted from 1975 persists, there will be 1.12 billion obese and 2.16 billion overweight adult population increased from today's 1.9 billion overweight and 650 million obese adult population (Kelly, Yang et al. 2008). Conclusively, there is little time left for curb the diseases' advance and even reverse the worldwide trend.

This urgency limits our options and pushes us into using model organisms to increase our understanding the roots of obesity and insulin resistance and makes short lived *Caenorhabditis elegans* as an attractive experimental medium over mice and rats. *C. elegans* non parasitic nematode with its adults reaching 1 mm and over 500 of them can easily fit a small confined space such a 60 mm petri dish and lives median life span of 12 to 17 days (Stiernagle 1999). Moreover there a little over 40 % human diseases have orthology to *C. elegans* (Markaki and Tavernarakis 2010). In addition to that, insulin-like signaling pathway is evolutionarily conserved across the species, from simple yeasts to the humans (Kaletsky and Murphy 2010).

The oat (*Avena sativa*) has shown to have various health benefits such as alleviating extra weight obese or overweight persons which it exerts this effect by having low glycemic index (GI) or lowering the GI of the food that it is combined with. GI lowering property comes from its high β -glucan content which is a glucose polysaccharide, mainly increases the viscosity of the foods and slowing down gastric emptying resulting with slower absorption of nutrients and lower glycemic load (Jenkins, Jenkins et al. 2002). Since different sources of β -glucan polymers results with different molecular weight, branching of the polymer therefore different properties hence rendering usage of other than oat β -glucans' useless which does not show the weight loss property. (Zeković, Kwiatkowski et al. 2005). Since oat is edible, there is

no known data or effort of producing oat β -glucans separately using microbial production techniques.

On the other hand, levan is a fructan polymer which can also be produced by wide variety of organisms from bacteria to higher organisms like plants (Lasseur, Lothier et al. 2010, Öner, Hernández et al. 2016). Levan polymer have already been consumed within Japanese fermented beans Natto produced by *Bacillus subtilis natto* as a result of fermentation and safe for human consumption therefore can also be used as food additive (Shih, Yu et al. 2005). *Halomonas* levan is the polymer produced by halophilic bacteria *Halomonas smyrnensis* AAD6T (Sarilmiser, Ates et al. 2015). *Halomonas sp.* enable us to produce levan polymer in non-sterile batch or fed-batch system which is advantageous over oat β -glucans (Erkorkmaz, Kirtel et al. 2018).

1.1. Aim

The aim of the study is to evaluate *Halomonas* levan's lipid lowering effect on 3 mutant and a wild type strains of *C. elegans* comparing it to the oat β -glucans' lipid lowering effect. If hypothesized effect exists, we aim to further evaluate levans lipid lowering effect while glucose is present in the medium. Furthermore, *Halomonas* levan's effect on *C. elegans* lifespan also will be evaluated.

1.2. General Background

1.2.1. The Obesity Epidemic

For the first time in known history there are more people dying from obesity linked diseases than people dying from malnutrition annually (Lozano, Naghavi et al. 2012, Collaborators 2017). Obesity is a complex, multifactorial medical condition characterized by storing excessive amounts of fat to the point excess fat leads to increased risk of diabetes (Control and Prevention 2017), hypertension (Beevers, Lip et al. 2014), cancer (Anand, Kunnumakara et al. 2008), bone fractures and osteoporosis (Rosen and Bouxsein 2006), heart disease (Fox, Golden et al. 2015), stroke (Benjamin, Virani et al. 2018), dementia (Qizilbash, Gregson et al. 2015, Albanese, Launer et al. 2017, Kivimäki, Luukkonen et al. 2018), sleep apnea (Wolk, Shamsuzzaman et al. 2003) and asthma (Farah and Salome 2012). Obesity is often described by the help of "Body Mass Index" (BMI) which is the estimated

quantification of fat tissue of an individual. It is defined as “A person’s weight in kilograms divided by the square of the person’s height in meters (kg/m^2)” by World Health Organization (WHO) (WHO 1995, World Health Organization 2019). According to WHO guidelines adults with above 30 BMI are defined as obese and adults that have 25.0–29.9 BMI are defined as pre-obese or overweight in adults older than 20 years (WHO 1995, World Health Organization 2019).

As shown in Figure 1.1, since 1980 there is increased prevalence of obesity across the globe, especially in developed and developing countries (Collaborators 2017). According to findings from NCD (Non Communicable Diseases) Risk Factor Collaboration group research, which curates data from 128.9 million individual; between 1975 and 2016, there is a steady increase of BMI from all age groups for both genders (Collaboration 2017). Both male and female adults combined adult obesity prevalence more than doubled from 4.12% in 1975 to 8.73% in 2016. Considering that world population also increased from 4.04 billion in 1975 to 7.444 billion in 2016 global adult obese population almost quadrupled from around 165 million people to around 650 million people (Collaboration 2017).

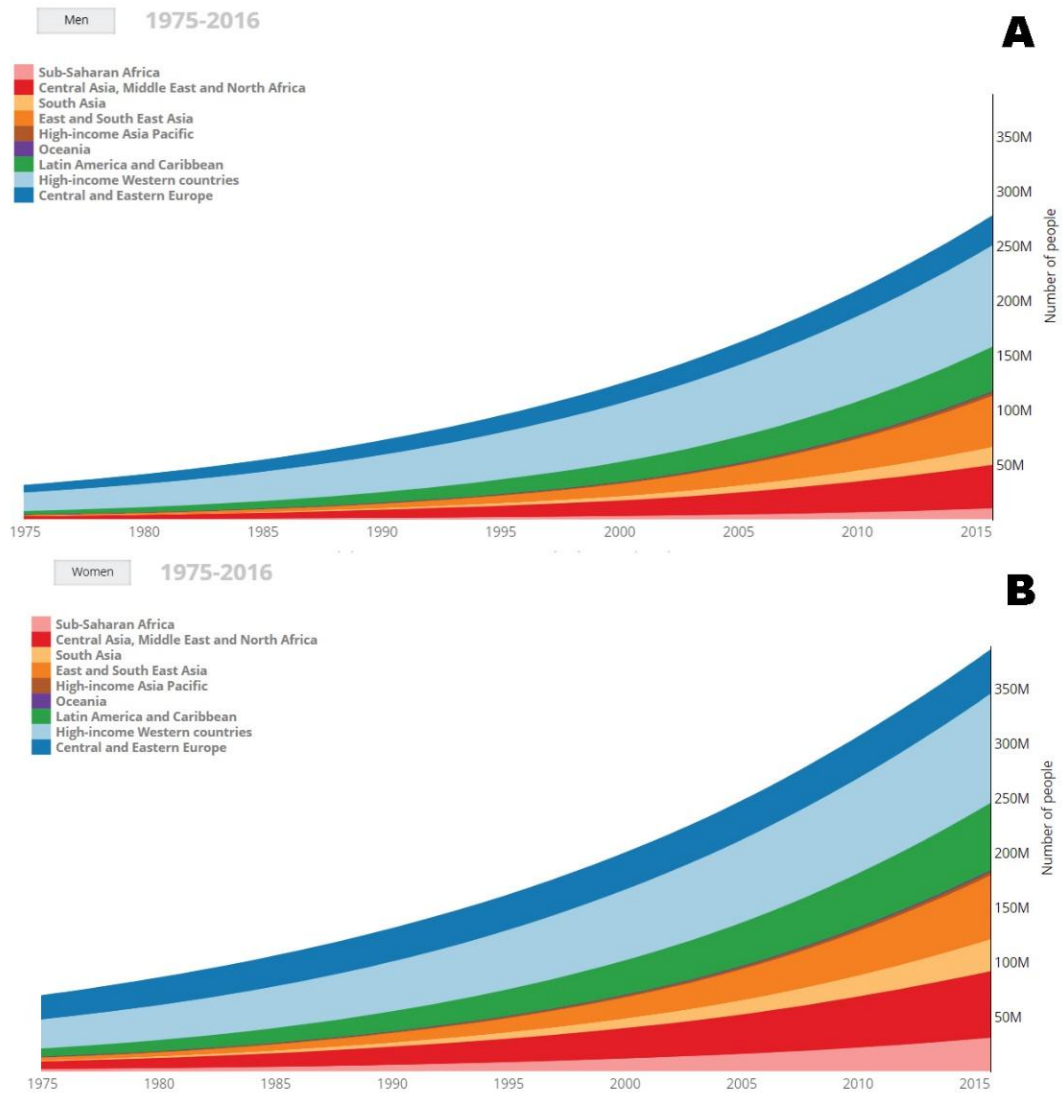


Figure 1.1. Stacked graph of obese population separated by color coded regions. Obese men (A) and women (B) population persistently increased between 1975 to 2015 (Collaborators" 2019).

A drastic change occurred during 1975 to 2016 time period in juvenile (5-19 years old) obese population. Global standardized prevalence of obesity in girls increased from 0.7 % to 5.6 % and in boys' prevalence of obesity increased from 0.9 % to 7.8 % (Collaboration 2017). This younger population is also susceptible to similar diseases like their adult counterparts, namely, insulin resistance (Caprio 2002), glucose intolerance (Invitti, Guzzaloni et al. 2003), Type 2 diabetes mellitus (T2DM) (Hannon, Rao et al. 2005), cardiovascular disease (Franks, Hanson et al. 2010), hepatic steatosis (Chan, Li et al. 2004) and high cholesterol (Chan, Li et al. 2004). Last but not least, studies show that gained weight and condition of obesity in childhood are likely to persist into adulthood (Singh, Mulder et al. 2008) and children with obese parents are

more likely to be obese (Bahreynian, Qorbani et al. 2017). This can be interpreted as continuation of the current trend.

A different aspect of this uptrend can be seen on 2017 OECD obesity update report, in OECD countries, more than 50 % of adult population is overweight and approximately 19.5% of general population is considered obese. On the other hand, back in 1980s, in these countries, obese people in population was less than 10%. Similarly, in Turkey 29% of the adult female population and 15.3% of male population are considered obese (2017). According to Turkish Statistical Institute's 2016 Turkish Health Survey press release, obesity prevalence in the adult population increased from 15.2% to 19.9% which equals to 31.1 % change, compared to 2008 data. In the same report, 15.3% of the adult male population, 24.5 % adult female population is considered obese and it is stated that 38.2 % of the adult male population and 29.3 % of the adult female population is overweight (Günel 2016) Many scientists define this increase and persistent trend as an epidemic (James, Leach et al. 2001, Low, Chin et al. 2009, Apovian 2016) even a pandemic (Frank 2016, Lee, Paz-Filho et al. 2016, Berger 2018).

1.2.2. Obesity and its Comorbidities

Obesity and weight gain do not simply happen overnight. Over time, excess weight gain usually caused by poor diet (Guo, Warden et al. 2004) combined with lack of exercise (Hu 2003) decreases mobility (Forhan and Gill 2013) causing even further weight gain leading to a vicious cycle of weight increase and mobility decrease (Pietiläinen, Kaprio et al. 2008) further exacerbated by insulin resistance and diabetes (Kahn, Hull et al. 2006). Other contributing factors are poor genetics such as having Prader-Willi (Cassidy and Driscoll 2009) or Bardet–Biedel (Mykytyn, Nishimura et al. 2002) syndromes or alternatively having “monogenic obesity” which might be caused by changes in genetic structures namely for example *PC1*, *PMOC*, *MC4R* genes *et cetera* (Farooqi and O’Rahilly 2006).

People with obesity have higher risk for developing T2DM. Risk of developing T2DM in overweight man increases 3.5-fold while overweight women have 4.6-fold more chance to develop T2DM than their same-sex normal weight counterparts. For obese women and man (with BMI above 35) this risk increases compared to 20-fold to 17-fold, respectively (Field, Coakley et al. 2001). After an obese person develops insulin

resistance and gains weight; excess insulin in bloodstream prevents glucose to be transported into muscle and brain cells which we can simply call as “starving on full stomach” (Perry, Caldow et al. 2016). This condition leads to muscle atrophy (Roy, Curtis et al. 2016), increased vulnerability of nervous system (Sriram, Benkovic et al. 2002) and in its full manifestation, causes diabetic ketoacidosis which is a life threatening condition in which liver breakdowns fat so fast, excessive ketones produced as a result of this decrease blood pH into acidic conditions (Elsheikh, Abdullah et al. 2018).

In addition to this, most brain cells use GLUT1 or GLUT3 receptors for glucose reuptake from blood flow which doesn't require insulin to work properly (Vannucci, Clark et al. 1998). In contrast to this, hippocampus which is responsible to memory formation and one of first brain regions to be affected by disease pathology of Alzheimer's disease (Llorens-Martín, Blazquez-Llorca et al. 2014); uses GLUT4 which employs insulin to function (Duarte, Santos et al. 2018). Prolonged insulin stimulation leads to downregulation of GLUT4 receptors in brain (Ma, Nakagawa et al. 2014) which impairs neuronal cell metabolism and most probably causes apoptosis of neurons in hippocampus by causing glucose starvation (Garrido, Osorio et al. 2015). In supporting fashion, brain GLUT4 expression knockout mice have glucose intolerance and decreased insulin sensitivity (Reno, Puente et al. 2017). Moreover, excess insulin in brain also prevents amyloid- β plaques, one of the hallmarks of the Alzheimer's disease, to be cleared properly; overburdening the Insulin Degrading Enzyme, which also clears amyloid- β plaques regularly. Excess insulin also inhibits negatively affects prognosis of Alzheimer's disease (Qiu and Folstein 2006, Li, Wu et al. 2018).

1.2.3. Possible Underlying Causes of Obesity

There are wide variety of hypotheses still investigated by research groups worldwide to shed a light on underlying causes of this obesity epidemic. These hypotheses include protein searching behavior (Simpson and Raubenheimer 2005, Gosby, Conigrave et al. 2014) (protein leverage hypothesis), antibiotic usage in livestock animals and poultry (Cox and Blaser 2015), socioeconomic status (McLaren 2007), modern life stressors (Razzoli, Pearson et al. 2017, van der Valk, Savas et al. 2018, Xenaki, Bacopoulou et al. 2018), increases in protein price (Brooks, Simpson et al. 2010) and lifestyle

differences that took place in last 3 decades (Owen, Sparling et al. 2010).

1.2.4. Protein Leverage Hypothesis

While most research effort focuses on lipids and carbohydrates, protein leverage hypothesis focuses on protein availability in foodstuff. From the advent of obesity epidemic to this day protein consumption remained constant while total energy consumption from fats and lipids are increased (Simpson and Raubenheimer 2005) (Figure 1.2) Protein leverage hypothesis explains increased fat and carbohydrate intake with decreased protein content in food. With another wording; according to this hypothesis a person tends to continue to eat until the person satisfies its appetite for protein and decreases of protein content in food over the last 40 years might be the underlying cause of obesity (Simpson and Raubenheimer 2005). Supportingly, there is increased evidence on decreased nutrition content in fruits and vegetables including the protein content over the last 3 decades (Davis, Epp et al. 2004, Myers, Zanobetti et al. 2014). Along with the plant research there is also evidence on poultry meat revealing that fat to protein ratio increased from 0.8 in 1970 to 3.2 in 2004 (Wang, Lehane et al. 2010). Another supporting pillar to this hypothesis is, evidence on protein consumption's positive role in appetite suppression (Batterham, Cowley et al. 2002, Batterham, Heffron et al. 2006)

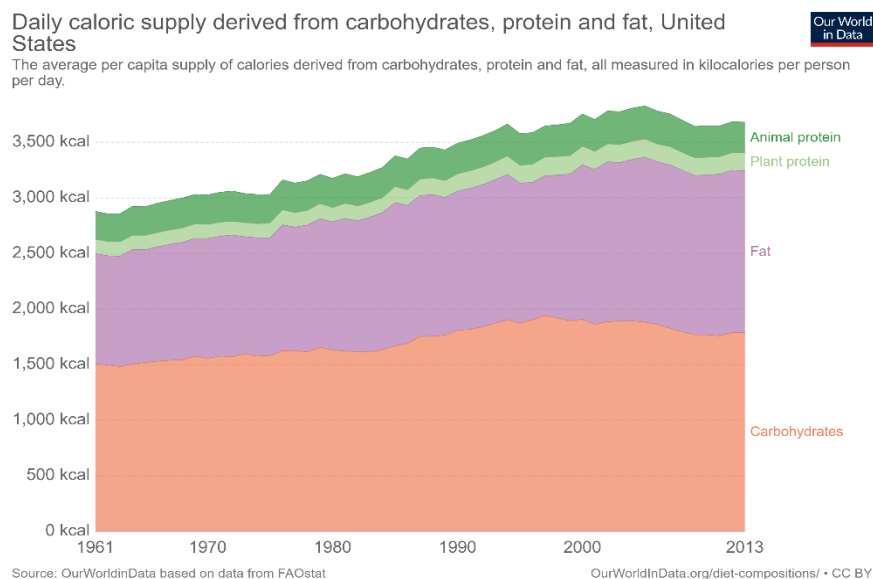


Figure 1.2. Graph of caloric supply and its sources. Daily average energy intake from food increased in a persistent trend since 1960 which correlates with obesity trends worldwide. Data shown here limited to USA but many other developed and developing countries follow the same trend (Roser and Max 2019).

Another aspect of this phenomenon can be examined by following increasing protein prices and decreasing availability of protein as driving factor in obesity epidemic (Brooks, Simpson et al. 2010). These socioeconomic dynamics affects the poor people most, almost positioning obesity and being overweight as “poor strata disease” (Brooks, Simpson et al. 2010). With non-existent protein content, fast consumption rate and being less filling option than other food; studies proved that sugary drinks contribute to being overweight and obesity (Hu and Malik 2010). To counteract increased consumption of carbohydrates, there is a “sugary drink tax” implemented in some cities and countries. For example, 10 % tax for sugary food implemented by Government of Mexico decreased sugary drink consumption by 12 % (Colchero, Rivera-Dommarco et al. 2017). Another instance to this one cent price increase per 30 grams of sugary foods decreased consumption from 9.6 % to 52.3 % in Berkeley, California (Lee, Falbe et al. 2019).

1.2.5. Nutritional Changes in Foodstuffs

Chickens getting fat over the years can be interpreted in two ways; whatever the animals eating is making them fat also effects humans and their meat might be causing humans to be fat. Some research groups believe that widespread antibiotic usage is to blame. Widespread antibiotic usage was adopted after it’s discovered that, antibiotic treated animals for infection prevention also gained weight in 1950s. Along with the antibiotics’ therapeutic properties its mainly used as weight gain agent in animal husbandry since 1970s. Large proportion of antibiotics, about 75%, are not retained by animals’ body and excreted in urine and manure (Riley, Raphael et al. 2013). Farm animals’ manure is used as fertilizer globally. Therefore, plants are also affected, retaining and accumulating wide variety of antibiotics in their tissues (Kumar, Gupta et al. 2005). Antibiotic-contaminated plant and animal-based food in turn might cause weight gain like they already do in farm animals. How the unintentional antibiotic consumption by humans exerts its weight gain affect remains unclear. However there is research associating antibiotic usage for the purpose for treating bacterial infection with higher BMI later in life (Thuny, Richet et al. 2010) and antibiotic usage in first 24 months in life significantly increases BMI in childhood (Saari, Virta et al. 2015).

1.2.6. Effect of Food Additives

Another suspect to obesity epidemic is changes in food additives that considered safe over the course of last four decades. While most of the food additives are in the at least United States Food and Drug Administration's "generally recognized as safe" category, their long-term effect is mostly unknown and could possibly exert its effects on population over the years of long-term use. A recent research on the food additive propionate, which is widely used as mold inhibitor, findings indicate; acute consumption of this additive by both mice and humans causes them to secrete glucagon which increases gluconeogenesis by glycogenolysis resulted with insulin resistance, hyperinsulinemia and weight gain (Tirosh, Calay et al. 2019). Also widely used food additive monosodium glutamate (MSG) is commonly used in lab conditions to create MSG induced obesity in mice since 1976 (Bunyan, Murrell et al. 1976). Although, the methodology used in mice studies administers MSG subcutaneously (Yoshida, Nishioka et al. 1984, Wang, Li et al. 2015, Pelantová, Bártoová et al. 2016) which doesn't reflect the human oral consumption rate, results of long term MSG consumption in humans is still unknown. Not surprisingly, common food emulsifiers like carboxymethylcellulose (E466) and polysorbate 80 (E433) are associated with induction of inflammation, obesity and metabolic syndrome in wild-type mice. These substances cause metabolic disturbances by altering gut microbiota similar to the unintentional antibiotic ingestion (Chassaing, Koren et al. 2015).

1.2.7. Addictive Properties of Food

In the obesity epidemic framework, human behavior also needs to be addressed. Regulation in food intake can be rooted from biological needs as well as it can root from hedonistic desires (Pandit, de Jong et al. 2011). Evidence on refined sugar's addictive properties is piling up in the recent years (Avena, Rada et al. 2008, Avena, Rada et al. 2009, Davis, Curtis et al. 2011, Finlayson 2017). Mice studies indicate that refined sugar is associated with changes in opioid and dopamine binding, enkephalin expression levels, release of acetylcholine and dopamine in brains rewards center: nucleus accumbens which indicate its addictive properties. Therefore sugar's addictive properties resemble psychostimulants and opioids, albeit in a lesser extent (Avena, Rada et al. 2008). Most packaged foods have added sugars in their ingredient list. A research surveying 40000 packaged food in Canada retail stores found that; 66 % of

these foods had at least one added sugar in their ingredients and their mean sugar content was significantly higher. A survey in United States has also shown similar results; 68 % of packaged food have found to have added sugars in it (Popkin and Hawkes 2016). It's safe to speculate other countries in the world would yield similar results.

1.2.8. Glycemic Load of Foods

While there are more opinions and hypotheses these hypotheses are intertwined and more than one could be true at the same time once we fully understand all of the reasons contributing to the prognosis of obesity. But there is one thing we are certain about: energy from excess sugar consumption is stored as fat *via* glycolytic pathway or pentose phosphate pathway (Hall 2016). Glucose's deposition into cells happens through insulin/insulin like growth factor I (IGF-I) signal pathway which is evolutionarily conserved from nematodes, fruit flies and even yeasts to mammals (Barbieri, Bonafè et al. 2003). In mammalian physiology though, mechanism is more complex than simpler organisms and insulin signaling in human body is closely related to the blood glucose levels which is largely affected by glycemic load of the foods consumed (Barbieri, Bonafè et al. 2003).

The glycemic index (GI) was first coined in 1981 as a tool for comparative measurement of blood sugar rising potential of various foods (Jenkins, Wolever et al. 1981). In 1997, another type of criterion named glycemic load (glycemic index x amount of carbohydrate in a portion) was developed which can be described as “measuring blood sugar rising potential of a food based on quality and quantity of carbohydrates it contains” (Liu and Willett 2002). Various studies showed that refined sugars and cereals have higher glycemic index compared with their raw states or raw alternatives (Thorburn, Brand et al. 1987). In addition to this, studies proved that consumption of high GI food for a long time period have adverse effects on health and metabolism (Liu and Willett 2002, Ludwig 2002, Cordain, Eades et al. 2003).

More specifically, consumption of high glycemic load foods may cause physiologic and hormonal changes, which result in chronic hyperglycemia, hyperinsulinemia and most importantly, insulin resistance. Moreover; as mentioned before, insulin resistance may lead to obesity, coronary heart diseases, type 2 diabetes and dyslipidemia (Liu and

Willett 2002, Ludwig 2002, Cordain, Eades et al. 2003). In addition to this, a diet with food containing low fibers and high glycemic index may increase type 2 diabetes risk (Miranda and Horwitz 1978, Schulze, Liu et al. 2004). One of the fundamental differences between high GI food and low GI foods is fiber content in these foods. More fiber in the foodstuff means slower gastric emptying, in other words slower absorption rate of nutrients than food with less fiber in it (Jenkins and Jenkins 1985) therefore lower GI. This is also evident when we compare raw fruit's and fruit juice's GI in which insoluble fibers are left out in pulp of juiced fruits. For example, while raw apple and raw orange have GI of 36 ± 2 and 43 ± 3 respectively; apple juice and orange juice have GI values of 41 ± 2 and 50 ± 2 (Atkinson, Foster-Powell et al. 2008).

1.2.9. Health Benefits of Fiber Rich Foods

Dietary fibers can be divided into two groups; soluble fibers and insoluble fibers. In a review, it is shown that soluble fibers have a greater hypolipidemic effect than insoluble fibers, inferred from the result from 68 positive results out of 77 human studies (Glore, Van Treeck et al. 1994).

One of most consumed soluble fibers is the β -glucans which are naturally found in cell walls of fungi, plants and bacteria. The oat (*Avena sativa*) is a fiber rich plant that contains approximately 3 to 8 grams of β -glucan fibers 82% of which was water soluble (El Khoury, Cuda et al. 2011). β -glucan fibers consist from six sided D-glucose which are linearly connected by 1-3 glycosidic links but connection points and their physicochemical properties like molecular weight, polymer charge, conformation in solutions and solubility vary greatly depending on the source organism of β -glucan (Zeković, Kwiatkowski et al. 2005). For example while bacterial (Mcintosh, Stone et al. 2005), fungal (Han, Han et al. 2008) and yeast (Volman, Ramakers et al. 2008) β -glucans are insoluble, cereal β -glucans are generally soluble (Chu 2014). Out of all fiber types and β -glucan types, cereal especially oat β -glucans are the most extensively studied in the scientific literature (El Khoury, Cuda et al. 2011).

Most importantly and not surprisingly, increased content of β -glucan significantly decreased GI and insulin response from test meals in proportion to the amount of β -glucan fibers added to the meals (Mäkeläinen, Anttila et al. 2007). On another instance β -glucans are shown to decrease postprandial glucose response by 33 %, 58 % and 62

% when added to breakfast meals 4.0, 6.0 and 8.4 grams, respectively, compared to the control meal. This result also implicate β -glucans effect on decreasing GI when added to these meals (Tappy, Gügolz et al. 1996). β -glucan supplemented bread are also shown to have significantly lower GI than bread made from white flour in a dose dependent manner (Cavallero, Empilli et al. 2002). Functional foods, a breakfast cereal and a bar supplemented with oat β -glucan are shown to reduce GI of the said foods compared to commercially available controls by 4 points for every gram of β -glucans added for every 50 grams of carbohydrate (Jenkins, Jenkins et al. 2002). In a study on 100 hypercholesteremic subjects, glucose-sucrose beverages supplemented with oat β -glucan significantly decreased total cholesterol by 7.4 % and postprandial glucose and insulin while compared to the control group while barley oats did not (Biörklund, Van Rees et al. 2005). While adding 4 grams of oat β -glucans to muesli also decreased the GI of the food (Hlebowicz, Darwiche et al. 2008) adding oat β -glucans to the pasta did not (Holm, Koellreutter et al. 1992). This might have happened due the fact that oat β -glucans are soluble and after cooking pasta, excess water is discarded as well as with the soluble fibers in it.

1.2.10. Levan As Soluble Fiber

Levan is an homopolymer, which consists from β -(2,6) linked fructose residues, and can be produced by bacteria (Shih, Yu et al. 2005), archaea (Poli, Kazak et al. 2009, Kirtel, Lescrinier et al. 2019), plants (Lasseur, Lothier et al. 2010). In addition to its very interesting features like, adhesiveness, biodegradable film forming, levan is also soluble in water and can be counted as a soluble fiber (Öner, Hernández et al. 2016). Levan in fiber form already has been consumed by humans in the form of Japanese traditional food, Natto. Natto is a fermented bean meal, which is fermented by *Bacillus subtilis* sp. Natto and produces levan that gives the sticky characteristics of this meal (Shih, Yu et al. 2005).

As in the β -glucans, properties of the levan polymer largely change with the molecular weight, branching, fermentation process and as well as the source organism (Wu, Chou et al. 2013). This provides to the levan polymer to have wide variety of applications such as; hair care (Gunn, Gabbianelli et al. 2009), skin whitener (Kim, Chung et al. 2005), wound and burn healing (Sturzoiu, Petrescu et al. 2011), anti-oxidant, anti-inflammatory (Srikanth, Siddartha et al. 2015) and anti-irritant (Kang, Jang et al.

2009).

One of the hallmarks of obesity is the increase in serum cholesterol and triglyceride levels due to the disturbance in lipid metabolism (Singla, Bardoloi et al. 2010). In a study on rats, levan decreased abdominal fat accumulation while rats were on lipid-rich diet. In the test group with its diet supplemented with 10 % levan, weight gain was 1.61 ± 0.21 g/day, in contrast to control group with 2.58 ± 0.29 g/day. In addition to this, in levan fed rats' serum triglyceride, free fatty acid and cholesterol levels significantly decreased compared to the control group (Kang, Hong et al. 2004).

Only known human trial for of the levan polymer for weight loss was on 29 Korean women and their diet was altered for the experiment for 12 weeks. After the 12-week period, subjects that consumed 6 g a day showed significant reduction on weight and body fat mass compared to the control group. Levan also decreased waist to hip ratio by 7 %, and serum triglyceride levels by 15 % in the levan consuming group compared to the control group (Kang, Jang et al. 2003).

In a study on obese C57BL/6J mice on a high fat diet, effect of levan (100 mg/kg/d), fermented ginseng (150 mg/kg/d) and combination of both were used for treating mice for 11-week period. At the end of the experiment, combination of levan and red ginseng significantly lowered the fat mass, body weight, total cholesterol and fasting blood glucose levels compared to the control group. This can be interpreted, combination with red ginseng, levan might have anti-obesity effect if consumed regularly (Oh, Lee et al. 2014). In a study on rats, high molecular weight levan decreased serum cholesterol levels significantly by 17 % to 51 % in response to the feeding with 1 % and 5 % levan respectively compared to the control group after 4 weeks (Yamamoto, Takahashi et al. 1999). In a study using *Bacillus natto* levan; rats treated with levan, cholesterol rich diet and cholesterol rich diet combined with levan; levan attenuated the serum levels of total cholesterol, LDL, HDL and triglycerides significantly when also fed with cholesterol rich diets compared to the rats treated with high cholesterol diets only (Belghith, Dahech et al. 2012). In a comprehensive study on rats, animals fed with high fat diet with 40 % fat for 6 weeks and various percentages of levan (1 %, 5 % or 10 % (w/w)) has been added to their diet and this new diet continued for 4 more weeks. After fed with the levan supplemented diet, the key enzymes for fatty acid synthesis, acetyl CoA carboxylase and hepatic fatty acid synthase mRNA expression

were downregulated. Most importantly, levan attenuated excess insulin and leptin levels disturbed with the high fat diet in a dose dependent manner which implied that it may also be used on obese and overweight people (Kang, Hong et al. 2006).

1.2.11. Prebiotic Properties of Levan

Soluble fibers are non-digestible carbohydrate polymers that can be fermented by human gut microbiota into its monomers and increases viscosity of foodstuff resulting with delayed gastric emptying and providing fullness, prebiotic effect (Slavin 2005). In this sense, levan is also a soluble fiber (Niv, Shapira et al. 2012). Despite extensive studies on the prebiotic effects of the another fructose polymer inulin (Kelly 2008, Kelly 2009), studies on prebiotic effects of levan are highly limited. In a study employing male Wistar rats fed with sucrose added basal diet and 10 % (w/w) levan supplemented diet has shown that in the colon levan was converted into lactate and short chain fatty acids; namely acetate and butyrate. Compared to the control group, levan increased the cecal weight and wall weight, and also increased total number of bacteria by 5-fold and lactic acid bacteria by 30-fold, proving prebiotic activity (Jang, Kang et al. 2003). In an *in vitro* fecal microbiota study with levan supplemented bacterial growth media, compared to the control groups, levan changed the fecal microbiota by increasing *Bacteriosides*, *Faecalibacterium*, *Escherichia* and *Streptococcus* species indicating the prebiotic activity of levan (Adamberg, Tomson et al. 2015).

As mentioned further above, antibiotic usage in livestock animals and increased antibiotic availability poses health hazards for the world population and might be one of the underlying cause of obesity (Cox and Blaser 2015). Antibiotics thought to exert is weight gaining effect on the organisms by altering the gut microbiota which is called dysbiosis (Riley, Raphael et al. 2013). Two different experiments on pigs have shown that, after supplementing their corn-soybean based diets with 0 %, 0.05 %, 0.10 %, or 0.20 % levan, average daily gain and gain to feed percentage increased significantly. Parallel to this finding, total digestibility of food measured by gross energy and nitrogen as well as *Lactobacillus* counts increased significantly in a dose dependent manner. In the other experiment, pigs were challenged with *E. coli* LPS (lipopolysaccharide) for immune reaction. For 42 days, pigs were fed with same diet above with 0 % or 0.10 % levan. Levan fed pigs were found to have increased white

blood cell count, lymphocyte percentage significantly increased and attenuated the cortisol, TNF- α and IL-6 concentrations significantly compared to the control group (Li and Kim 2013). In a study on male Ross broilers fed with 0.25 % or 0.50 % levan supplemented CON basal diet for 31 days and the result of this compared to the control group, levan supplemented group increased *Bifodobacteria* and *Lactobacillus* concentrations and decreased *Clostridium perfringens* and *E. coli* concentrations in cecum as well as later stage growth performance significantly compared to the control group (Zhao, Wang et al. 2013). These studies indicate that levan might be used in livestock industry as a cleaner alternative to the antibiotics for improving immune system, and livestock food digestibility.

Although research papers on levan's prebiotic properties are limited, there are a number of patent applications utilizing levan's prebiotic potential. As a prebiotic, levan can be found in composition of; a patent for improving intestinal disorders by supporting growth of gut microbiota (Dutta 2018), patents for optimized and individualized prebiotic supplement (Madsen, Oswald et al. 2019), for prebiotic composition for gut-based therapies (Ranganathan, Dickstein et al. 2004), for Levan-producing *Lactobacillus* strain for the prebiotic use (Vincent, Brandt et al. 2005) and for prebiotic mixture for the prevent and treat gastrointestinal disease conditions (Vincent, Brandt et al. 2005) were reported.

1.2.14. *Caenorhabditis elegans* as Model for Obesity

Caenorhabditis elegans is a free-living nematode with its adults being 1 mm long and smaller larvae being 0.25 mm long. With its small size, ability to grow in petri dishes and to feed on *Escherichia coli* OP50 strain, *C. elegans* is an ideal model organism for wide range of studies with no need for ethics committee approval for research (Corsi, Wightman et al. 2015). Wild-type *C. elegans* have a lifespan of 21 days and can become an adult in 48 hours from larval stage 1. With a generation time of 3 days, *C. elegans* can hatch approximately 300 eggs per hermaphrodite, which will eventually turn into adults (Baumeister and Ge 2002). *C. elegans* is the first multicellular organism with its genome sequenced and more than 65 % of its genes related to human diseases are conserved (Baumeister and Ge 2002).

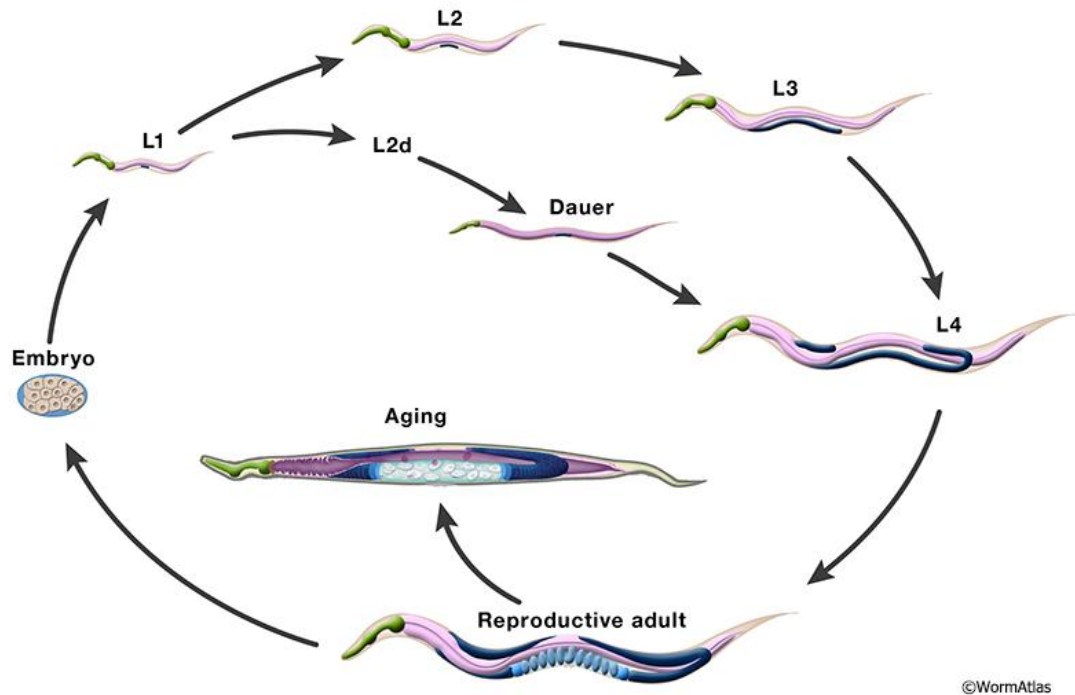


Figure 1.3. *Caenorhabditis elegans* life cycle. Adapted from WormAtlas website (Herndon 2019).

In 20 °C after 9 hours after an egg leaves the adult hermaphrodite nematodes vulva, it hatches into L1 (larval stage 1) larvae. During this period, depending on food availability or crowding it can turn into stress resistant dauer larvae (Fielenbach and Antebi 2008). Alternatively, L1 larva can turn into L2 larva after 12 hours by molting. After L2 stage, molting into L3 stage larva takes 8 hours and this stage also lasts 8 hours before molting into L4 stage. After 8 hours larva enters the L4 stage, it hatches into young adults and in 8 hours turns into an adult capable of egg laying without molting (Hall and Altun 2007).

C. elegans stores fat under its skin orthologue cuticle layer and in its intestinal cells and this fat can be stained with lipid affinity dyes like Nile Red, 8 Sudan Black and Oil Red O (ORO). Fluorescent light emitted from stained tissue can be observed easily thanks to *C. elegans*' transparent body and light intensity correlates with fat mass (Ashrafi 2007). *C. elegans* have around 20.000 genes and evaluating the gene silencing mutations for every gene is possible by observing their effect on the phenotype in mutant strains. Consequently, genome-wide RNAi technology studies were done to detect genes related to fat deposition (Liu, Spoerke et al. 1999). As a result of that, inactivation of more than 300 genes resulted with a decrease in fat deposition and inactivation of more than 100 genes resulted in an increase in the fat deposition

suggesting a very complex mechanism (Ashrafi 2007).

1.2.15. *Caenorhabditis elegans* as Model for Longevity

C. elegans have a median lifespan of 20 days, counted from the hatched egg to end of its life. Nematodes can be counted as dead if there are no bodily movements such as pharyngeal pumping and locomotion (Sutphin and Kaeberlein 2009). Nematodes don't have exact equivalent of mammalian sleep behavior but in some cases they can stay in a restful inactivity and should be lightly prodded by platinum wire to encourage movement, especially when assaying the nematodes for their lifespan (Raizen, Zimmerman et al. 2008). Lifespan analysis is labor intensive and it consists of counting the number of the nematodes each day or every other day which means including weekends (Sutphin and Kaeberlein 2009). Moreover, there is no way to extract knowledge of nematodes healthiness known as health span, while utilizing lifespan assay. Contrary to this, recording nematodes pharyngeal pumping rate (PPR) provides useful information about the animals' healthiness and lifespan at the same time while it can be recorded in every 3 to 4 days (Bansal, Zhu et al. 2015). Pharynx is pumped almost always during a nematode's lifespan and has a vital role through its life because most important organ in feeding of *C. elegans* (Avery and Shtonda 2003). Being a muscular organ, pharynx is susceptible to decay over time mainly because of muscles getting older known as sarcopenia. Caloric restriction known to increase lifespan in *C. elegans* which also increases pharyngeal pumping through the life of a nematode. Research shows death from old age correlates with the pharyngeal deterioration which is evident by slower movement than younger animals and it eventually stops moving which effectively ends energy intake (Zhao, Gilliat et al. 2017). Usually, dead animals found with no fat tissue indicates food intake failed and energy deposits are consumed until the end of its life (personal observation). In short using PPR as a surrogate marker for lifespan is advantageous over classical lifespan assays because it provides more information and have more relaxed experimental procedure.

1.1.16. Molecular Mechanisms Involved in Fat Metabolism and Longevity in *C. elegans*

C. elegans can be used in research on the molecular mechanism of obesity by exposing the nematodes to substances that might increase/decrease body weight (Dwyer,

Donohoe et al. 2005). Genes are conserved through evolution, and there are many similarities between distinct species. *C. elegans* daf-16 gene encodes “Fork head transcription factor” (FOXO) orthologue protein (Dwyer, Donohoe et al. 2005, Eijkelenboom and Burgering 2013). daf-2 encodes human insulin receptor (INSR) orthologue protein which is 35% similar with human insulin receptor, 34% similar with human insulin-like growth factor-1 and 33% human insulin receptor-related receptor (Zheng and Greenway 2012). Mutations on daf-2 and daf-16 may alter the metabolism in *C. elegans* similar to the insulin resistance in humans, causing *de novo* fat synthesis (Yu and Larsen 2001). sir-2.1 encodes the NAD-dependent protein deacetylase orthologous with mammalian SIRT1. Mutations on sir-2.1 may result with insulin resistance in *C. elegans*, similar to the SIRT1 downregulation causing insulin resistance in human (Zheng, Greenway et al. 2014). While sir-2.1 overexpression increases lifespan dependent to daf-16 manner (Tissenbaum and Guarente 2001) deletion sir-2.1 slightly decreases lifespan (Wang, Oh et al. 2006). But, deletion of sir-2.1 don’t have any effect on long lived daf-2 mutants indicating SIR-2.1 functions parallel to the insulin/IGF-1 signal pathway in which affects DAF-16 in the end (Wang and Tissenbaum 2006).

Table 1.1 : Genetic properties of strains used in this research.

Strain Name	Genotype
N2	Wild Type
GR1307	daf-16(mgDf50) I
VC199	sir-2.1(ok434) IV.
GA158	daf-16(mgDf50) I; daf-2(m65) III.

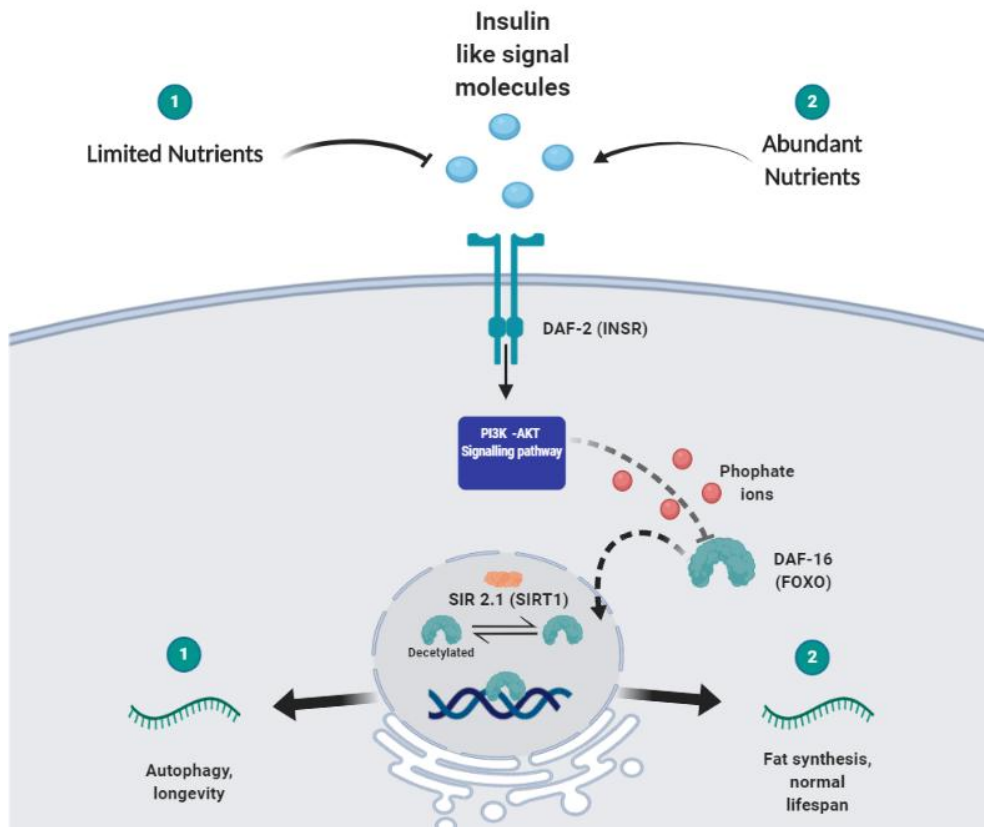


Figure 1.4. A simplified diagram of Insulin signaling pathway of the *Caenorhabditis elegans*. If nutrients in the environment are limited, DAF-2 insulin like signaling pathway stagnates leaving DAF-16 transcription factor uninhibited resulting with autophagy and longevity decreased fat accumulation. On the contrary, when there is abundant nutrients DAF-2 pathway activates leading to normal lifespan fat accumulation. Note that human orthologue of shown proteins written in the parentheses. Diagram adapted from multiple sources (Murphy and Hu 2005, Berdichevsky, Viswanathan et al. 2006, Tissenbaum 2018).

daf-2 deletion increases lifespan (Dorman, Albinder et al. 1995) and daf-16 null mutations completely reverses this increased life span (Dorman, Albinder et al. 1995); implying that increased DAF-16 protein located in nucleus essential for this pathway. Also as mentioned above, deletion of sir-2.1 don't have any effect on daf-2 mutation induced lifespan increase and it acts independently of insulin like signaling pathway (Wang and Tissenbaum 2006). Moreover, 2 % glucose added to the *C. elegans* medium shown to reduce lifespan of the nematodes by upregulating insulin like signaling pathway and downregulating daf-16 pathway (Lee, Murphy et al. 2009). It is found that while glucose addition to the medium nullifies long living daf-2 mutants prolonged lifespan and reduces the wild type nematodes lifespan; glucose addition cannot further reduce the lifespan of short-living daf-16 mutants (Lee, Murphy et al.

2009). In short, using various nematode strains containing null mutations of pathways mentioned above is effective strategy for investigating the effects of treatments on nematode lifespan and pathways involved can be easily detected.

2. MATERIAL AND METHOD

2.1. Materials

2.1.1. Levan Polysaccharide

In our study purified *Halomonas* levan polymer was obtained by culturing the *Halomonas smyrnensis* AAD6T bacteria. Bacteria culture originated from Çamaltı Saltern located in İzmir, Turkey (Poli, Kazak et al. 2009, Poli, Nicolaus et al. 2013). Purification of *Halomonas* levan conducted by Industrial Biology and Systems Biology (IBSB) research group of Marmara University Bioengineering department.

2.1.2. Chemicals

All chemicals and solutions used in this study were supplied by Sigma-Aldrich (USA), MERCK (Germany), Biofroxx (Germany). All of the experiments were conducted in Department of Molecular Biology at İstanbul Kültür University and Bioengineering Department and Chemical Engineering Department at Marmara University.

2.1.3. Standard Solutions

Solutions used in C. elegans Handling

M9 Solution

3.0 g KH_2PO_4

6.0 g Na_2HPO_4

0.5 g NaCl

1.0 g NH_4Cl

dH₂O

3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl , 1 ml 1 M MgSO_4 , H₂O to 1 liter, sterilized by autoclaving.)

1M KPO_4 Buffer

108.3 gr KH_2PO_4

35.6 gr K_2HPO_4

Adjust volume to 1L with dH₂O and sterilize with autoclaving.

Trace Metals Solution

0.346 g FeSO₄·7H₂O

0.930 g Na₂EDTA

0.098 g MnCl₂·4H₂O

0.144 g ZnSO₄·7H₂O

0.012 g CuSO₄·5H₂O

Complete up to 500 mL dH₂O. Sterilize by autoclaving, keep in aluminum foil.

Cholesterol Solution

250 mg cholesterol was dissolved in 50 mL 99% Ethanol and kept in 0-8 °C.

S- Basal Solution

5.8 g NaCl

1 g K₂HPO₄

6g KH₂PO₄

Adjust volume to 1 L with dH₂O and add 1 ml cholesterol solution.

S-Complete Solution

1 liter of S-Basal Solution

Add the following solutions:

3 ml 1 M CaCl₂

3 ml 1 M MgSO₄

10 ml trace metals solution

10 ml 1 M potassium citrate (pH 6.0)

Nematode Growth Media

3 g NaCl

20 g Agar

2.5 g Bacto-peptone

Add dH₂O up to 975 mL

Autoclave and after solution is cooled down to 55 °C add:

50 µl 0.1% cholesterol solution in 95% ethanol

1 ml of 1 M CaCl₂

1 ml of 1 M MgSO₄

25 ml of 1 M KPO₄

1 mL of Ampicillin solution

Optionally add 1 mL of Nystatin solution to prevent fungal contamination.

20% Sodium Hypochlorite Solution

2 mL domestic bleach

0.5 mL 10 M NaOH

Add dH₂O up to 10 mL

10x Phosphate Buffered Saline

80g NaCl

2.0g KCl

14.4g Na₂HPO₄

2.4g KH₂PO₄

Dissolved in 800 mL dH₂O and pH adjusted to 7.4. Volume adjusted to 1L with dH₂O and autoclaved.

1x Phosphate Buffered Saline

5 mL 10x PBS was diluted with 45 mL of sterile dH₂O solution.

Soft Agar Solution

0.58 g NaCl

0.68 g KH₂PO₄

30 g glycerol

0.56 ml 1 M NaOH

0.4 g agar

Volume adjusted to 100 mL with dH₂O and autoclaved.

Oil Red O Stock Solution

0.5 gr of Oil Red O dissolved into 100 mL isopropanol

Oil Red O Working Solution

30 mL of stock solution was diluted by 20 mL of water.

Ampicillin Solution

To make 50 mg /mL stock solution with 89.6% pure ampicillin salts, dissolve 558 mg ampicillin in 10 mL dH₂O, aliquot and keep in -20 °C freezer for further use.

5-fluorodeoxyuridine Solution

2mM main stock solution has been prepared by dissolving 49.2 mg FUdR into 10 mL dH₂O and kept in 4 °C with the shelf life of one month. Main stock solution is diluted 1 to 10 with final concentration of 200 µM.

2.1.4. Laboratory Equipment

Marmara University Laboratory:

Autoclave:

- Mod3870 Elv, Systec, Germany,
- Nüve OT 032, Turkey
- Nüve S-Class EN 13060, Turkey

Microplate reader:

- Promega GloMax®-Multi+ Microplate Multimode Reader, USA

Refrigerator:

- +4°C Arçelik, Turkey;
- +4°C, Indesit, Turkey

Deepfreeze:

- -80°C Heto Holten, Denmark
- -20°C Heto Holten, Denmark
- -20°C Uğur Derin dondurucu, Turkey

Water bath:

- Polyscience, USA

pH meter:

- Mettler Toledo, Switzerland

Balance:

- Mettler Toledo Ab204-S, Switzerland
- Mettler Toledo Pg403-S, Switzerland
- Mettler Toledo Pg40002-S, Switzerland

Centrifuge:

- Nüve NF400R, Turkey
- Sigma 3K30, Germany
- Sigma 8K10, Germany

Water purification system:

- Primary Grade Water Purification System, Purelab Prima 30 Ultrapure Water System, P.Maxima Ls, (Usf Elga, U.K.)

Heating Magnetic Stirrer:

- Heidolph Mr3003s, Germany

Microliter pipette:

- Finnpipette, Finland

Multichannel microliter pipette:

- Rainin L8-200XLS+, 8-channel, USA

Automatic pipettor:

- Witeg, Germany

Freeze dryer:

- Lyovac GT2, Steris, USA

Orbital shaker:

- B.Braun Certomat BS1, Germany

- New Brunswick Scientific, Excella E24, USA

Oven:

- Binder, Germany

Spectrophotometer:

- Perkin Elmer UV/Vis, Lambda35, USA

Vortex:

- Heidolph, Germany

FT-IR Spectrometer:

- Thermo Scientific Nicolet 6700, USA

Fermenter:

- Sartorius, Biostat B, Germany

NMR:

- Varian, USA

Istanbul Kültür University Laboratory:

Autoclave:

- Nüve, OT 4060, Turkey
- Nüve OT 90 L

Inverted microscope:

- Olympus, IX71– 1B, Japan
- Zeiss, Discovery V.12, Germany

Biological safety cabinet:

- Nüve, LN90, Turkey

Liquid nitrogen storage system:

- Air Liquide Arpege 40, France

Refrigerator:

- +4°C Arçelik, Turkey

- +4°C, Indesit, Turkey

Deepfreeze:

- -80°C New Bruinswick Scientific, U725, United Kingdom

- -20°C Arçelik, Turkey

Water bath:

- Nüve, BM302, Turkey

pH meter:

- Mettler Toledo, Switzerland

Balance:

- Sartorius, LE62025, Germany

Centrifuge:

- Eppendorf, 5810R, Germany

Water purification system:

- Millipore, Direct Q 5UV, USA

Heating Magnetic Stirrer:

- Sigma Aldrich, Stuart hotplate stirrer SB162, USA

Microliter pipette:

- Finnpipette, Finland

Orbital shaker:

- Labnet, Gyrotwister, USA
- Heidolph, Unimax 1010, Germany

Oven:

- Nüve, FN-120, Turkey

Vortex:

- Velp Scientifica, RX3, Italy

2.2. Experimental Methods

2.2.1. Culturing Conditions of *E. coli* OP50 Bacteria

E. coli (OP50) Culture Medium: Twenty-five grams of LB broth, 1 L of dH₂O (autoclave), one scoop (10 µL of *E. coli* (CGC), and 10 µL/mL streptomycin were mixed for 16 h at 37 °C in a shaker-incubator and stored at 4 °C. A final concentration of 5×10^8 – 5×10^{11} cells/mL was obtained. After that, *E. coli* OP50 stock was aliquoted and heat killed at 75 °C for 40 minutes, as optimized from Jan Gruber et al. (Gruber, Tang et al. 2007). Before every experiment, 50 µl of heat killed bacteria were added to the petri dishes and allowed to dry for at least overnight.

N2 (Wild Type), VC199 (sir-2.1(ok434) IV.), GR1307 (daf-16(mgDf50) I.), GA158 (daf-16(mgDf50) I; daf-2(m65) III.) were acquired from *Caenorhabditis* Genome Center (CGC), University of Minnesota, USA. Nematodes were cultured inside petri dishes containing nematode growth media (NGM) and transferred into fresh petri dishes every 3 day to prevent overcrowding. NGM petri dishes were seeded with 5×10^8 – 5×10^{11} CFU/ml *E. coli* OP50 before transferring the worms. All strains were kept in 20 °C incubator except GA158 strain which was kept at 15 °C.

2.2.2. Freezing and Defrosting *C. elegans* Strains

All nematode strains were frozen inside soft agar solution upon delivery from CGC as described below. Nematodes were collected into cryovials with washing plates with 0.6 mL S-Complete solution each, 4 days after they were transferred into new NGM media, when they reached at L1 stage, right after food depletion and before larvae were transformed into dauer stage. Soft agar solution has been boiled inside microwave oven 30-40 minutes before the freezing procedure started and kept into room temperature to cool it down. Cryovials were kept on ice and 0.6 mL soft agar solution was poured into solution and mixed horizontally to ensure worms are frozen through whole solution, not just the bottom. After mixing, cryovials were placed inside 7-8 layers of napkin and were placed inside an ice box before the box was kept in -20 °C freezer for 40 minutes to one hour. Right after that, cryovials were relocated into -80 °C overnight. Before relying on the frozen nematode cultures, cryovials were removed

from the deep freezer and part of the frozen mix was transferred into fresh NGM with the back of a sterile surgical blade and quickly returned into deep freezer. This procedure can be repeated 5-6 times for each frozen vial. While worms preferably should be kept in -196 °C liquid nitrogen stock for longer term storage but in this study, worms were kept in -80 °C freezer and success of defrosting worms was sufficient.

2.2.3. Age Synchronization of Nematode Cultures

2.5-3-day old petri dishes with gravid adults and unhatched eggs were washed with M9 and solutions were transferred into microfuge tubes. Tubes were centrifuged at 400 rcf for 2 minutes to clear bacterial presence. This step was repeated until sufficient number of worms were collected from petri dishes and supernatant had transparent appearance. After this step, supernatant was removed in order to add freshly prepared 1mL sodium hypochlorite solution. After sodium hypochlorite addition, tubes have been mixed by shaking vigorously between 5 to 8 minutes until adult and larvae populations were completely dissolved. After this step, tubes were centrifuged at 2000 rcf for 2 minutes to precipitate eggs, supernatant was aspirated afterwards. Following this, 100-150 µl of M9 buffer was added to tubes and eggs inside the buffer were relocated to the new microfuge tubes. Consequently, tubes were filled with M9 buffer until 1.5 mL and centrifuged at 2000 rcf for 2 minutes with supernatant aspirated afterwards. This step was repeated at least thrice to clean residual bleach which damages newly hatched larvae even in low concentrations. Finally, after the last centrifuge supernatant was aspirated all but 200 µl, and the remaining liquid was transferred into fresh NGM plates preferably along the outer circle of the petri dish.

2.2.4. Oil Red O Staining Procedure

4 days before the experiment, animals were prepared by age synchronization. After the animals reached L4 / young adult stage, 100 µl of treatment solutions were added onto bacteria lawn and animals were added inside of the puddle. Worms were treated 48 hours before collected for staining.

Nematodes were collected by washing their petri dishes with 1xPBS and collected in 1.5 mL microfuge tubes and then centrifuged at 400g for 2 minutes. Supernatant was aspirated to cleanse the excess bacteria. This step should be repeated until the supernatant looks clear. To fixate the nematodes, 120 µL methanol was added and

incubated for 2 minutes on ice. After that, fixated nematodes were centrifuged for 1 minute at 1000 rcf and supernatant aspirated. 600 μ L of 60% isopropanol was added and incubated for 15 minutes to dehydrate the worms. After 15 minutes, ORO solution was added to stain worm fat tissues. Worms were incubated in this solution for at least 40 minutes in an orbital shaker. To clean up the excess stain, 1xPBS containing 0.1% Triton-X100 solution were added to fill the entire microfuge tube, settled down by gravity for 3 minutes. This step also eliminates eggs and larvae, centrifuging defeats the purpose. This step was repeated until there was no visible dye particles or laid eggs. Worms were then mounted on 0.5% agarose filled 60 mm petri dishes and their images were captured.



Figure 2.1 A nematode after staining procedure.

2.2.5. Light Microscopy

Worm images were captured under 640 times or 400 times zoom if not applicable with Olympus, IX71 stereomicroscope. For both IFD and PPR experiments, we aimed to capture at least 25 worms. Main challenge was lighting conditions of the petri dishes as pear shaped stage prevented us to capture worms efficiently when we try to capture the worms near borders of the plate (picture XXX), to resolve this problem we simply designed two stages for 35-mm and 60-mm petri dishes and commissioned Çuhadaroğlu Aluminum Factory (İstanbul, Turkey) to produce an alternative stage which doesn't block the lights near the sides of the plates. After that, custom made stages were used during the experiments.



Figure 2.2. Uneven lightning near the borders of a petri dish caused by default stage of IX-71 microscope.



Figure 2.3. On the left, pear shaped stage that casts dark zones on the plates, on the right custom-made stage designed for 35-mm petri dishes.

2.2.6. Pharyngeal Pumping Rate Experiment

Before every experiment worms age was synchronized as described above. Meanwhile, freshly prepared 400 mM FUDR was added 200 μ l on NGM agars before they have been seeded by bacteria. After FUDR was dried out, plates were seeded with bacteria. After the worms have reached L4 stage before transferring the worms to the petri dishes experimental solutions (glucose, oatmeal, levan or HL) added to the petri dishes and worms placed into puddle. 15 nematodes has been transferred to the petri dish and 8 of them recorded randomly. First PPR recording has been done the day after, counted as 4th day. After that, for every 3- or 4-days worms were transferred into

fresh dishes by using worm picks, in which experimental solutions, bacteria, FUDR were renewed. After changing the petri dishes, PPR were recorded with Free Screen Recorder (Digital Wave Ltd., Great Britain) software since microscope's default software DPController couldn't provide sufficient frame per second requirements. In short, PPR were recorded at 4th, 7th, 10th, 13th, 17th, 21st days using IX71– 1B stereomicroscope equipped with a transmitted light for at least 15 seconds for each worm. Dead worms were recorded for 5 seconds. Pharyngeal pumping rate was counted with two independent examiners and average of their counts was divided by length of the video (usually 15 seconds) and multiplied by 60.

2.2.7. Image Analysis

Images have been analyzed using ImageJ v1.52j (National Institute of Health, USA). To calculate optical density of a flat image, we had to use an external standard too in order to apply Beer-Lambert law on 2D images as described by Mark Vivino (Vivino 2019) Preferably we have used David Rodbard's formulae (Rodbard 2019) and "Kodak No. 3 Calibrated Step Tablet" provided in the related NIH website description. Step tablet is an image used for calibrating densitometric devices, and in this case, we have measured 21 steps of the image by using rectangular selection tool (Figure 2. 1). These measurements were than matched with OD values by using "Calibrate..." function in the software (Figure 2. 2).

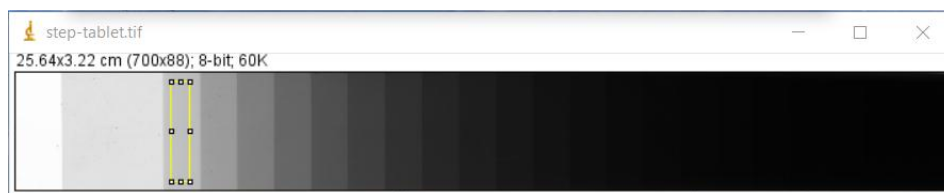


Figure 2.3. Selection of steps in the step tablet.

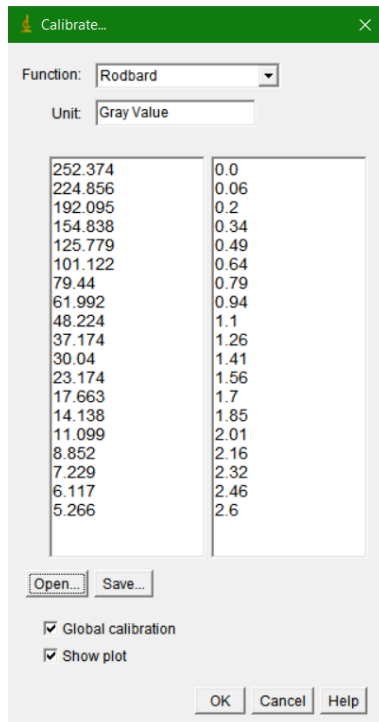


Figure 2.4. Calibration function of the software. Measurement results of each step given on the left column and respective OD value given on the right column. “Rodbard” function was selected.

After calibration step, worm images were opened in app simply by dragging the files on the application. Before analyzing each RGB image; red, green, blue channels were splitted using “Split Channels...” command. After closing green and blue channels, “Make Binary” command was used in order create high contrast image which provides ease of selection of the worm with “Wand” tool. After selection, high “Split Channels...” effect was removed by using “Ctrl + Z” shortcut and selection of pharynx and tail removed by using selection brush tool and finally OD values were measured using “Measure” command or by pressing “Ctrl + M” shortcut.



Figure 2.5. Image of nematode after its pixels was inverted and selected with wand tool. Yellow borders designate selected parts of the image.



Figure 2.6. An example of properly selected worm.

2.2.8. Statistical Analysis

Statistical analysis has been done by using GraphPad Prism 8.0.1 (California, USA) software. All ORO stain results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$.

3. RESULTS AND DISCUSSION

Oatmeal shown to have lipid lowering and lifespan elonging properties on *Caenorhabditis elegans*. Although not many, for levan polymer there are studies exist for its weight loss effects mainly on mice and rats. To our knowledge there are no studies exist comparing *Halomonas* levan with oatmeal for its lipid lowering effects using *C. elegans* as model organism.

3.1. Oil Red O Staining Results

To compare levan with oatmeal for their lipid lowering effects, for first batch of Oil Red O staining experiments we used 4 strains; N2, GR1307, GA158 and VC199 and 5 experimental condition; control (no treatment), 2 % glucose, 2 % levan, 2 % hydrolyzed levan and 3 % oatmeal. After we got significant results on N2, VC199 and GA158 strains for lipid lowering effect which is comparable to the oatmeal's lipid lowering effect we started second batch of experiment using only levan and glucose combinations compared to the control group. All experimental groups fed with 200 μ l LB medium heat killed *E. coli* which confirmed to have 5×10^9 to 5×10^{11} cells/ml which calculated from its optical density.

3.1.1. N2 Results

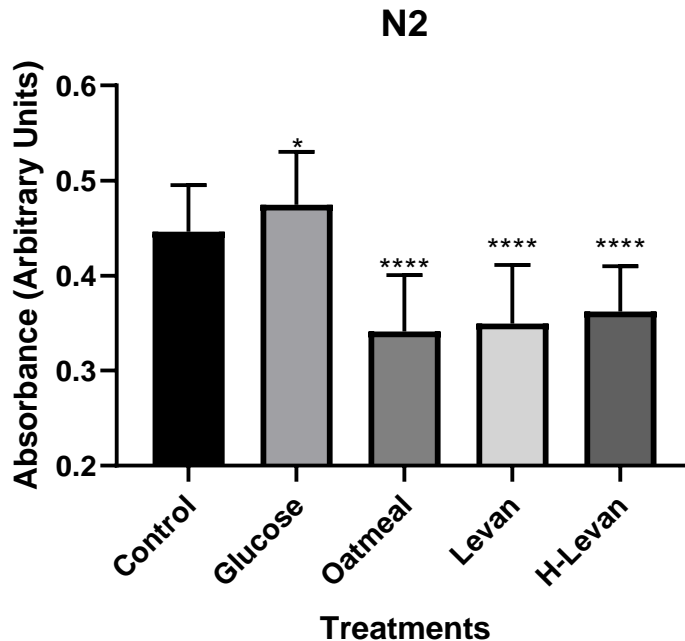


Figure 3.1. Result of N2 treatments. * indicates $P \leq .05$ and **** indicates $P \leq .00005$ 2% Levan and 2% Hydrolyzed Levan treatment significantly reduced intestinal fat similar to the 3% oatmeal treatment while 2% glucose treatment significantly increased fat deposition compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE (Standard Error) statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.1).

Our first result (Figure 3.1) indicate that levan and hydrolyzed levan decrease the fat deposition similar to the oatmeal around 22 %, 19 % and 24 % respectively while adding glucose to the diet increased the intestinal fat 11 %. This increase alone can be perceived with looking at redness to the representative pictures (Figure 3.2.). But it is noteworthy to add these pictures just a small fraction of 468 nematodes imaged for this experiment and exhibited with the sole purpose of better visualization.



Figure 3.2. Aligned pictures of N2 strain representing the experiment results shown in the figure above. A: control group, B: glucose treated group, C: oatmeal treated group, D: levan treated group, E: hydrolyzed levan group.

Another research group also reported decreased intestinal fat deposition by around 30 % after oatmeal added to the nematode medium 0.5 to 1 % (Gao, Gao et al. 2015). Although their research provided great insights which led to this research, small sample size ($n=5$) compared to the minimum standard maintained ($n=20$) during Oil Red O staining experiments; usage of Nile red as fat staining dye render their data rather unreliable which will be further explained (Gao, Gao et al. 2015).

Usage of Nile red stain for visualizing fat deposits also exercised by our laboratory before switching to the Oil Red O (data not shown), and it is found that Nile red staining have relatively high day to day variability due to usage of acetone which is more volatile than isopropanol used in Oil Red O. Another reason to choose Oil Red O over Nile red dwarfs preceding one is that, it has been clearly shown that Nile red intensity does not correlate with fat storages in *C. elegans* proved by comparing biochemical analyses and quantification of mutant strains' triglyceride content and their Nile red intensity. Within the same study, it has also been shown that 6 hours of fasting state increased Nile red intensity by 40 % while same amount of fasting decreased Oil Red O intensity by 17 % which is more reliable compared to the Nile red staining (O'Rourke, Soukas et al. 2009).

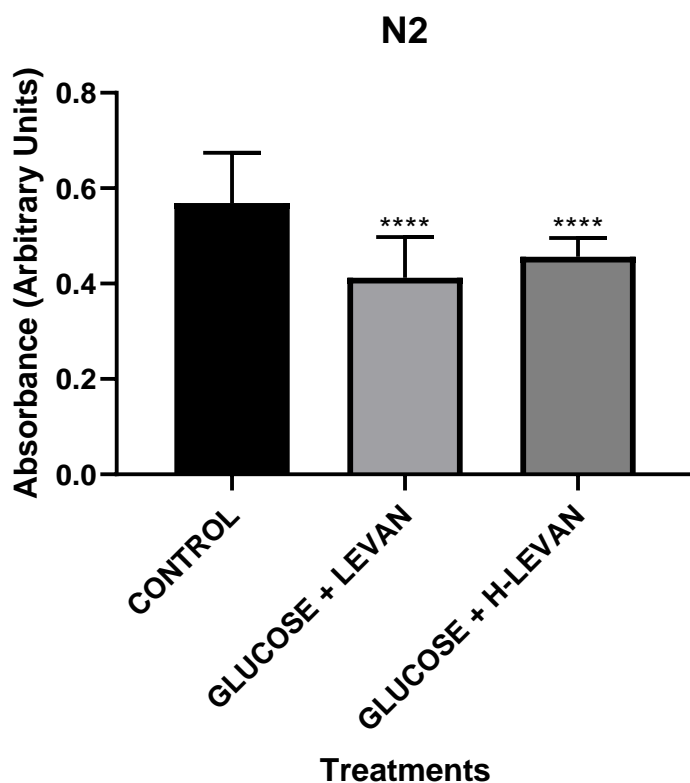


Figure 3.3. Result of N2 combined treatments. **** indicates $P \leq .00005$

After these finding combined active compound experiments conducted. When combined with 2% glucose; 2 % levan and 2 % hydrolyzed levan shown to reduce intestinal fat in N2 strain significantly compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed

using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.3). This data shows levan and hydrolyzed levan alleviate the fat increasing effect of glucose of *C. elegans* in wild type strains which can't be able to observed in N2 strain when nematodes treated with both 2 % glucose or 0.5 to 1 % oatmeal combined together in Gao et al.'s work(Gao, Gao et al. 2015).

3.1.2. GR1307 Results

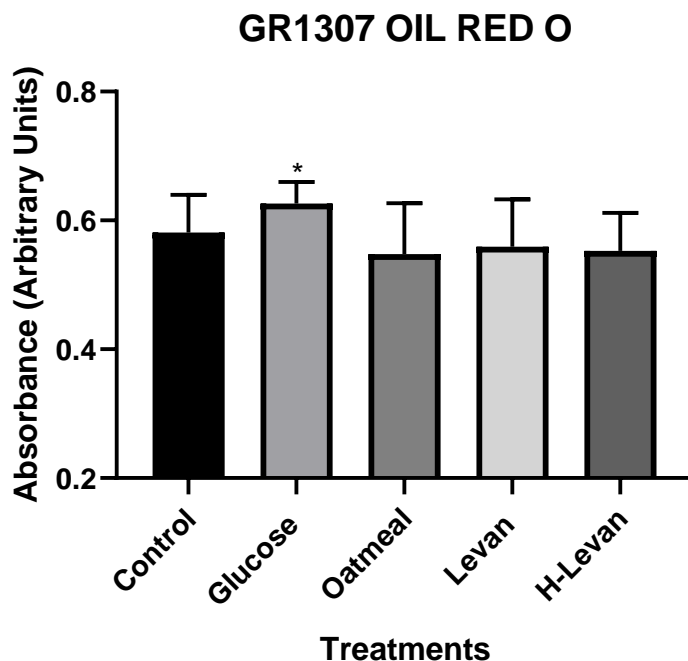


Figure 3.4. Result of GR1307 treatments. * indicates $P \leq .05$

2% levan, 2% hydrolyzed levan and 3% oatmeal treatment (non significantly) reduced intestinal fat while 2% glucose treatment significantly increased fat deposition compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.4).

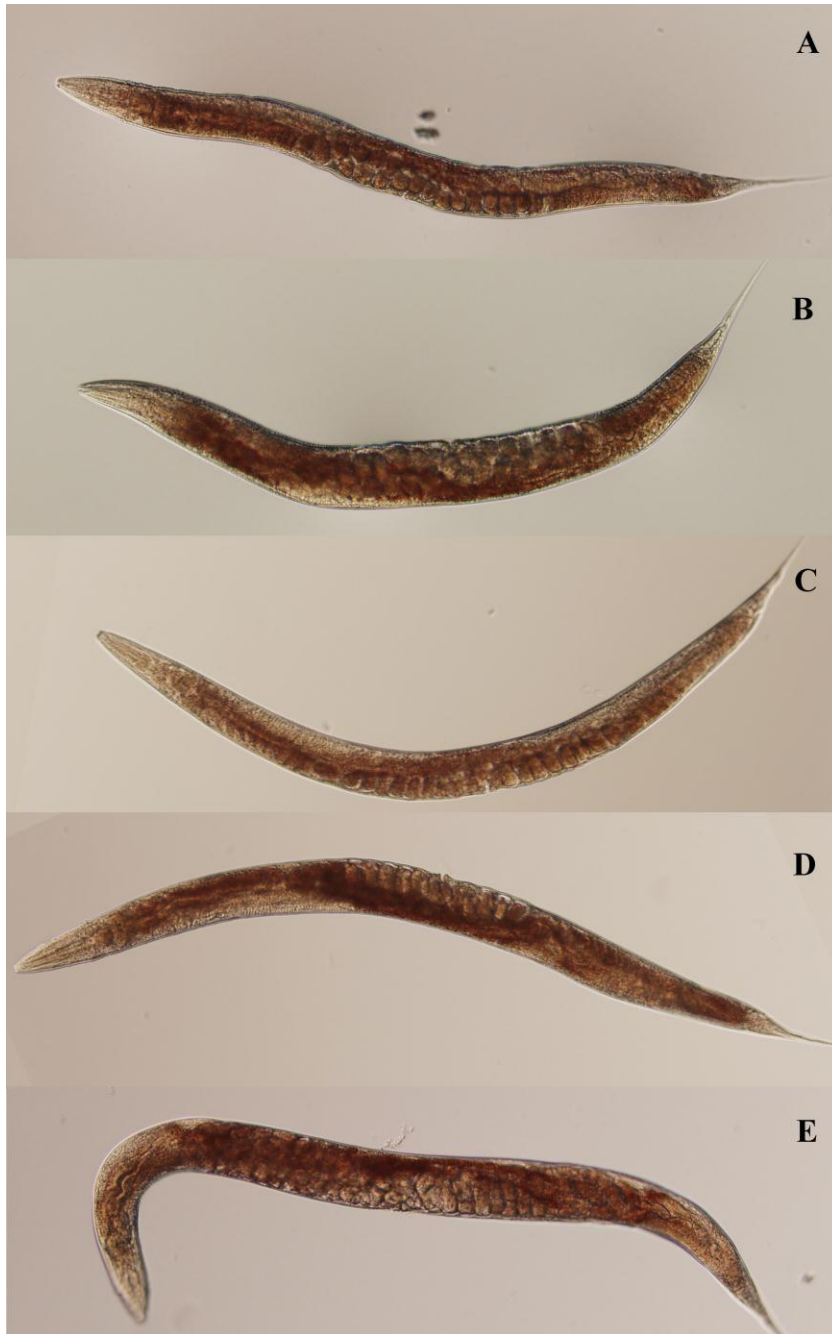


Figure 3.5. Aligned pictures of GR1307 strain representing the experiment results shown in the figure above. A: control group, B: glucose treated group, C: oatmeal treated group, D: levan treated group, E: hydrolyzed levan group.

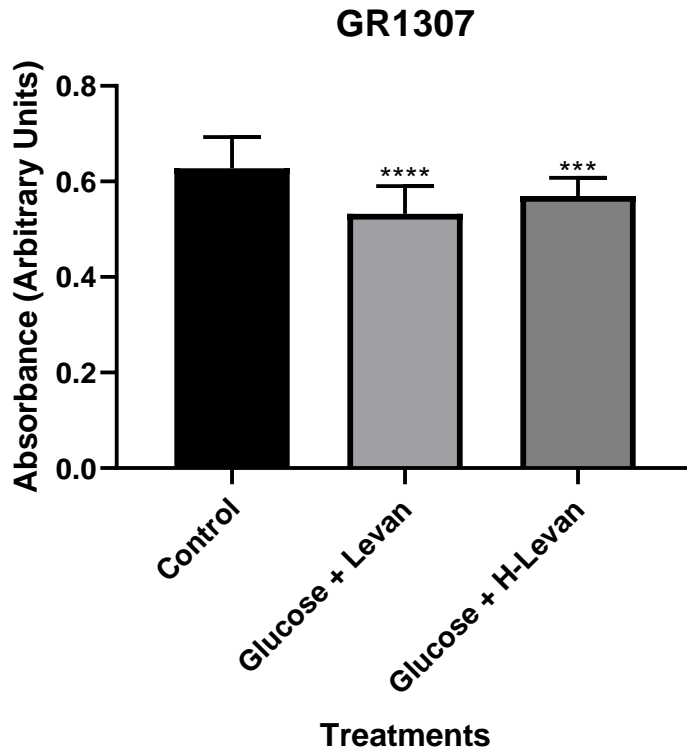


Figure 3.6. Result of GR1307 combined treatments. *** indicates $P \leq .0005$

When combined with 2% glucose, 2 % levan and 2 % hydrolyzed levan shown to reduce intestinal fat in GR1307 strain significantly compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.6).

Closest work to compare the results is again Gao et al.'s and their results shows decreased intestinal fat deposition by 17 % by treating 0.5 % and 37 % by treating % 1 oatmeal with worms respectively. And again this finding can disregarded easily and can be tied to Nile red usage in their experiment (Gao, Gao et al. 2015). But when compared to VC199, GA158 and N2 strains' results, GR1307 (daf-16(mgDf50) I.) results contradicts and this is only experimental group in which oatmeal, levan and hydrolyzed levan fail to reduce intestinal fat significantly. While levan and hydrolyzed levan usage does not significantly reduce fat deposits in the scope of first experiment (Figure 3.5), these substances shown to reduce intestinal fat deposits when they are combined with lipid increasing glucose (Figure 3.6) makes these experiments not only contradicts within the experiment set-ups also contradicts across the research papers.

This result could be credited of DAF-16 proteins' central position in the IIS pathway in which a deletion shows unpredictable results, as well as can be credited to simple overlooked experimental errors and repeating experiments which form "Figure 3.5" can relieve this contradiction.

3.1.3. VC199 Results

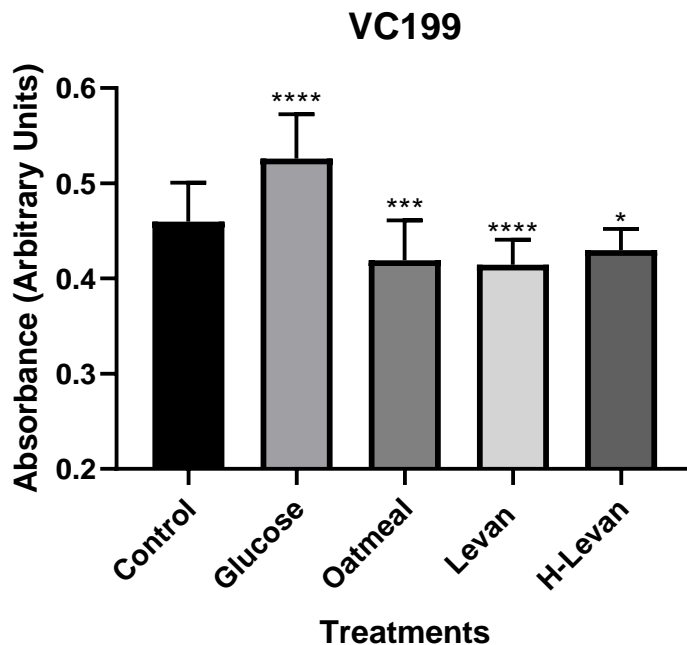


Figure 3.7. Result of VC199 treatments. * indicates $P \leq .05$ *** indicates $P \leq .0005$ and **** indicates $P \leq .00005$.

2 % levan and 2 % hydrolyzed Levan treatment significantly reduced intestinal fat similar to the 3 % oatmeal treatment while 2% glucose treatment significantly increased fat deposition compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.7).

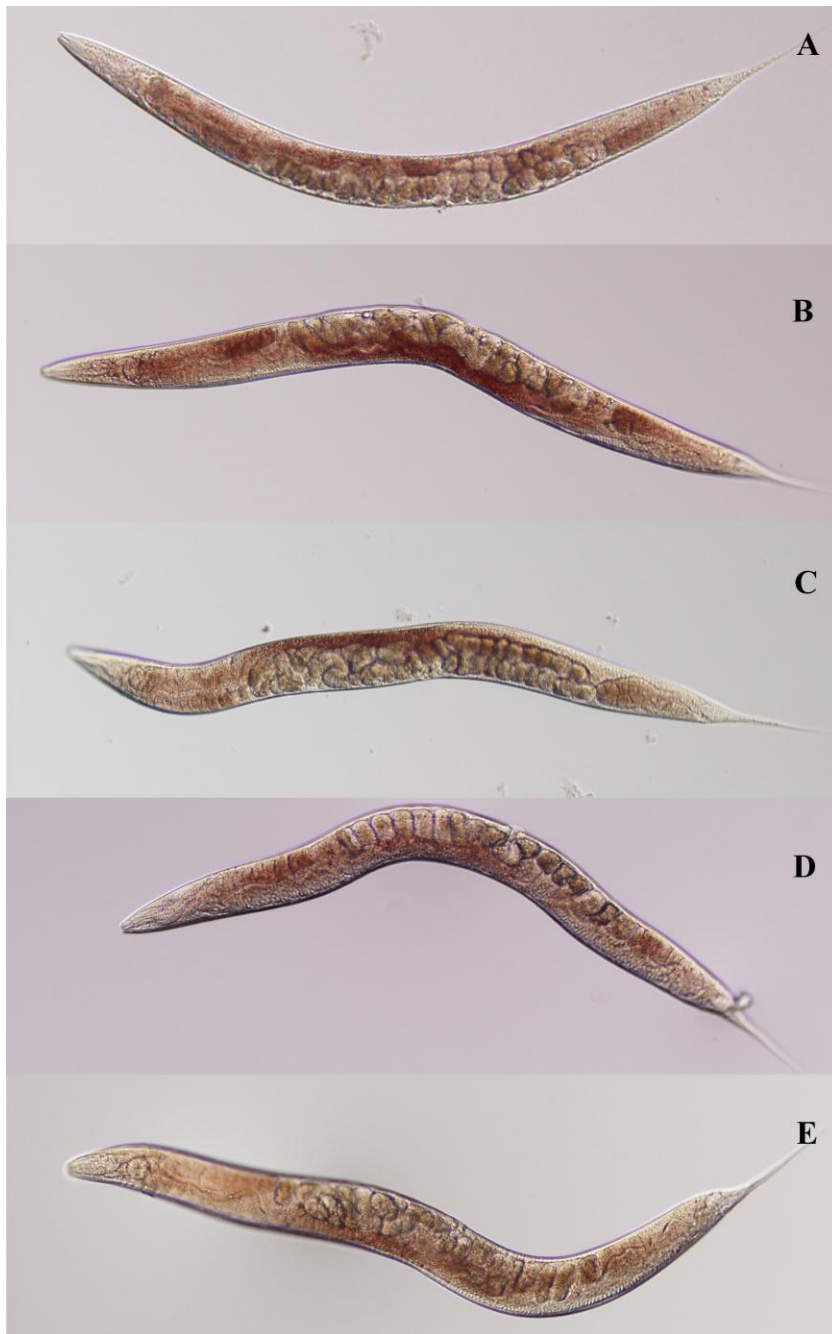


Figure 3.8. Aligned pictures of VC199 strain representing the experiment results shown in the figure above. A: control group, B: glucose treated group, C: oatmeal treated group, D: levan treated group, E: hydrolyzed levan group.

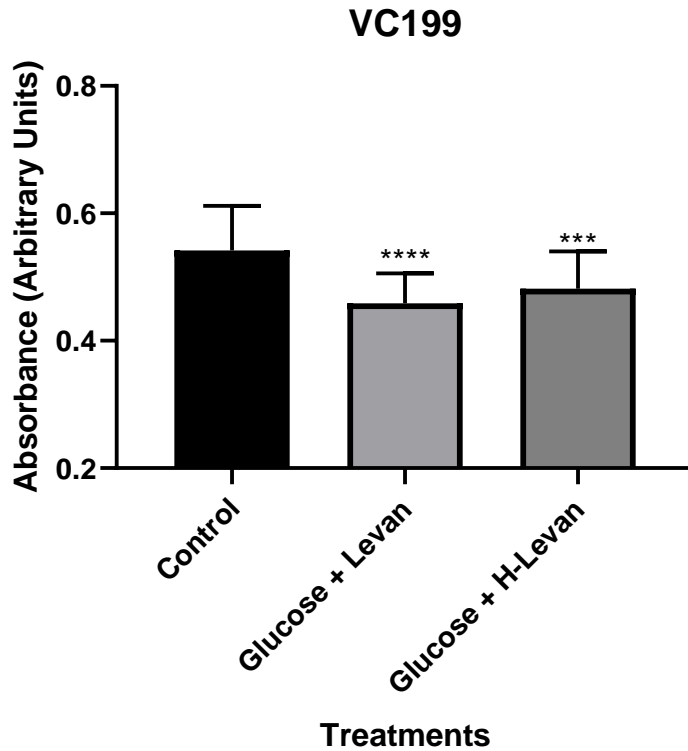


Figure 3.9. Result of GR1307 combined treatments. *** indicates $P \leq .0005$.

When combined with 2% glucose, 2 % levan and 2 % hydrolyzed levan shown to reduce intestinal fat in VC199 strain significantly compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.9).

VC199 is sir-2.1 (ok434) deletion mutant lacks SIR2.1 protein which affect IIS pathway by deacetylation of DAF-16 as well as acting parallel to the this pathway and sir-2.1 deletion leads to suppression of slow-feeding caloric restriction mutant eat-2 and unc-13 strains (Wang and Tissenbaum 2006). Considering this, significant reduction of intestinal fat deposits by treating the worms with oatmeal, levan and hydrolyzed levan in VC199 strain implicates, assuming levan also causes some form of caloric restriction, completely bypasses effect of the sir-2.1 deletion. Gao et al. also reported reduction of intestinal fat deposits in VC199 strain after treatment of 3 % oatmeal by around 15 %. Our results agree (Figure 3.7 and Figure 3.9) with the scientific findings that indicate higher dose of treatments needed in the absence of sir-2.1 gene in which in this case levan and hydrolyzed levan found to be high enough

even in the presence of glucose (Burnett, Valentini et al. 2011, Gao, Gao et al. 2015).

3.1.4. GA158 Results

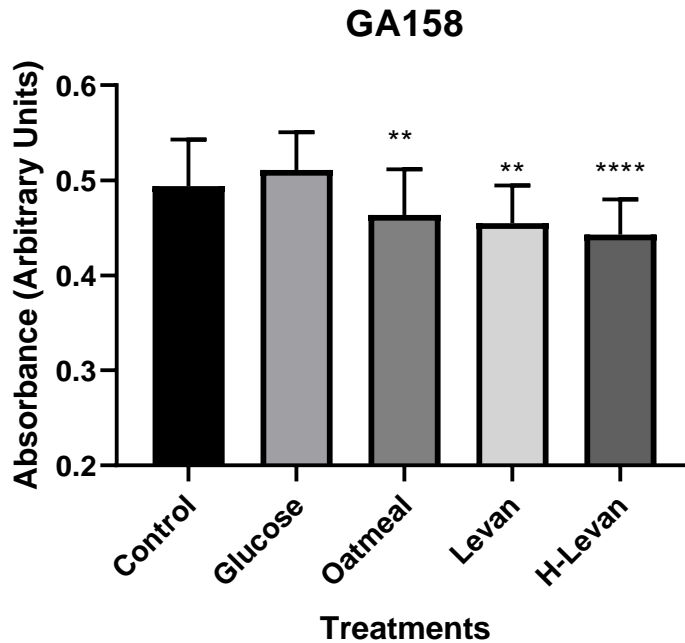


Figure 3.10. Result of GA158 treatments. ** indicates $P \leq .005$ and **** indicates $P \leq .00005$.

2% Levan and 2% hydrolyzed levan treatment significantly reduced intestinal fat similar to the 3% oatmeal treatment while 2% glucose treatment non-significantly increased fat deposition compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as duplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.10).



Figure 3.11. Aligned pictures of GA158 strain representing the experiment results shown in the figure above. A: control group, B: glucose treated group, C: oatmeal treated group, D: levan treated group, E: hydrolyzed levan group.

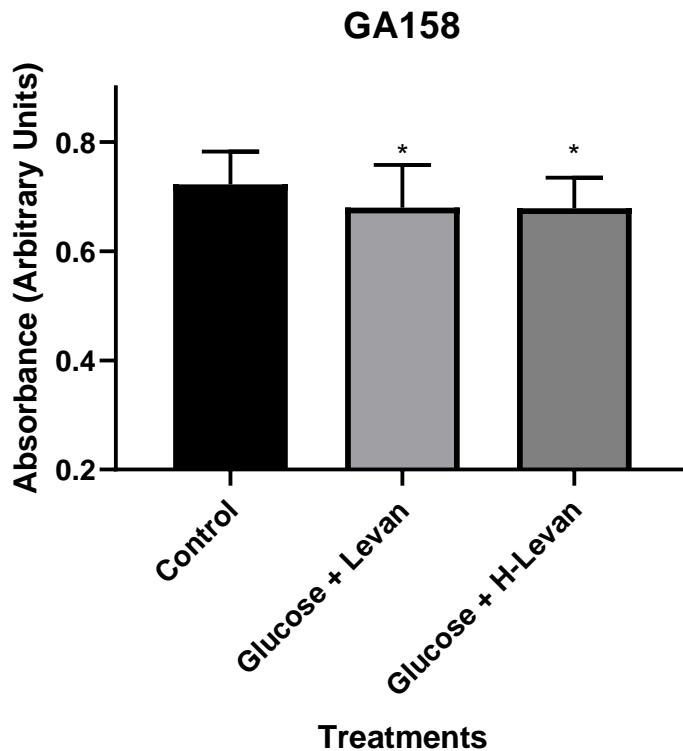


Figure 3.12. Result of GA158 combined treatments. * indicates $P \leq .05$.

When combined with 2% glucose, 2 % levan and 2 % hydrolyzed levan shown to reduce intestinal fat in GA158 strain significantly compared to the control group. Results presented here are the mean value of at least 20 animals and experiment conducted as triplicate. All results are presented as mean \pm SE statistical analysis was performed using a one-way analysis of variance (ANOVA). Statistical significance was set at $P \leq .05$ (Figure 3.12).

3.2. Pharyngeal Pumping Rate Experiment Results

3.2.1. N2 Results

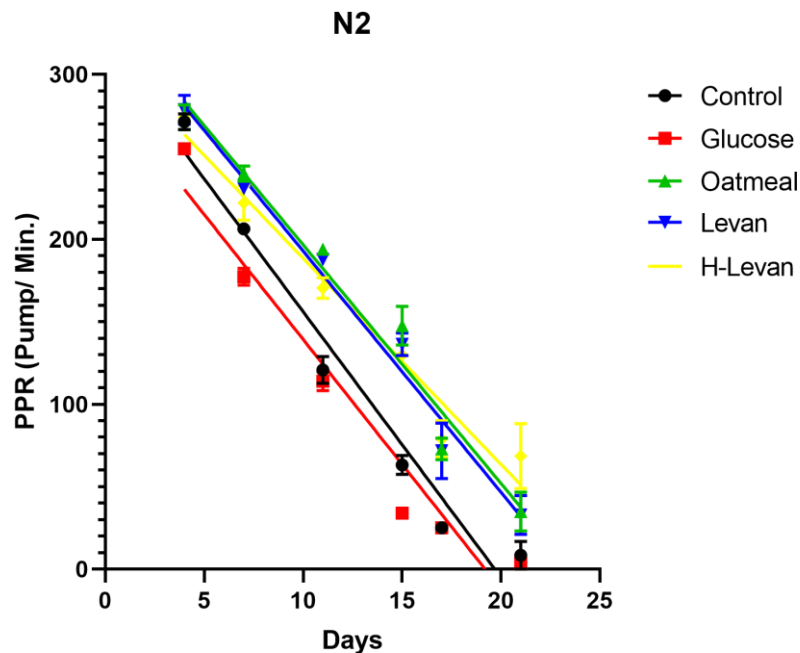


Figure 3.13. Pharyngeal pumping rate results of N2 strain.

Graph shows median PPR values of duplicate experiments containing 15 animals each and PPR of 8 of the randomly selected animals has been recorded every 3 or 4 days. Data here indicate that; HL, levan and oatmeal group shown to have increased PPR through the experiment compared to the control and glucose group. Linear regression lines fitted into the graph shows higher elevation of HL, levan and oatmeal than glucose and control group which is a result of higher PPR value these of experimental treatments. This elevation differences are statistically extremely significant ($p < 0.0001$) and difference between glucose and levan, difference between glucose and oatmeal and difference between glucose and hydrolyzed levan are individually significant ($p < 0.05$) calculated by one-way ANOVA. Moreover; HL, levan and oatmeal shown to have positive impact on nematode lifespan evident by 21st day's PPR values and more nematodes were alive compared to glucose and control groups.

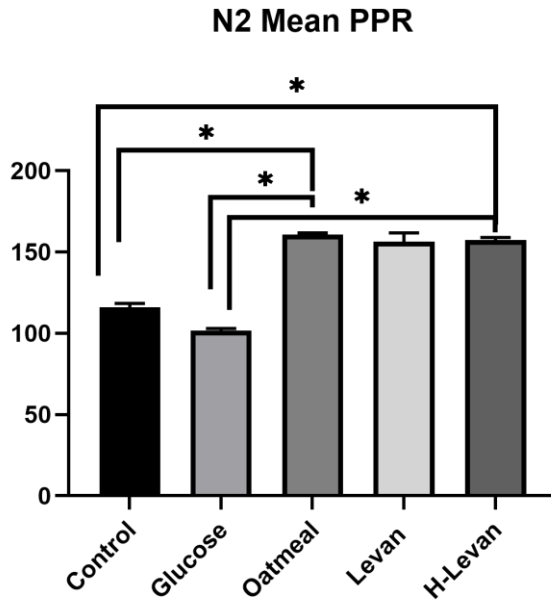


Figure 3.14. Mean PPR value comparisons between experiment groups in the results of N2 strain.

To better visualization of the data (Fig 3.14), mean values of PPR including every measurement, calculated and made into a graph, error bars plotted with SEM (Standard Error Mean). After that one-way ANOVA is applied to determine degree of significance of the relation between each group. Difference between control and oatmeal, control and HL, glucose and oatmeal, glucose and HL found to be statistically significant ($p < 0.05$).

3.2.2. GR1307 Results

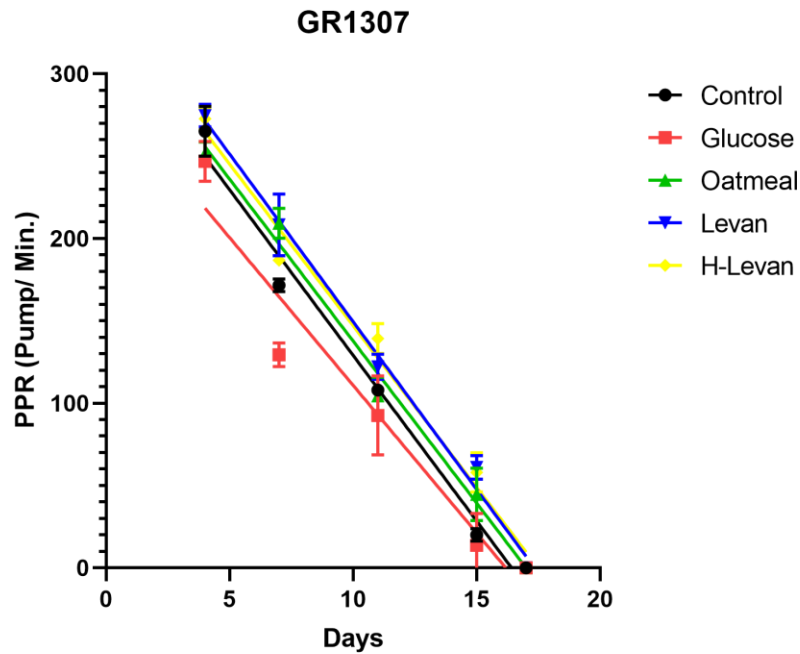


Figure 3.15. Pharyngeal pumping rate results of GR1307 strain.

Data here (Fig 3.15) indicate that; HL, levan and oatmeal group shown to have increased PPR through the experiment compared to the control and glucose group. Linear regression lines fitted into the graph shows higher elevation of HL, levan than oatmeal and oatmeal group's elevation higher than glucose and control group which is a result of higher PPR value of the former group of experimental treatments. This elevation differences are statistically extremely significant ($p < 0.0001$) but there are none individual significant differences between each group when calculated by one-way ANOVA. None of the experimental treatments increased the lifespan and short lived *daf-16* null mutants' nematodes PPR measured 0 at day 17, with none of the animals surviving that day.

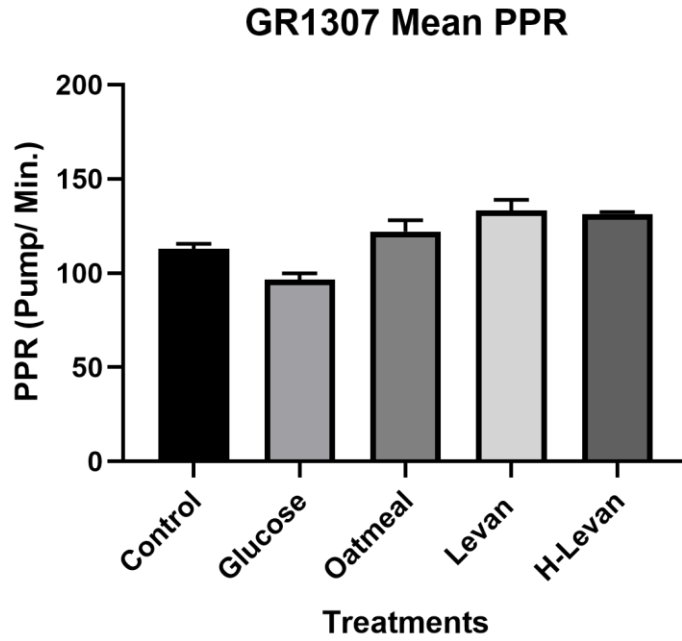


Figure 3.16. Mean PPR value comparisons between experiment groups in the results of GR1307 strain.

Data here indicates (Fig 3.16) the mean values of PPR including every measurement, calculated and made into a graph, error bars plotted with SEM. After that one-way ANOVA is applied to determine degree of significance of the relation between each group. Although oatmeal, levan and HL group have higher mean PPR value than control and glucose group, no statistical significance can be found between groups.

3.2.3. VC199 Results

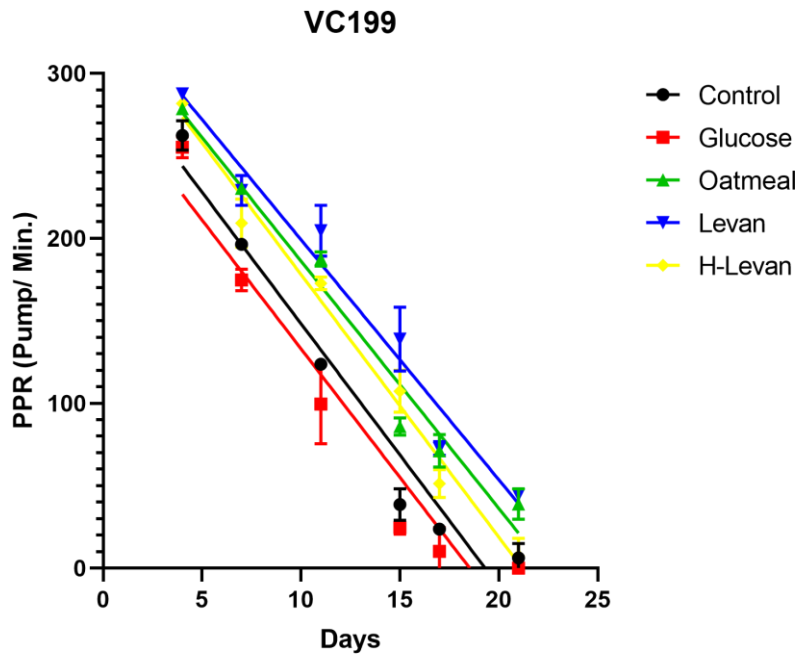


Figure 3.17. Pharyngeal pumping rate results of VC199 strain.

Data here (Fig 3.15) indicate that; levan and oatmeal group shown to have increased PPR through the experiment compared to the HL, control and glucose group. Linear regression lines fitted into the graph shows clear elevation differences between each group and value of each group can be arranged from higher to the lowest; with groups aligned into levan, oatmeal, HL, control and glucose group respectively. Higher elevation is a result of higher PPR value these of experimental treatments. This elevation differences are statistically extremely significant ($p < 0.0001$). When individually calculated differences between levan and glucose, levan and control, control and glucose, levan and HL are found statistically significant ($p < 0.05$); differences between oatmeal and control, oatmeal and glucose found statistically very significant ($p < 0.01$).

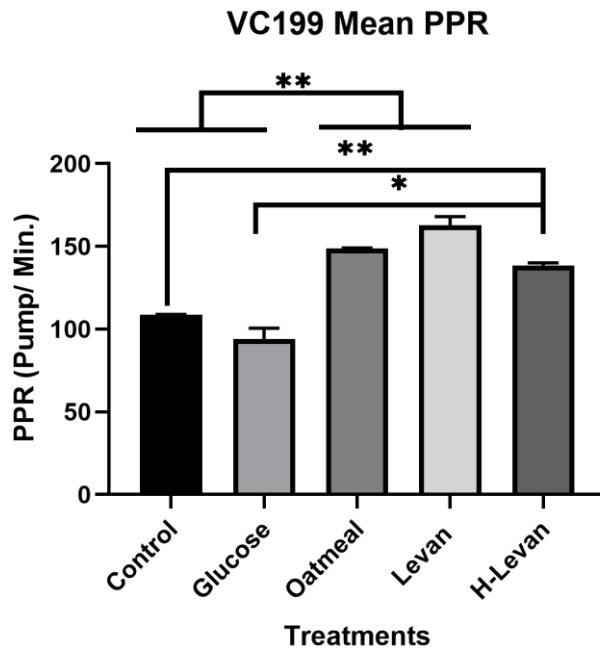


Figure 3.18. Mean PPR value comparisons between experiment groups in the results of VC199 strain.

Column graph indicates (Fig 3.18) the mean values of PPR including every measurement, error bars plotted with SEM. After that one-way ANOVA is applied to determine degree of significance of the relation between each group. As shown above; differences between oatmeal and control, oatmeal and glucose, levan and glucose, levan and control, HL and control found to be statistically very significant ($p < 0.01$). while differences between HL and glucose found to be statistically significant ($p < 0.05$).

3.2.4. GA158 Results

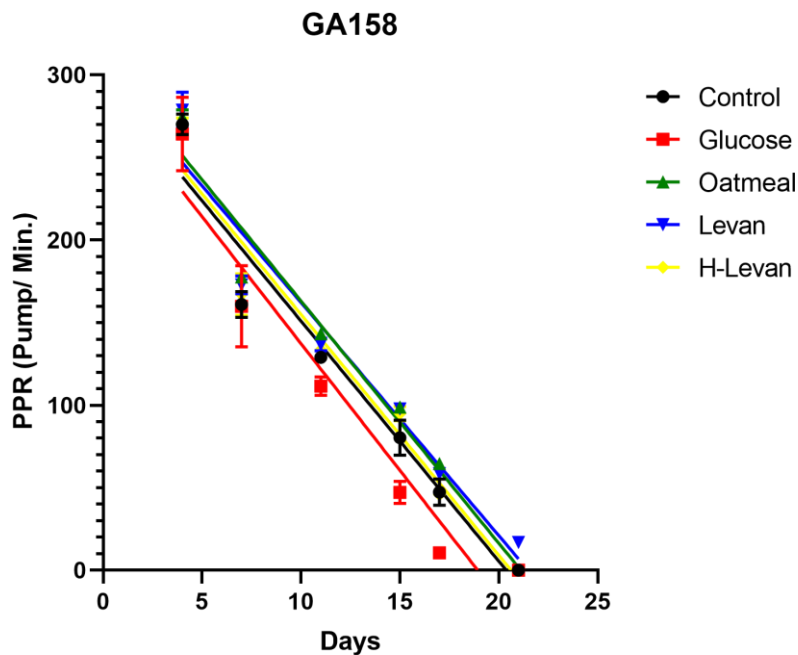


Figure 3.19. Pharyngeal pumping rate results of GA158 strain.

Data here (Fig 3.19) indicate that; HL, levan and oatmeal group shown to have increased PPR through the experiment compared to the control group and control group's PPR is value is higher compared to the glucose group. Linear regression lines fitted into the graph shows higher elevation of HL, levan and oatmeal than control group, control group have higher elevation than glucose group; which is a result of higher PPR value these of experimental treatments. This elevation differences are statistically significant ($p < 0.0163$) and difference between control and levan; and difference between control and levan are individually significant ($p < 0.05$) calculated by ANOVA.

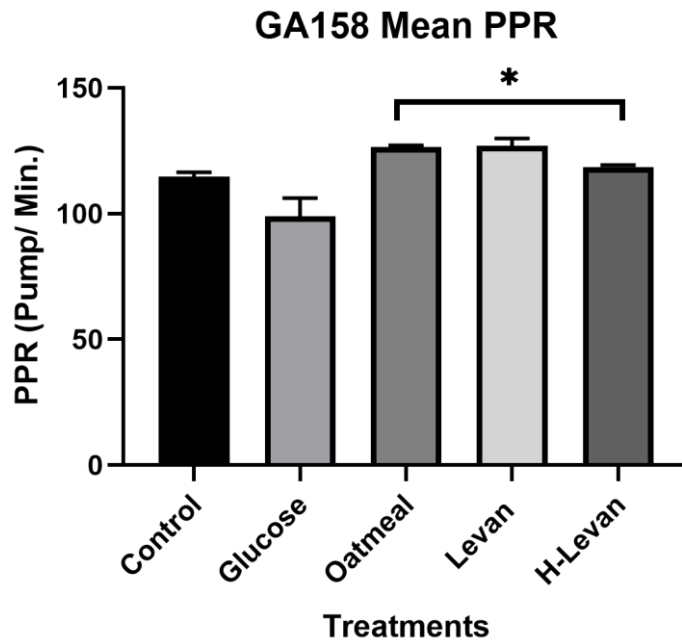


Figure 3.20. Mean PPR value comparisons between experiment groups in the results of VC199 strain.

Column graph indicates (Fig 3.18) the mean values of PPR including every measurement, error bars plotted with SEM. After that one-way ANOVA is applied to determine degree of significance of the relation between each group. As shown above; differences between oatmeal and H found to be statistically significant ($p < 0.05$).

Since pharynx is used for nutrient intake, rate of the of the pharyngeal pumping is decisive factor for caloric intake in nematodes. It is known fact that, caloric restriction and caloric deprivation increases median PPR along with the lifespan (Lee, Wilson et al. 2006). Nematodes deprived from food source such as bacteria for prolonged periods of time have higher PPR than nematodes feeding *ad libitum*, when they transferred into petri dishes containing food. This can be explained as a compensating behavior, simply consuming more calories until the organism can achieve its median caloric intake. This is also evident from the fact PPR goes back to normal in food deprived after couple of hours after the transfer into petri dish with bacteria (Luedtke, O'Connor et al. 2010).

3.3. Discussion of Experimental Results

In our experiments levan, HL and oatmeal groups shown to have increased PPR than control and glucose whether it is statistically significant or not. Hypothetically, these

findings might indicate four possibilities; levan polymer and oatmeal β -glucans might be blocking pharyngeal opening partially, they might be preventing nutrient absorption by sticking into intestines, and finally they might be absorbed with food and excreted without digestion only to be ingested again, effectively reducing food intake per pump which means dilution of caloric source. In fact, all of them might be true at the same time but even one of the possibilities is enough for causing a caloric reduction in nematodes. To directly link high PPR with low caloric intake we should also take into account of Oil Red O staining results.

When we look at N2 ORO results and PPR results it is shown that PPR and ORO data correlates; while PPR is increased over the course of the experiment fat tissue is reduced by HL, levan and oatmeal compared to the control and glucose. Glucose increased fat deposition, decreased PPR especially after 7 days and worms of this are aged faster than the other groups. These results indicate nematodes might be affected by either caloric deprivation or caloric restriction which begs the question “which molecular mechanisms might be involved?”.

To understand molecular mechanisms involved we should look at mutant strains' results. *daf-16* deficient mutant strain GR1307 (*daf-16(mgDf50)* I) indicates that levan, HL and oatmeal has shown no significant effect on fat tissue reduction, on PPR and on lifespan. During the normal course of the nematode lifespan, it is known that when IIS pathway is activated DAF-16 is inhibited increasing fat deposition to allow nutrient taken stored in intestines (Eijkelenboom and Burgering 2013). *daf-16* null mutants known to have higher fat content and shorter lifespan. Our result indicates fat reduction and PPR increase is dependent on DAF-16 transcription factor. This assumption only contradicts with the glucose-levan/glucose-HL combined treatment with glucose in which levan and HL significantly reduced fat tissue but this glucose known the affect *C. elegans* gene expression patterns (Lee, Murphy et al. 2009) and it is safe to assume there might be other molecular mechanisms involved result of the altered expression pattern.

When we look at another strain, sirtuin deficient VC199 (*sir-2.1(ok434)* IV.) we can see significant fat reduction by oatmeal, levan and HL; significant fat tissue reduction by HL and levan when combined with glucose; and significant PPR increase by HL,

levan and oatmeal compared to the glucose and control groups. From these results we can assume fat reduction and PPR increase caused by oatmeal, levan and HL are exerting their effects SIR-2.1 independent manner. This result is particularly important to understand degree of caloric deficiency caused by our treatments because apparently even there are many overlapping pathways between the effect of caloric deprivation and caloric reduction; there are experiments showed that caloric deprivation exerts its effect by SIR-2.1 dependent and DAF-16 independent manner (Lee, Wilson et al. 2006) while it is shown that caloric reduction exerts its effect by DAF-16 dependent and SIR-2.1 independent manner (Greer, Dowlatshahi et al. 2007). From these results we can postulate oatmeal, levan and HL imposes the nematodes some kind of caloric restriction.

GA158 (*daf-16(mgDf50) I; daf-2(m65) III.*) is *daf-2* and *daf-16* double mutant which lacks insulin receptor orthologue DAF-2 and FOXO orthologue DAF-16 protein (Patel, Garza-Garcia et al. 2008). While DAF-2 acts as receptor and found in the upstream of IIS pathway, DAF-16 acts as transcription factor and found in downstream of the same pathway. Hence GA158 mutant lacks 2 important component of IIS pathway and it is useful to observe whether IIS pathway involved in intestinal fat deposition. Our findings indicate even in the absence of the main components of IIS pathway oatmeal, levan and HL reduces fat depositions in *C. elegans*; the latter two lowers fat deposits even in the presence of 2 % glucose. Even though this strain also lacks DAF-16 transcription factor, lacking DAF-2 relieves the *daf-16* null mutant's resistance to the fat reducing, PPR increasing experimental treatments. This might be the due the fact DAF-2 plays important role to inhibiting other fat burning pathways not limited to just inhibiting DAF-16 transcription factor. It useful to note that while there are no significant PPR increase by oatmeal, levan and HL compared to the glucose and control groups; former groups' median PPR still higher than the latter groups' values.

From these experiments we can postulate that levan and hydrolyzed levan decreases fat deposits by caloric restriction provided by caloric dilution. Because of *C. elegans* feeds by pharyngeal pumping and cannot selectively "eat" the bacteria; any substance with no apparent caloric value added to the nematodes can reduce caloric intake by diluting food source and reduce energy intake per pump. As first law of

thermodynamics dictates that energy neither created or destroyed can only be transferred and fat deposits is just a form of stored energy; we postulate levan and hydrolyzed levan might also have slow down energy intake by either slow down gastric emptying or by reducing absorption of foods by covering the cell membranes in intestines and not by triggering form of response from the IIS pathway. Lastly and most importantly, being a surrogate marker lifespan; increased PPR values observed in levan and HL treated groups can be translated into these polymers anti-aging properties and may also positively impact humans' health. Whether these views are viable or not are not in the scope of this research and can further investigated in future.

4. CONCLUSIONS

As experimental evidence derived from GA158, N2 and VC199 strains clearly shows *Halomonas* levan and its hydrolyzed derivative shown to reduce fat depositions significantly compared to the control group comparable to the oatmeal experiment group. On all strains we have observed increased PPR in levan, HL and oatmeal groups compared to control and glucose groups. Our findings indicate these experimental treatments exert their effect on PPR, fat deposition and lifespan DAF-16 dependent and SIR-2.1 independent manner, and DAF-2 nullifying mutations reverts the effect of DAF-16 mutations on fat tissue and PPR. Although it is statistically non-significant, in GA158 and VC199 strains levan treatment shown to reduce fat tissue even more than oatmeal treatment. Also, for all strains while glucose was present, levan and hydrolyzed levan significantly reduced fat depositions compared to the control groups. Inferred from this *Halomonas* levan have lipid lowering effect as strong as oatmeal and can also be used in human experiments in future. Also, from our finding it can be also inferred that levan may also provide benefiting humans with its anti-aging properties as it shown in nematodes. Recent findings from Kirtel et al. shown that levan from *Halomonas sp.* can be produced up to 18 g/L with relatively high bioconversion efficiency compared to the other species of bacteria (Erkorkmaz, Kirtel et al. 2018). Moreover, usage of byproducts of sugar production such as sugar beet and starch molasses and usage of high salt in the production in the fermentation system provides low cost non sterile system which is sustainable and clearly advantageous over usage of oatmeal. As mass production of penicillin made possible in 1940 and started saving millions of people ever since, not the discovery of it in 1928; cost efficient levan production can enable large scale usage in food industry in which levan has already been under the spotlight and can be found inside ingredients of ever-increasing amounts of patents. This hopefully would lead to increase the variety of healthier food alternatives and provide a safe haven from today's obesogenic environment. And before this step, of course more research should be conducted on mice or rat models and finally on humans to confirm these findings.

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