2015

Fermentable Non-Digestible Dietary Fiber and Bioactive Compounds Affect Aging and Fat Deposition in Caenorhabditis elegans

Chenfei Gao

Louisiana State University and Agricultural and Mechanical College

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FERMENTABLE NON-DIGESTIBLE DIETARY FIBER AND BIOACTIVE COMPOUNDS
AFFECT AGING AND FAT DEPOSITION IN *CAENORHABDITIS ELEGANS*

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition & Food Sciences

by
Chenfei Gao
B.Sc. Hebei University of Science & Technology, 2006
M.Sc. Louisiana State University, 2011
December 2015
• To my parents Mr. Yunshen Gao and Mrs. Zhenhua Chen: without your sacrifices any of this would not be possible.
• To my wife Ms. Tian Shu, the center of my life: thank you for being my best friend.
• To my Aunt Ms. Yuncai Gao: for her encouragement, support, and suggestions.
ACKNOWLEDGMENTS

First and foremost I would like to thank my advisor Dr. Jolene Zheng for kindly providing me the opportunity of my Ph.D. degree. From day one, she has been a wonderful mentor and a great advisor. I greatly appreciate her patience, encouragement and supports. I am deeply indebted to Dr. Jack N. Losso for his support, advice, guidance, and patience throughout my graduate studies. I enjoyed the many discussions we had. I am very grateful to Dr. John W. Finley for all his support and expertise throughout the project.

I wish to express my most sincere and humble gratitude to Dr. Michael J. Keenan as my Graduate committee. He is wonderful, caring, and compassionate. I am also thankful for the Dean’s Representative, Dr. Adam T. Melvin, for being the witness in the completion of the dissertation. I also appreciate his wonderful suggestions for my study.

Thanks to Mr. Mingming Wang, my lab mate, for helping me in my projects. I am thankful to all the lab student workers, Michael King, Jason King, Zach Fitzpatrick, Cindy Kim, Mai Phuongmai Tran, Jenny Nguyen, Melanie Kaltakjian, Tuan Cao, and Nancy Mai for helping in research. I would like to thank Dr. Mark LeBlanc and Ms. Tammy Smith (Agricultural Chemistry Department, College of Agriculture, Louisiana State University) for their assistance on PWB, oats, and wheat bran samples preparation. I also would like to thank Ms. Anne Raggio for her technical support and help in performing bacteria DNA extraction and PCR study.

The projects received support from LSU AgCenter, grants awarded to Dr. Jolene Zheng by LA BoR (LEQSF(2010-13)-RD-B-02), ConAgra Foods® Inc., ConAgra Mills™, and PepsiCo Inc. The nematode strains used were provided by the Caenorhabditis Genetics Center (University of Minnesota, MN), which is funded by the NIH National Center for Research Resources (NCRR).
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ABSTRACT

Overweight and obesity have been imposing $147 billion a year to the health care system in the United States. Limited medications are available in the market with side effects. Surgical treatments are second-line obesity treatments. Short chain fatty acids (SCFAs) produced from the fermentable resistant starch improves the secretion of satiety hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) from L-endocrine cells of cecum and colon. We hypothesized that consumption of fermentable non-digestible dietary fiber and bioactive compounds will increase insulin sensitivity, reduce body fat, and improve healthspan in Caenorhabditis elegans (C. elegans), and hyperglycemia (2% glucose) that may cause insulin resistance and impair lipid metabolism will attenuate these effects. Wild type C. elegans N2 or sir-2.1(ok434)IV, daf-16(mgDf50)I, and daf-16(mgDf50)I;daf-2(m65)III mutants were used. The control animals were fed with E. coli OP50. Experimental groups were fed with additional treatments: butyrate (0.3mM, 0.6mM), sodium acetate (5mM, 50mM), sodium propionate (7mM), or tributyrin (0.5mM, 3mM); PWB, oats or wheat bran (0.5%, 1.0%, or 3.0% w/v) with or without additional 2% glucose. SCFAs increased the lifespan of N2 and daf-16(mgDf50)I, but reduced lifespan in the daf-16/daf-2 deficient and sir-2.1(ok434)IV mutants. PWB or wheat bran sustained the pharyngeal pumping rate (PPR) in N2, sir-2.1(ok434)IV, daf-16(mgDf50)I, and daf-16(mgDf50)I;daf-2(m65)III. The N2, daf-16, or sir-2.1 mutant increased the PPR following oat consumption. This increase persisted in the presence of glucose at a low dose in daf-16 or daf-16/daf-2 mutant. The Nile red stained intestinal fat deposition (IFD) was reduced by butyrate (0.3, 0.6mM), acetate (100mM), propionate (0.3mM), and tributyrin (0.1, 1mM) in N2; and was increased in sir-2.1 mutant. PWB reduced IFD in N2, sir-2.1 or daf-16 mutants. Hyperglycaemia attenuated the effects on IFD in N2 or daf-16/daf-2 mutant. Oat-feeding
decreased IFD in N2, and daf-16 or daf-16/daf-2 mutant with or without hyperglycaemia. Wheat bran reduced IFD in N2, and in daf-16 or daf-16/daf-2 mutants without hyperglycemia, while hyperglycemia increased IFD in sir-2.1(ok434)IV. In summary, PWB, oats, wheat bran, and SCFAs reduced the IFD and improved the healthspan in *C. elegans*, and these effects were mediated by the sir-2.1, daf-2, or daf-2/daf-16 pathways.
CHAPTER 1: INTRODUCTION

1.1 General Introduction

Obesity are affecting more than one-third of the adults (34.9% or 78.6 million) and 17% of the youth in the United States. Obesity is related to the increasing rate of chronic diseases including cardiovascular diseases, type 2 diabetes, fatty liver diseases, atherosclerosis, hypertension, insulin resistance, stroke, asthma, sleep apnea, osteoarthritis, neuro-degeneration, gall-bladder disease and cancers (Hotamisligil et al., 1994). The increasing prevalence of obesity associated diseases is responsible for the increased medical cost in the U.S. The estimated annual medical cost of obesity in the U.S. was $147 billion in 2008; the medical costs of the obese people were $1429 (estimated to be 42%) greater than normal weight people in 2006 (Finkelstein et al., 2009).

Anorectic agents and other anti-obesity drugs have been available for more than half a century (Snow et al., 2005). The systematic side effects and safety concerns of obesity drugs, however, have limited their clinical use. For example, The side effects of anti-obesity drugs include: Exenatide causes headache, nausea, and vomiting (Fineman et al., 2004), Sibutramine raises blood pressure, and Orlistat is associated with gastrointestinal in origin (Maahs et al., 2006). Surgical treatments also have been used as treatment interventions (Snow et al., 2005, Sinha et al., 2015, Zheng et al., 2010), but the high cost, potential risk, and weight regain have limited the utilization of surgery (Cirocchi et al., 2013). Therefore, safe and effective obesity treatments such as functional foods are in a growing demand. Functional foods provide medical benefits beyond their essential function as nutrients (Bray and Greenway, 2007, Hotamisligil et al., 1994). For example, the consumption of higher amounts of complex carbohydrate can lower
the prevalence of obesity, coronary heart disease, type 2 diabetes, and cancers (Anderson, 2004, Hauner et al., 2012). Dietary resistant starches (RS) present an emerging solution to obesity. Gut bacteria have been demonstrated to ferment RS, high-amylose maize (Tachon et al., 2013).

Dietary resistant starches (RS) present an emerging solution to obesity. Gut bacteria have been demonstrated to ferment RS, high-amylose maize (Tachon et al., 2013).

Average daily consumption of dietary fiber in the U.S. is currently 3 to 10 g/day, 4 to 13 fold less than recommended (Goldring, 2004, Anderson and Akanji, 1991). RS commonly exists in foods such as legumes, potato, and whole grains. RS can escape digestion by the digestive enzymes in small intestine and can be fermented by some intestinal anaerobic bacteria in large intestine as energy source for these bacteria and produce short-chain fatty acids (SCFAs, C2-C6) which have been demonstrated as component in the maintenance of health (Keenan et al., 2007, Fuentes-Zaragoza et al., 2010).

Dietary RS dilute energy density, reduce body fat, increase gut satiety hormones, elevate fat oxidation and lower plasma cholesterol/triglyceride (Dethlefsen et al., 2007, Keenan et al., 2006, Zhou et al., 2006). Feeding C. elegans with amylose starch significantly reduced the fluorescent intensity of Nile red staining for the intestinal fat deposition (IFD) and increased the locomotion of C. elegans (Zheng et al., 2010). The endogenous compounds filtered from the microflora of cecal contents of mice fed high amylose starch showed similar trends to reduce the fluorescent intensity of the Nile red positive IFD and to increase locomotion in C. elegans. Keenan et al. has demonstrated that RS feeding to rodents reduces abdominal fat significantly (Keenan et al., 2007). Zhou et al. has demonstrated that total glucagon-like peptide 1 (GLP-1) and total peptide-YY (PYY) in rats fed a RS diet were significantly increased compared with controls; Plasma PYY and gene transcription for PYY in the cecum from rats were significantly increased (Zhou et al., 2008). RS can also augment food digestion and GI motility, stimulate the growth of beneficial microorganisms in the colon and maintain bowel health (Burnell et al., 2005,
Zheng et al., 2010). Furthermore, RS promote insulin sensitivity, boost mitochondrial function, and enhance hosts’ health (Zhou et al., 2008, Tachon et al., 2013, Keenan et al., 2013, Dethlefsen et al., 2007, Flint, 2012, Forsberg et al., 2014, Sekirov et al., 2010).

Endogenous SCFAs are major products from the fermentation of RS by intestinal anaerobic bacteria. The endogenous SCFAs produced mainly include acetate, propionate, and butyrate (Haarman and Knol, 2006, Duncan et al., 2003, Louis et al., 2007, Dethlefsen et al., 2007, Sekirov et al., 2010). These SCFAs contribute to normal large-bowel functions including the stimulation of fluid, the absorption of electrolyte and the modulation of muscular contraction by the perfusion of visceral circulation (Topping and Clifton, 2001). SCFAs produced by gut bacteria from the fermentable RS also increase the secretion of satiety hormones PYY and GLP-1 from L-endocrine cells of the cecum and colon (Keenan et al., 2013, Zheng et al., 2008).

Barley is the fourth most important cultivated foodstuff. It contains 62-77 % starch (w/w) which is composed of 25-35% amylose starch with 3-5% RS₃ and β-glucan (Asare et al., 2011, Vasanthan and Bhatty, 1998), a mixture that provides a distinctive amylose–amylopectin interaction (Tang et al., 2001, Lifschitz et al., 2002, Behall et al., 2005, Rendell et al., 2005, Dongowski et al., 2002). Barley also contains high levels of functional lipids, such as, total phytosterols (1.2-9.6% of barley oil) and total tocotrienols (0.3-0.6% of barley oil) which are 6-12 fold higher than in palm oil (0.05%) and 4-8 folds higher than in rice bran oil (0.08%) (Moreau et al., 2007). Barley also provides vitamins, trace minerals, and bioactive compounds (Inglett et al., 2011, Cui and Wang, 2009). In addition, there are many phenolic compounds in barley, such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (Holtekjølen et al., 2008, Verardo et al., 2008). These phenolic compounds in barley serve as natural antioxidants with
antiradical and antiproliferative potentials for disease prevention and health promotion (Madhujith and Shahidi, 2007).

The nematode *Caenorhabditis elegans* (C. elegans) is a multicellular eukaryote with small size and short lifespan. *C. elegans* conserves 65% of the genes associated with human disease and 35% of genes homologue to human (Villegas et al., 2008). The C. elegans model is considered as an convenient organism to screen different sources of prebiotics and pharmaceutical compounds and study human diseases or study of aging and test of the therapeutic compounds that may curtail aging (Zheng et al., 2010, Finley et al., 2013, Zheng et al., 2014, Zheng and Greenway, 2012). C. elegans is a model organism that is less expensive, more time-efficient, and a convenient option for initial high-throughput screening and detailed mechanism research (Villegas et al., 2008).

Insulin/Insulin-like growth factor Signaling (IIS) pathway is a primary mechanism for controlling metabolic physiology not only in humans, but also in C. elegans (Van Dyke and McCarthy, 2002). Many components of the IIS pathway are conserved from nematodes to humans (Barbieri et al., 2003, Zheng and Greenway, 2012). Reduction in the activities of the components of the IIS pathway leads to the activation of a series of genes involved in lifespan control, stress tolerance and protein misfolding suppression, collectively resulting in life-span extension (Kenyon, 2010, Lin et al., 1997). dauer formation-2 gene (daf-2) C. elegans mutants cause fat accumulation and glycogen production in adult (Burnell et al., 2005, Kimura et al., 1997). daf-16 is critical in mediating the downstream insulin signaling pathways and is the major target of the daf-2 pathway, a homolog of the insulin and insulin-like growth factor (IGF) receptors (Zheng and Greenway, 2012, Rizki et al., 2011, Von Stetina et al., 2007). ‘Aging’ has been reported to be related with the downstream effects of IIS pathway (Benedetti et al., 2008).
C. elegans has both an aerobic and an anaerobic metabolism, and can survive under a hypoxic condition (Burnell et al., 2005). Oxygen or “hypoxia gradient”-oxygen deprivation diffuses bidirectionally between the intestinal tract and the external environment (Burnell et al., 2005). John Finley, et al in 2013 have demonstrated that high amylose maize RS$_2$, legume (Finley et al., 2013), and the endogenous fermentation products from RS reduced the fluorescent intensity of the Nile red positive intestinal fat deposition (IFD) in C. elegans in vivo (Zheng et al., 2010, Zheng and Greenway, 2012, Dethlefsen et al., 2007, Flint, 2012, Finley et al., 2013). Our data suggest a possibility that the anaerobic bacteria in the C. elegans intestinal tract could ferment dietary fibers. Direct identification of C. elegans intestinal microbiota has not been reported. We hypothesize that feeding fermentable dietary fibers to C. elegans should closely resemble the effect of colonic fermentation products by the intestinal anaerobic microbiota in the large intestine in rodents or in humans. Likewise, a potential relationship of microbiome with C. elegans has been proposed (Cabreiro and Gems, 2013).

The objectives of this study were to determine the effects of consumption of Prowashonupana barley (PWB), Oats, or wheat bran byproduct which are rich in fermentable dietary fibers and other bioactive components on the IFD, lifespan, insulin resistance, and hyperglycemia-impaired lipid metabolism using high through-put C. elegans model. In addition, a pilot study was performed to investigate whether PWB increases the intestinal anaerobic microbiota in C. elegans.
CHAPTER 2: REVIEW OF LITERATURE

2.1 Background of obesity

2.1.1 Prevalence of obesity

Obesity is an accumulation of excessive body fat. A popular acceptable definition of obesity is the body mass index (BMI), which is the body weight in kilogram divided by the square of body height in meter, exceeding 30 kg/m$^2$. Overweight is defined as a BMI of 25 or higher. Extreme obesity is BMI more than 40 kg/m$^2$. The prevalence disorders of obesity have been increasing at a rapid rate in both developed and developing countries. The obese population is affecting more than one-third of the United States adults aged 18 years and over (34.9% or 78.6 million) and 17% of youth aged 0-17 (Zheng and Greenway, 2012, Ogden et al., 2014). The prevalence of obesity distribution map showed that no state in the U.S. has a prevalence of obesity less than 20% (Figure 1). The West had the lowest prevalence of obesity (24.9%), followed by the Northeast (26.5%), the Midwest (30.1%), and the South (30.2%). The States of Mississippi and West Virginia had more than 35% of obesity prevalence. The Louisiana’s adult obesity rate in 2014 is 34.9 percent as the fourth highest rate in the nation (Health, 2015). The increasing prevalence of obesity is responsible for the increased medical cost in the U.S. The estimated annual medical cost of obesity in the U.S. was $147 billion in 2008; the medical costs of the obese people were estimated to be $1,429 (42%) greater than normal weight people in 2006 (Finkelstein et al., 2009). Obesity predisposes to diabetes and/or other health care costs to an even greater extent (Scheen, 2000).
2.1.2 Obesity related health issues

Obesity is associated with an increased risk for developing diabetes, cardiovascular diseases, stroke, osteoarthritis, liver and gallbladder disease, sleep apnea and respiratory problems, and certain types of cancer (WHO, 2000, Must et al., 1999). Obesity also induce to hypertension, hyperlipidemia and hyperinsulinemia/insulin resistance (Kotsis et al., 2010). Additionally, obesity is correlated to impaired glucose tolerance, high levels of circulating insulin, and reduced insulin sensitivity. Obesity and insulin resistance are recognized as the leading causes of type 2 diabetes and the obese people have higher risk factors of insulin resistance than normal weight people (Kadowaki et al., 2003). Moreover, obese people display higher blood pressure levels than normal weight individuals even in the normotensive range; the obesity and hypertensions increase the probabilities of coronary diseases (Kotsis et al., 2010). In
addition, excess weight has a positive relationship with mortality which may differ among racial and ethnic groups (Must et al., 1999). The health risk increase is associated with a relatively small body weight gain, even not marked as obese (Health, 1998).

2.1.3 Causes of obesity

Obesity is a complex and incompletely understood chronic disease. Varieties of factors play roles in obesity including behavior, society, environment, metabolism, and genetic factors. One of the commonly acceptable reasons for obesity is energy imbalance that is energy or calorie intake higher than energy expenditure that a person uses to breathe, circulate blood, digest food, and be physically active (Danforth, 1985). The excess energy will be accumulated as fat or glycogen in the body. Overweight and obesity happen over time when the calories intake is always more than the expenditure. Environment and behavior factors include lack of physical activities, lack of healthy foods, oversized food portions, smoking, and stress associate with overweight and obesity (Health, 1998). Over the last 50 years, there has been a trend of increased intake of calorically dense foods that are high in fat, sugars, and salt but low in vitamins, minerals, and other micronutrients. There also has been a simultaneous trend of decreasing physical activity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization (French et al., 2001, Kumanyika, 2001). In addition, family history also influences the amount of fat stored in body because family members may share the similar habits, foods, and similar physical activities. Genes play an important role in the development of obesity; genes determine that some races have higher risk than others. Hormone problems such as underactive thyroid (hypothyroidism), Cushing’s syndrome, and polycystic ovarian syndrome (PCOS) may also cause overweight/obesity (Health, 1998).
2.1.4 Obesity treatments

Obesity is an epidemic chronic disease that requires effective prevention and management in long-term strategies. Although pharmaceutical, medical, and surgical interventions to treat obesity are available, the practical use of these treatments remain rare (Finkelstein et al., 2009). A reduced energy intake through reduced dietary energy density, increased energy expenditure through exercise, lifestyle modification, anorectic agents, other drugs to treat obesity, and bariatric surgery have all been used as treatment interventions (Wirth et al., 2014, Zheng et al., 2010). Anti-obesity pharmaceuticals have limited efficacy on weight loss and the safety concern also curtailed the usage. Two obesity drugs including sibutramine and orlistat have been approved by FDA for long-term use (Bray and Greenway, 2007, Zheng et al., 2010). However, both of them cause side effects. Sibutramine may cause blood pressure increase, dizziness or blurred vision; back pain, dry mouth, headache, nausea, nervousness, etc. Orlistat is associated with embarrassing gastrointestinal side effects such as anal leakage and incontinence. Phentermine is approved as an appetite suppressant to reduce weight for short-term use. The adverse effects of phentermine include nervousness, vomiting, headache, etc. (Nelson and Gehlert, 2006). Lorcaserin that is another weight-loss drug has side effect of causing headache, dizziness, nausea, anxiety, and pain in muscles (Rankin and Wittert, 2015, Halpern and Halpern, 2015). Liraglutide that is a long-acting glucagon-like peptide-1 receptor agonist has side effects including headache, dizziness, nausea, and tired feeling (Rankin and Wittert, 2015, van Velsen et al., 2014). Another medicine used to manage weight in obese or overweight is Contrave that contains a combination of bupropion and naltrexone (Drugs.com, 2015). Side effects of this medicine is blurred vision, dizziness, nervousness, headache,
tiredness, and irritability (Drugs.com). Other anti-obesity drugs in the U.S. such as benzphetamine, diethylpropion, and phendimetrazine are also associated with side effects (Hampp et al., 2013, Drugs.com, 2015, Cerner Multum, 2013). The weight loss effects of these anti-obesity treatment groups are modest, which reduced body weight less than 5% compared to the placebo groups (Li et al., 2005). Therefore, anti-obesity drugs available in the market are not efficient and safe strategies for treating and preventing obesity.

Extreme obese patients (BMI of 40 kg/m² or greater) with obesity-related comorbid conditions could consider surgical intervention. Comorbid conditions include hypertension, impaired glucose tolerance, diabetes mellitus, hyperlipidemia, and obstructive sleep apnea (Khaodhiar et al., 1999). Lifestyle modification including diet and behavior should always be recommended to all the obese patients. Before surgery, patients should undergo an assessment that includes metabolic, cardiovascular, psychosocial, and dietary details (Wirth et al., 2014). Early mortality rates for Roux-en-Y gastric bypass (RYGB) range from 0.3% to 1.9% and an evident learning curve for the operator (Snow et al., 2005). Additionally, the cost of such surgeries cannot be covered by insurance. Commonly used surgical methods include sleeve gastrectomy, gastric banding, and gastric bypass (Torres and Rubio, 2011). After bariatric surgery, lifestyle modification is required for weight management.

2.1.5 Treat obesity by modified diet

Obesity is largely preventable through lifestyle modifications which enhance human and animal insulin sensitivity, reduce visceral adiposity, lower triglyceride levels, and reduce the risk of impaired glucose tolerance (Mann, 2000). The lifestyle modifications include reducing the saturated fatty acid and carbohydrate intake, increasing the consumptions of polyunsaturated fatty acid, fruits, vegetables, whole-grain, increasing physical activity, and decreased sedentary
activity (Mann, 2000, Kamath et al., 2008). Energy dilution in the diet with fruits, vegetables, and whole grains is considered an suggested approach to manage of body weight because the ample amounts of fiber and water in these food products dilute the energy content per volume of food (USDA, 2010, Darmon et al., 2004). Weight loss in overweight and obese people reduces the risk of diabetes and coronary heart disease, reduces blood pressure and serum triglycerides, increases high-density lipoprotein (HDL)-cholesterol, and generally results in the total serum cholesterol reduction and low-density lipoprotein (LDL)-cholesterol (Health, 1998).

Plant source foods or their carbohydrate components have functions in the management and prevention of non-infectious chronic illnesses. The consumption of higher amounts of complex carbohydrate such as whole-grain foods lowers the prevalence of obesity, coronary heart disease, type 2 diabetes, and certain cancers (Anderson, 2004, Hauner et al., 2012). Whole-grain foods are high in RS and dietary fiber, and they are less energy dense compared with refined grain foods. Whole grain foods have components that resist intestinal digestive enzymes, which helps to explain their effectiveness in increasing fecal bulk, an important component of their laxative action (Topping and Clifton, 2001). Previous studies have reported a negative relationship between fiber consumption and the risk of obesity and diabetes (Haenen et al., 2013) since consumption of dietary fiber decreases the accumulation of fat mass, increases insulin sensitivity, and it can enhance feelings of satiety (Haenen et al., 2013). Whole grain cereals contain nutrients and bioactive compounds that have health promoting benefits (Rebello et al., 2014). Additionally, epidemiological studies demonstrated that cereal fiber and whole grain consumption lower the risk factors such as inflammation, insulin resistance, elevated blood pressure, and dyslipidemia, of chronic diseases including obesity, diabetes, and CVD (Rebello et al., 2014). Bioactive compounds from plants, animals, and marine foods have attracted much
attention because of their potential benefits to human health including anti-obesity, anti-inflammation, and anti-oxidant properties (Wang et al., 2008, Jayaprakasam et al., 2005). These compounds could be free radical scavengers and that act as potential chemo preventive agents (Rebello et al., 2014, Fardet et al., 2008). Therefore, the consumption of compounds containing RS and dietary fibers may play a positive role in weight management.

2.2 Health benefits of resistant starch and dietary fiber

2.2.1 Resistant starch

Starch is classified as rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS in order to characterize its nutritional property. The RS is a form of dietary fiber. RS exists in several foods such as legumes, potato, and whole grains. RS can escape from digestion by the digestive enzymes in the small intestine and can be fermented by gut microbiota in large intestine as energy source for these bacteria and produce SCFAs (Keenan et al., 2007, Fuentes-Zaragoza et al., 2010). A Dietary Reference Intakes Committee has recommended more research into dietary energy dilution for weight control and weight loss (Health, 1998, Health, 2015). Adding RS to the diet has the effects of diluting dietary energy density and a bulking effect similar to non-fermentable fiber. The RS from whole-grain has shown increased post-prandial satiety in humans (Anderson et al., 2010). Four main types of RS have been identified based on the structure or source (Murphy et al., 2008): RS type 1 (RS₁) bound by indigestible plant cell walls; found in whole-grains such as beans, grains, and seeds. RS type 2 (RS₂) that is resistant to digestion in the raw state and under low heat treatment due to its high amylose content. This type of RS is found in potatoes, bananas, plantains, and high amylose corn. RS₂ becomes accessible upon high heating that destroy the starch granulate. RS type 3 (RS₃) forms from retrograded amylose and amylopectin during food processing. When some starches are cooked,
cooling them (fridge or freezer) changes the structure and makes it more resistant to digestion; found in cooked and cooled potatoes, grains, bread, cornflakes, and beans. RS type 4 (RS4) doesn’t occur naturally and has been chemically modified. RS has a low calorific (8 kJ/g) value compared with fully digestible starch (15 kJ/g) (Fuentes-Zaragoza et al., 2010). Fermentable RS dilute the dietary energy in the diet and increases energy expenditure and plasma levels of PYY, increases GLP-1, and attenuates body fat accretion in rodent models (Keenan et al., 2006, Zhou et al., 2006). RS also provides health benefits in reducing body fat in rodents and humans (Zhou et al., 2006). It may lower plasma lipid levels and triglyceride concentration, especially plasma cholesterol, by delaying gastric emptying, thereby limiting hepatic lipogenesis owing to less glucose as substrate and less insulin as an activator (Higgins et al., 2004). RS has been shown to promote colon function by alleviating infectious diarrhea and promoting colonic mineral absorption due to the interactions between RS and the microflora (Keenan et al., 2013). RS, a prebiotic, intakes correlate negatively with colorectal cancer risk (Topping and Clifton, 2001). High amylose Maize starch is fermented by Ovariectomized (OVX) rats with increased bacteria levels, especially promotes the survival of Lactobacillus species (Keenan et al., 2013).

2.2.2 β-glucans

β-glucan is a major component of water soluble cereal fiber (Figure 2). Soluble oat fiber, oat β-glucan, consists mainly of linear polysaccharide (1→3), (1→4)-β-D-glucan (Zhang et al., 2012). The (1→3)-link prevents close packing of the molecule and makes the molecule partly soluble in water (Morgan, 2000). The molecular weight of β-glucan varies between 50 and 3000 kDa. β-glucan usually comprises 2-8% (dry weight) of the oat whole grain. Barley grains rich in β-glucan provides an excellent source of soluble dietary fiber for attenuating blood glucose and reducing low-density lipoprotein cholesterols (LDL). Barley β-glucan is a functional ingredient
implicated to lower glycemic response, plasma cholesterol and food intake. Health benefits associated with β-glucan might depend on their polyphenol and antioxidant contents that vary with the method of preparation and purity (Zhang et al., 2012).

Figure 2 Structure of β-D-glucan.

2.2.3 SCFAs and gut microbiota

The gastro-intestinal tract harbors a vast majority of microbiota in human and other mammals’ cecum and colon that count for an estimated mass of 250–750 g of digestion and equal to at least $10^{10}$ to $10^{11}$ cfu/g wet weight (Topping and Clifton, 2001). More than 50 genera and over 300-1000 species of bacteria have been identified in human feces (Hill, 1995, Nagpal et al., 2015). The dominant anaerobic organisms include *Bacteroides, Bifidobacteria, Eubacteria, Streptococci,* and *Lactobacilli,* while others, such as *Enterobacteria,* may also be found (Hill, 1995, Topping and Clifton, 2001). Some bacteria grown on intermediate products of fermentation such as H$_2$, lactate, succinate, formate, and ethanol, and convert them to the end products such as SCFAs (Cummings et al., 1987). Some other bacteria convert CO$_2$ to acetate or methane (CH$_4$). Microbes in the digestive system have a homeostatic symbiosis relationship in which the host provides a stable environment with nutrients and the microbes provide benefits as well to the host (Leser and Molbak, 2009). Gut microbiota are directly related to mechanisms
including defense against pathogens, synthesis of vitamins, fermentation of dietary fibers and dietary proteins; priming the immune system early in life; and stimulating the gut motility (Leser and Molbak, 2009, Topping and Clifton, 2001). The colonic microflora are affected by the gross nutritional shifts, aging, or variations in food intake. Aged people have relatively lower amounts of *Bifidobacteria* but the count for *Escherichia coli* (*E. coli*), *Streptococci*, and *Clostridia* are increased (Mitsuoka, 1996).

The SCFAs including acetate (C\textsubscript{2}), propionate (C\textsubscript{3}), and butyrate (C\textsubscript{4}) are the major fermented metabolic products of dietary fiber and RS in the human gastrointestinal (GI) tract (Vinolo et al., 2011). Animal studies, principally with rats and pigs, have shown that large bowel SCFAs are increased by the provision of fermentable carbohydrates (Keenan et al., 2007, Keenan et al., 2006). Various anaerobic bacterial populations are involved in the production of SCFAs and conversion of polysaccharides, oligosaccharides, and disaccharides to their constituent sugars. This fermentation also provides energy for microbial growth and maintenance and also metabolic end products such as SCFAs, H\textsubscript{2}, lactate, succinate, formate, and ethanol (Topping and Clifton, 2001). Intestinal microbial communities hydrolyze non-digestible plant polysaccharides such as lignin, hemicelluloses, pectin, cellulose, and RS into SCFAs which are readily absorbed and used as energy sources by colonocytes, and also by other tissues including liver and muscle (Vinolo et al., 2011).

SCFAs have been demonstrated for their role in colonic health and reducing the risk of metabolic diseases, including irritable bowel syndrome, inflammatory bowel disease, obesity, cardiovascular disease, and cancer (Reimer et al., 2014, Vinolo et al., 2011). These fatty acids as anti-inflammatory agents affect the function of leukocytes, inhibit stimuli-induced expression of adhesion molecules, chemokine production and consequently suppress monocyte/macrophage
and neutrophil recruitment (Vinolo et al., 2011). SCFAs produced by gut bacteria from the fermentable RS increase secretion of satiety hormones PYY and GLP-1 from epithelial cells of the cecum and colon (Keenan et al., 2013, Zhou et al., 2008, Zheng et al., 2008).

Increasing lines of evidence support the hypothesis that the total beneficial anaerobic bacterial population in fecal samples directly associates with prebiotics intake as an energy source, produces fermentation products, and has the capability of controlling type 2 diabetes, obesity, and aging (Turnbaugh et al., 2009, Van der Meulen et al., 2006, Flint, 2012). Meanwhile, prebiotics alter the composition and population of intestinal microbiota by enhancing the growth of beneficial bacteria (Turnbaugh et al., 2006), which regulate host metabolism by the xenobiotic compounds perceived in the intestine (Cabreiro and Gems, 2013, Shen et al., 2009). Colonic anaerobic microbiota *Bifidobacteria, Bacteroides, Lactobacilli, Clostridium* cluster IV, and *Clostridium* Cluster XIVa/XIVab are necessary for the fermentation of dietary fibers in humans or rodents (Keenan et al., 2006). *Clostridium* cluster IV primers for PCR include subgroups that represent a mixture of genera including *Clostridium, Eubacterium,* and *Ruminococcus* (Van Dyke and McCarthy, 2002). *Clostridium* cluster XIVa and XIVab comprises more than 20 clostridial species (Matsuki et al., 2002, Van Dyke and McCarthy, 2002). *Bacteroidetes* primers are used to enumerate the *Bacteroides* group including *Prevotella* and *Porphyromonas spp. Bacteroides spp.* are anaerobic Gram-negative bacillus bacteria (Duncan et al., 2003, Bartosch et al., 2004). *Bifidobacteria* primers are used to identify *Bifidobacterium spp.* which are classified under phylum *Actinobacteria* (Bartosch et al., 2004), a strictly Gram-positive anaerobic branched rod producing lactic and acetic acid but not CO₂ (Van der Meulen et al., 2006). *Lactobacillus* primers can detect a genus of bacteria under phylum *Firmicutes* (Haarman and Knol, 2006), which consume wide varieties of carbohydrate as fermentation
energy source and grow under aerobic conditions or intestinal anaerobic conditions (Van Dyke and McCarthy, 2002, Silva et al., 1987).

2.2.4 SCFAs

2.2.4.1 Butyrate

The health benefits of butyrate have attracted the most attention from investigators. Butyrate is a major energy source for epithelial cells of colonic mucosa that stimulate cell proliferation (Scheppach et al., 2001). Sodium butyrate is the sodium salt of butyric acid, which is a four carbon normal fatty acid and is a natural metabolite in many organisms including bacteria populating the gastrointestinal tract. The health benefits of butyrate include the prevention and inhibition of colon carcinogenesis, protection against mucosal oxidative stress, and strengthening of the colonic defense barrier; butyrate also has anti-inflammatory properties (Hamer et al., 2008). Studies in vitro demonstrated that exposure of many tumor cell lines to butyrate leads to anticarcinogenic effects by induction of apoptosis, inhibition of proliferation and promotion of a more differentiated phenotype (Hamer et al., 2008). The anti-inflammatory effect of butyrate is demonstrated by the suppression of nuclear factor kappa-B (NF-κB) and inhibition of HDAC (Andoh et al., 1999, Place et al., 2005). NF-κB is a transcription factor that controls the expression of genes encoding proinflammatory cytokines, chemokins, inducible inflammatory enzymes (Jobin and Sartor, 2000). Moreover, butyrate may affect DNA repair systems and levels of enzymatic or non-enzymatic antioxidant systems (Hamer et al., 2008). Dietary RS increase PYY and GLP-1 satiety gene and hormone expression which are also associated with increased butyrate levels in the cecum in rats (Keenan et al., 2006).

2.2.4.2 Acetate
Acetate is the most abundant SCFA product by the colonic microflora; it is approximately 60-75% of the total SCFAs in feces; it is quickly absorbed soon after production and transported to the liver (Louis et al., 2007). Acetate is essential for cholesterol synthesis in the body (Hijova and Chmelarova, 2007). The major benefit of acetate is that it acts as an energy substrate for muscles as acetate reaches the systemic circulation (the portion of the cardiovascular system which carries oxygenated blood away from the heart, to the body, and returns deoxygenated blood back to the heart) (Kiviluoma et al., 1988). Acetate has been shown to suppress harmful bacteria growth (Araya-Kojima et al., 1995).

2.2.4.3 Propionate

Propionate has previously been indicated as another health-promoting metabolite being produced by the gut microbiota fermented RS in the large intestine. Propionate has the potential to reduce cholesterol concentrations in blood (Hosseini et al., 2011). Propionate is metabolized by the liver and used for gluconeogenesis, whereas acetate is a substrate for cholesterol synthesis and lipogenesis (Haenen et al., 2013).

2.3 Prowashonupana barley

Barley is a healthy cereal grain with a rich nutlike flavor. Barley is one of the domesticated grains since the ancient time. Barley has been widely distributed in more than 100 countries in the world. Barley has been used in animal feed, fish feed, beverages, alcohol, and foods. Barley is nutritious and popular health food. Hullness barley is a form of domesticated barley. Barley has attracted much attention for the health promoting properties.

Table 1 Average nutrient content of hullness barley, oat groats, and Prowashonupana barley.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Hullness barley</th>
<th>Oat groats</th>
<th>Prowash barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>13</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>3</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

18
### Table 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Prowashonupana</th>
<th>Barley</th>
<th>Rendell et al., 2005</th>
<th>Rendell et al., 2005</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>60</td>
<td>59</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>LMW sugars</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Total fiber</td>
<td>13</td>
<td>10</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>β-Glucan</td>
<td>5</td>
<td>5</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

*Prowashonupana* barley (*Hordeum vulgare*) is a waxy, hullness, shrunken-endosperm, short awn barley with low glycemic index, low starch, high fiber, high protein and relatively low calories (Rendell et al., 2005). The *Prowashonupana* barley has 30% dietary fiber and 21% starch, which are 2~3 times the amount of fiber and approximately half the amount of starch compared with other common cereal grains (Table 1). Approximately half of the dietary fiber is β-glucan. Hallfrisch et al. indicated that hydrocolloids (β-glucan) extracted from *Prowashonupana* were as effective in reducing glucose and insulin responses in non-diabetics as an oat extract, barley or oats (Hallfrisch J, 2003). Products containing barley flour improve glycemic control in type II diabetic subjects, reduce glycemic index (GI) values (GI=28 to 39), and lower absorption of energy nutrients (Lifschitz et al., 2002, Kim et al., 2011, Hole et al., 2012). Barley also provides vitamins, trace minerals, and bioactive compounds (Inglett et al., 2011, Cui and Wang, 2009). There are many phenolic compounds in barley, such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (Holtekjølen et al., 2008, Verardo et al., 2008). These phenolic compounds serve as natural antioxidants with antiradical and antiproliferative potentials for disease prevention and health promotion (Madhujith and Shahidi, 2007).

*Prowashonupana* barley can also be used in a variety of foods such as hot cereals, cookies, crackers, breads, tortillas, granola bars, fruit-filled cereal bars, extruded snacks, and pastas. It can enhance the flavor, texture, appearance and nutritional compositions of these foods.

### 2.4 Oats
A species of cereal grain, oat (*Avena Sativa*), is mainly used as oatmeal for human consumption as well as livestock feed. Oat has been demonstrated as a health promoting food that is able to attenuate blood postprandial glycemic and insulinemic responses, to lower blood total cholesterol and low-density lipoprotein (LDL) cholesterol, and to improve high-density lipoprotein (HDL) cholesterol and blood lipid profiles as well as to maintain body weight (Zhang et al., 2012). Oat is also capable of modifying immune response and reducing the risk of colon cancer (Mälkki and Virtanen, 2001). It is also known for its effect of satiety, retarding absorption of nutrients, as well as a deterrent of various disorders of the gastrointestinal tract (Mälkki and Virtanen, 2001). The oats high in soluble fiber content, low in saturated fat and cholesterol may reduce the risk of heart disease (Table 2) (Zhang et al., 2012). The main soluble fiber components in oat is a class of polysaccharides known as beta-D-glucan in a structure of \((1\rightarrow3), (1\rightarrow4)\)-\(\beta\)-D-glucan, while the main glucan in mushrooms and yeasts is \((1\rightarrow3), (1\rightarrow6)\)-\(\beta\)-D-glucan (Zhang et al., 2012). The effects of oat on GI tract are due to both soluble and insoluble fibers because soluble fiber has high swelling and water-binding ability, and as a substrate in colon fermentations and insoluble fiber has bulking effect (Mälkki and Virtanen, 2001).

Polyphenols have various biological functions, such as antioxidant, anti-inflammatory and anti-cancer activities that can protect the human body, which is constantly exposed to free radicals present externally, as well as produced internally. Antioxidants such as vitamin E, vitamin C, RS, lignans, flavonoids, and other phenolic compounds from foods could neutralize the environmental free radicals and slow down the process of aging (Lobo et al., 2010, Madhujith et al., 2004, Emmons et al., 1999, Rebello et al., 2014, Jung et al., 2011). Oats as one of the important dietary antioxidant contributors (Emmons et al., 1999, Zhang et al., 2012),
vitamin E (tocopherols), phytic acid, phenolic compounds, and avenanthramides (Figure 3) are the most abundant antioxidants in oat, as well as the flavonoids and sterols are present in oat (Emmons et al., 1999). These antioxidants attenuate the DNA damage and lipid peroxidation caused by the free radicals in the human body (Emmons et al., 1999, Xiao et al., 2015).

Table 2 Macronutrient content of whole grain oat flour and oat bran.

<table>
<thead>
<tr>
<th>%, as is basis</th>
<th>Whole grain oat flour</th>
<th>Oat bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>10 - 17</td>
<td>13 - 24</td>
</tr>
<tr>
<td>Starch and sugars</td>
<td>56 - 62</td>
<td>10 - 50</td>
</tr>
<tr>
<td>Fat</td>
<td>4 - 9</td>
<td>5 - 14</td>
</tr>
<tr>
<td>Total dietary fiber</td>
<td>11 - 17</td>
<td>15 - 32</td>
</tr>
<tr>
<td>β-Glucan</td>
<td>2 - 7</td>
<td>6 - 20</td>
</tr>
</tbody>
</table>

Figure 3 Structure of Avenanthramide.

2.5 Wheat bran

Wheat bran is the by-product from milled refined grain production. Although refined grains have an extended shelf life and a finer texture, refined grain foods contain solid fats, added sugars, and sodium. Wheat bran is high in a non-digestible form of carbohydrates and presents a high concentration of vitamins, minerals, and dietary fiber while refined grains are low
in some of these essential nutrients (USDA, 2010). Wheat bran provides a good source of proteins and minerals apart from being a good source of dietary fiber (Table 3). Wheat bran is also low in saturated fat, trans-fat, mono- and oligo- saccharides, calories (H and Prakash, 2002). Wheat bran can be used to make many food products, such as cookies, bread, cereal, pasta, and cereal bar. Wheat bran is of relatively low fermentability, which helps to account for its relative superiority as a fecal bulking and laxating agent (Topping and Clifton, 2001). During the refining process of grains the bran and germ fractions are removed. Thus, most refined cereal products have lost the protective components (Slavin, 2004).

Table 3 Chemical composition of wheat flour and wheat bran samples (H and Prakash, 2002).

<table>
<thead>
<tr>
<th>Constituent (Per 100g)</th>
<th>Whole wheat flour</th>
<th>Whole wheat bran</th>
<th>Coarse</th>
<th>Medium</th>
<th>Fine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (g)</td>
<td>8.54±0.002</td>
<td>2.17±0.001</td>
<td>6.65±0.212</td>
<td>6.88±0.084</td>
<td>8.23±0.003</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>14.0±0.120</td>
<td>15.0±0.146</td>
<td>15.1±0.002</td>
<td>18.57±0.397</td>
<td>20.3±0.282</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.57±0.106</td>
<td>4.08±0.002</td>
<td>4.13±0.003</td>
<td>2.40±0.001</td>
<td>2.79±0.006</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>67.6</td>
<td>28.1</td>
<td>16.2</td>
<td>34.0</td>
<td>48.7</td>
</tr>
<tr>
<td>Total ash (g)</td>
<td>1.52±0.001</td>
<td>4.99±0.004</td>
<td>5.22±0.001</td>
<td>4.55±0.005</td>
<td>3.48±0.001</td>
</tr>
<tr>
<td>Insoluble fiber (g)</td>
<td>5.45</td>
<td>42.8</td>
<td>49.9</td>
<td>37.05</td>
<td>15.2</td>
</tr>
<tr>
<td>Soluble fiber (g)</td>
<td>1.3</td>
<td>2.8</td>
<td>2.8</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>8.0±0.0</td>
<td>15.1±0.0</td>
<td>12.0</td>
<td>10.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Phosphorous (mg)</td>
<td>435</td>
<td>560</td>
<td>920</td>
<td>1200</td>
<td>1400</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>40</td>
<td>78.3</td>
<td>96</td>
<td>96</td>
<td>160</td>
</tr>
<tr>
<td>Bioavailable iron (mg)</td>
<td>1.83</td>
<td>3.16</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.6 C. elegans model

C. elegans is a small, free-living (as opposed to parasitic) soil nematode widely distributed around the world (Wood, 1988, Riddle et al., 1997). C. elegans is a multicellular eukaryotic organism, about 1mm in length, with transparent and round shape body (Riddle et al., 1997). C. elegans is the first animal to have its genome completely sequenced and more than 65% of the genes relating to human disease are conserved in C. elegans (Shen et al., 2009). Wild-type
C. elegans (N2) live for approximately 21-25 days. They grow to adult in 38-46 hours after hatching; C. elegans has a life cycle about 3 days under optimal conditions (Figure 4). C. elegans have large brood sizes of 300 progeny per hermaphrodite when grown in an agar dish and fed with non-pathogenic Escherichia coli (E. coli, OP50) as a standard laboratory food source.

Figure 4 Life cycle diagram of C. elegans at 22°C.

C. elegans can be maintained in the laboratory using Nematode Growth Medium (NGM) agar in petri dish or liquid culture fed with E. coli OP50. The pharynx is responsible for the ingestion of bacteria by crushing the bacteria in the grinder in the terminal bulb and passing the food into the intestine through the pharyngeal-intestinal valve through coordinated muscular contraction (Mango, January 22, 2007). Wild type and over 3,000 mutant strains of C. elegans are available in Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis,
C. elegans strains included: N2 Bristol (Wild type), null strains of sir-2.1(ok434) IV, daf-16(mgDf50)I and daf-16(mgDf50)I;daf-2(m65) III (Table 4).

C. elegans is a convenient model system in many research fields including genomics, cell biology, neuroscience and aging (Zheng and Greenway, 2012). It is also practical to use this animal studying the effect of bioactive materials on lifespan, fat metabolism, and Parkinson's disease. The pharyngeal pumping rate (PPR) of C. elegans is negatively correlated to their lifespan and is well accepted in the study of aging (Greer et al., 2007). Locomotion analyses have shown that there is altered or impaired C. elegans response to exogenous insults or toxins (Boyd et al., 2010). Using RNAi with Nile Red staining, Ashrafi et al. have reported that 305 genes are responsible for reducing body fat and 112 genes are responsible for increasing fat storage (Ashrafi et al., 2003). C. elegans deposits fat for energy storage along its intestinal tract. Lipid staining dyes such as Nile Red or BODIPY can be visualized directly and quantitated photometrically in the intact and transparent animal (O'Rourke et al., 2009, Zhang et al., 2010). C. elegans model serves as an intermediate step in research which will also reduce the number of rodents or other higher animals required (Zheng and Greenway, 2012, WormAtlas. Altun, 2005).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Gene deficient</th>
<th>Human homologs</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (N2)</td>
<td>-</td>
<td>Forkhead box protein O (FOXO)</td>
<td>- C. elegans daf-16 has a central role in mediating the downstream insulin signaling pathways and is the major target of the daf-2 pathway.</td>
</tr>
</tbody>
</table>
The receptor, which has preserved ligand-binding and tyrosine kinase domains.

The sir-2.1 transgene functions upstream of daf-16 in the insulin-like signaling pathway and was responsible for lifespan extension of *C. elegans*.

| sir-2.1(ok434)IV | sir-2.1 | NAD-dependent protein deacetylase siru1n-1 |

2.7 Insulin signaling pathway and related genes in *C. elegans*

Insulin/Insulin-like growth factor Signaling (IIS) pathway is a primary mechanism for controlling metabolic physiology not only in humans, but also in *C. elegans* (Figure 5) (Van Dyke and McCarthy, 2002). Under conditions of abundant food, the IIS pathway is active. dauer formation-2 gene (*daf-2*) activates the signaling cascade represses dauer formation-16 gene (*daf-16*) which codes for the Forkhead family of transcription factors (FOXO), through AGEing-related gene-1 (*age-1*) and *akt-1/2* (Zheng and Greenway, 2012, Wang and Tissenbaum, 2006). Reduction in the active components of IIS pathway as in *daf-2* mutants or upon decreased food availability leads to the activation of a series of genes involved in lifespan control, stress tolerance and protein misfolding suppression, and collectively resulting in life-span extension (Kenyon, 2010, Lin et al., 1997). Down-regulation of insulin signaling loss of function of *daf-2*, a homolog of the insulin and insulin-like growth factor (IGF) receptors in *C. elegans*, leads to the phosphorylation of *daf-16*, causes fat accumulation in adults and glycogen production (Lin et al., 2001, Berdichevsky et al., 2006a). *C. elegans age-1* is the signaling molecule downstream from the insulin receptor and is the homolog of human phosphatidylinositol-3-hydroxy kinase (PI3K) (Zheng and Greenway, 2012, Lin et al., 2001). DAF-18 inhibits the *age-1* downstream protein PDK-1 which phosphorylates the SGK-1/akt-1/2 complex that acts as an inhibitor of dauer formation-16 gene (*daf-16*) (Seo et al., 2015, Zheng et al., 2013, Rizki et al., 2011). The
functions of *daf-16* in biological processes include metabolism, life span, stress responses, and dauer formation and exit, by mediating downstream gene expression in response to environmental and nutritional conditions (Barbieri et al., 2003). *daf-16* is suppressed by *daf-2* and *age-1* through the SGK-1/akt-1/-2 complex (Seo et al., 2015, Zheng et al., 2013, Rizki et al., 2011). SGK-1/akt-1/-2 complex is the homolog of human serine/threonine protein kinase (AKT/PKB) and regulates multiple cellular process as well as glucose metabolism. ‘Aging’ has been reported to relate with the downstream effects of IIS pathway (Figure 5) (Benedetti et al., 2008). Insulin resistance that is a primary component in the pathophysiology of type 2 diabetes is a pathological condition in which cells fail to respond to normal action of the hormone insulin (Jayaprakasam et al., 2005, Chiu et al., 2007).

Figure 5 Summary of the insulin signaling pathway in *C. elegans*.
DAF-16 (FOXO transcription factor) is the major target of the DAF-2 pathway (homologue of insulin signaling pathway in *C. elegans*); DAF-18 inhibits PDK-1, which phosphorylates the SGK-1/akt-1/-2 complex; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3); PDK-1, 3-phosphoinositide-dependent kinase 1; DAF-18, homologous to human PTEN; AKT1/2 and SGK-1, orthologs of the serine/threonine kinase Akt/PKB. Activations are indicated by ‘→’ and inhibitions are indicated by ‘┴’.

### 2.8 *sir*-2.1 extends lifespan

Lifespan in *C. elegans* is controlled by many pathways independently or overlapping such as insulin/insulin like signaling pathway and TOR (target of rapamycin) signaling pathway (Greer and Brunet, 2009, Mair and Dillin, 2008). *sir*-2.1 like proteins (sirtuins), a family of NAD$^+$ dependent deacetylases, is related to gene silencing, DNA repair, rDNA recombination and aging in model organisms from *E. coli* to humans (Figure 6) (Wood et al., 2004, Lin et al., 2000, Hekimi and Guarente, 2003, Landry et al., 2000). *sir*-2.1 repressed the expression of ER stress genes and was responsible for lifespan extension of *C. elegans* induced by resveratrol (Figure 6) (Viswanathan et al., 2005).
Figure 6 Regulation of DAF-16/FOXO by SIR-2.1/SIRT1 in *C. elegans*. SIR-2.1 coordinate to fine-tune the transcriptional activity of DAF-16 on a distinct subset of potential target genes. DAF-16 target genes responsive to the SIR-2.1 pathway largely overlap with a small subset of IIS-regulated genes, and are specialized in longevity determination, cellular defense, and lipid/fatty acid/amino acid homeostasis.

Extra expression of *sir-2.1* extends the lifespan of budding yeast by 30%, apparently by mimicking caloric restriction (Wood et al., 2004) while deletion or knockdown of the gene shortens lifespan (Wang and Tissenbaum, 2006, Bamps et al., 2009). The lifespan of *sir-2.1* null mutant was not obviously shorter than the wild-type *C. elegans* N2 (Wood et al., 2004). The extension of lifespan by elevation of *sir-2.1* activity might also have a FOXO-independent function which codes *daf-16* in *C. elegans* (Wang et al., 2010, Berdichevsky et al., 2006a). SIR-
2.1 acts with 14-3-3 proteins to affect DAF-16 activity and life span and act in parallel to the insulin-like pathway to regulate DAF-16 and extend life span (Figure 6) (Berdichevsky et al., 2006b).

2.9 Gene regulation of satiety signals

The satiety signals, PYY, GLP-1, and cholecystokinin (CCK) are produced in the colon and increase in the blood stream in response to food intake (Zhou et al., 1985, Zhou et al., 2008). CCK that is a peptide hormone found in the central nervous system and gastrointestinal tract plays a central role in initiating satiety and modulating food intake (Moran and Kinzig, 2004, Gibbs et al., 1973). CCK regulates food intake by being released from enteroendocrine cells in response to the presence of lipid or protein in the gut postprandial (Moran, 2000). Exogenous administration of CCK decreases food intake in rats (Gibbs et al., 1973). However, mice lacking the gene for CCK have normal body weight, body mass, and fat absorption as compared with wild-type littermate controls, differences in longevity were not examined (Lo et al., 2008). Food-restricted rats showed increased plasma CCK, which suggests a physiological role for CCK in the adaptation to reduced food ingestion (Chowdhury and Rayford, 2001). Two distinct receptor proteins, CCK1R and CCK2R, mediate the action of CCK in mice, and both receptors play a regulatory role in the control of food intake (Chowdhury and Rayford, 2001). Two potential homologs of CCK receptors are found in the C. elegans genome, ckr-1 and ckr-2. RNAi knockdown of ckr-1 or ckr-2 also decreases the longevity of the eat-2 mutant significantly but has no effect on the life span of age-1(hx546) and clk-1(e2519) mutants (Park et al., 2010). Regarding obesity, the polymorphism of the cholecystokinin 1 receptor (CCK1R) gene may be related to an increase in body fat content in middle-aged and elderly people (Funakoshi et al., 2008).
CCK1R mediate the CCK-induced suppression of food intake (Peikin, 1989), and the peripheral administration of CCK1R antagonists increased food intake (Wolkowitz et al., 1990).

2.10 Quantitative real-time PCR for DNA and mRNA analysis

PCR or the polymerase Chain Reaction is a technique for amplifying DNA. Amplifying DNA create multiple copies of strands of DNA. Amplification of DNA is able to detect enough DNA signal for quantification. Polymerase is a thermostable enzyme and will synthesize a complementary sequence of bases. There are two types of PCR: conventional PCR and real-time PCR. Conventional PCR detects the amplified DNA on agarose by an end-point analysis while real-time PCR allows the detection of the accumulation of amplified product as the reaction proceeds. The advantage of real-time PCR over conventional PCR is the accuracy and high sensitivity. There are several ways to detect the fluorescent PCR product including Taqman PCR method and SYBER green method.

2.10.1 Taqman real-time PCR method for C. elegans mRNA analysis

The principle of Taqman qPCR is to use a fluorogenic labeled probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. Thermus aquatic DNA polymerase cleave fluorescence probe (TaqMan probe), which is designed to be complementary to a specific sequence spanned by the PCR primers. The Taqman oligonucleotide probe is constructed containing a reporter dye at its 5’ end and a quencher dye at its 3’ end (Figure 7). If the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. The Quencher dyes are usually FAM (6-carboxyfluorecein) and TAMARA (6-carboxyl- tetra methyl -rhodamin). Using DNA binding dye more DNA copies are created and florescence increases. If the target sequence is present, the probe anneals downstream from one of the primer sites and
is cleaved by the 5’ nuclease activity of Taq DNA polymerase as this primer is extended. Separation of the reporter dye from the quencher dye increases the reporter dye signal.

Figure 7 The principle of Taqman real-time PCR.
(1) Reporter and the quencher dyes are in close proximity, no fluorescence signal is emitted due to the quenching effect (black arrow in 1, 2, and 3), (2) Soon after the annealing of the TaqMan probe and the primers, (3) the primers are extended by the DNA polymerase. As the polymerase reaches the TaqMan probe, it uses its exonuclease activity to remove the probe one nucleotide at the time, (4) this releases the reporter from the proximity of the quencher and allows for the release of a fluorescence signal from the reporter.

2.10.2 SYBR green real-time PCR method for gut microbiota DNA analysis

SYBR green is another frequently used dye in RT-PCR. The SYBR Green I dye binds to all double-stranded DNA present in the sample. During the PCR, DNA polymerase amplifies the target sequence, or “amplicons”. The increase in fluorescence intensity is proportional to the
amount of amplicons produced (Figure 8). The advantages of the SYBR Green method over Taqman method is that it can be used to monitor the amplification of any double-stranded DNA sequence; No probe is required, which reduces assay setup and running costs. The disadvantage of SYBR green method is that this dye can bind to nonspecific double-stranded DNA sequences.

1. **Reaction setup**: The SYBR Green dye fluoresces when bound to Template ds DNA

2. **Template denaturation**: when the DNA denatured, the SYBR Green dye is released and the fluorescence is drastically reduced

3. **Amplification**: during extension, primers anneal and PCR product generated (annealing and extension)

4. **Polymerization completed**: when the polymerization is complete, the SYBR Green dye binds to the ds DNA increase in fluorescence detected by the 7900HT system

Figure 8 The principle of SYBR Green real-time PCR.
2.11 Reference


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CHAPTER 3: SCFAS AFFECTED THE LIFESPAN AND INTESTINAL FAT DEPOSITION IN C. ELEGANS MODEL

3.1 Introduction

The Western-style diet is high in fat and sugar, low in fiber intake that is far less than the recommended amount for daily diets (Eaton, 2006, Simpson and Campbell, 2015). Current daily fiber intakes are 10 to 20 grams (Eaton, 2006) lower than the national fiber recommendations, 30 to 38 grams a day for men and 25 grams a day for women between 18 and 50 years old (King et al., 2012). The dietary fermentable carbohydrates have relatively lower energy density and produce similar health benefits as calorie restriction. Dietary fermentable carbohydrate can escape the digestion in small intestine and are fermentable by anaerobic bacteria in the colon (Macfarlane and Macfarlane, 2003). There is a tight association between fermentable nondigestible carbohydrates and positive effects on host metabolism.

Diets and intestinal microbiota play important roles in maintaining the health of host, including preventing obesity and related metabolic complications (Korpela et al., 2014). A considerable content of microbial flora, which are about $10^{11}$ - $10^{13}$ bacteria per gram content, exist in the colon (Backhed et al., 2004, Sekirov et al., 2010). This microbial flora includes more than 1,000 bacterial species and most of the microorganisms are saccharolytic (Henningsson et al., 2002, Backhed et al., 2004). Firmicutes and Bacteroidetes account for more than 90% of the bacterial population in the colon (Ley et al., 2008). Obesity is a multifactorial state, where host genes, life style and the gut microbiota interact in a complex and largely unknown way (Everard and Cani, 2013, Flint, 2011). One study has reported that the abundance of Clostridium sphenoides was decreased in obese subjects as compared to healthy controls (Korpela et al., 2014). The “Western” style diet causes dysbiosis which affects both host GI tract metabolism
and immune homeostasis (Sekirov et al., 2010). In a study with mice, the microbiota composition of mice fed with a high fat and sugar diet is associated with increase of Firmicutes including Clostridium innocuum, Eubacterium dolichum, Catenibacterium mitsuokai and Enterococcus spp., as well as a significant reduction in several Bacteroides spp. (Walker et al., 2011). In contrast, low carbohydrate diets result in enriched populations of bacteria from the Bacteroidetes phyla (Walker et al., 2011), and calorie-restricted diets prevent the growth of Clostridium coccoides, Lactobacillus spp. and Bifidobacteria spp., which are major butyrate producers required for colonocyte homeostasis (Santacruz et al., 2009). Complex carbohydrates also increase the levels of beneficial Bifidobacteria spp. such as B. longum subspecies longum, B. breve and B. theraiotaomicron (Pokusaeva et al., 2011).

Human colonic bacteria ferment the indigestible fermentable carbohydrate to SCFAs, mainly acetate (C\(_2\)), propionate (C\(_3\)), butyrate (C\(_4\)) (Topping and Clifton, 2001) and gases (CO\(_2\), CH\(_4\), and H\(_2\)) (Cummings et al., 1987). SCFAs can be rapidly absorbed from the colon and can contribute as an energy source for intestinal epithelial cells (Vinolo et al., 2011). E. ruminantium belongs to the family Lachnospiraceae, is xylanolytic and produces mainly formic acid as well as butyrate (Louis et al., 2004). Clostridiaceae felsineum is a pectinolytic butyrate-producer (Rainey FA, 2009). Bacteroidetes and Clostridial species are active degraders of dietary polysaccharides (Korpela et al., 2014). Other SCFAs, such as propionic acid, are beneficial at low concentrations but have neurotoxic effects in high quantities and may play a role in the development and persistence of the symptoms of autism. Acetate: propionate: butyrate values were similar in different regions of the large intestine (approximately 57:22:21) (Macfarlane and Macfarlane, 2003, Cummings et al., 1987).
SCFAs prevent colonic diseases and metabolic diseases including irritable bowel syndrome, inflammatory bowel disease, obesity, cardiovascular disease and cancer (Wong et al., 2006). Butyric acid is important in the prevention and treatment of diseases of the colonic mucosa (Henningsson et al., 2002). SCFAs have been recognized as potential mediators involved in the effects of gut microbiota on intestinal immune function and inflammatory processes (Vinolo et al., 2011). Individual SCFAs may provide specific health benefits. Butyrate is the main energy substrate for the colonocytes and is metabolized by the cells in preference to glucose or glutamine, accounting for 70% of the total energy demand of the colonic mucosa (Roediger, 1982, Scheppach, 1994) and has been shown to protect against damaging inflammatory responses.

Feeding C. elegans with SCFAs showed similar effect on the nematodes as feeding with amylose starch or fermented RS from filtered cecal contents from amylose starch fed mice (Zheng et al., 2010, Keenan et al., 2013). The purpose of current study was to determine the effects of SCFAs, products of fermentable fibers, on the intestinal fat deposition, insulin-resistance, and lifespan using the C. elegans N2, and null mutants, sir-2.1(ok434)IV, daf-16(mgDf50)I, or daf-16(mgDf50)I;daf-2(m65) III.

3.2 Materials and methods

3.2.1 E. coli OP50 culture

An E. coli pellet was inoculated into LB (Luria-Bertani) broth containing 0.2% streptomycin. The culture was incubated at 37°C for 24 hours, and then stored at 4°C. The enumeration of the E. coli culture was carried out using the E. coli/coliform Petrifilm™ (Cat. # 6404, 3M Corp., Minneapolis, MN). The incubation time for the Petrifilm™ is 24 hours at 37°C (Zheng et al., 2010, Yuan et al., 2012).
3.2.2 C. elegans culture

The C. elegans model organism does not require approval by Institutional Animal Care & Use Committee (IACUC). All the C. elegans strains and E. coli, OP50, which is standard laboratory food source for C. elegans, were obtained from Caenorhabditis Genetics Center (CGC, University of Minnesota). The four C. elegans strains used in this study included: N2 Bristol (Wild type), null strains of sir-2.1(ok434)IV, daf-16(mgDf50)I and daf-16(mgDf50)I;daf-2(m65) III.

Mature gravid C. elegans were age-synchronized with a NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratios) to dissolve the C. elegans body and to release the viable eggs. Eggs were washed with S-complete solution 3 times and hatched overnight at room temperature. The age-synchronized C. elegans were diluted to 100 animals/ml, plated in liquid culture in 96-well plate (120μl/well, 10-15 animals) (Solis and Petrascheck, 2011) fed with E. coli OP50 (10^9 cfu/ml) (Zheng et al., 2010, Yuan et al., 2012) at 20 or 15°C (daf-16 mutant) in low temperature incubators (Fisher Scientific, Pittsburgh, PA). Thirty microliters of 5-Fluoro-2’-deoxyuridine (FUDR, 0.6mM) stock solution were added to each well at L4 stage.

The effect of SCFAs on lifespan and intestinal fat deposition (IFD) of C. elegans was tested using 96 well plate liquid culture method (Solis and Petrascheck, 2011). Sodium butyrate was purchased from Fisher Scientific (Cat#: AAA1107936, Pittsburgh, PA). Sodium acetate trihydrate (Cat#: S8625), sodium propionate (Cat#: P5436), and tributyrin (Cat#: 113026) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO). Control animals were fed with E. coli (OP50) in densities of 10^9 (cfu/ml). The treatment groups received additional sodium butyrate (0.3mM, 0.6mM), sodium acetate (5mM, 50mM), sodium propionate (7mM), or tributyrin
(0.5mM, 3mM). The numbers of live animal were manually recorded every other day under an Inverted microscope with a 4× objective (Nikon Eclipse Ti, Melville, NY, USA).

3.2.3 Resistant starch treatment and pharyngeal pumping rate

The experimental groups were fed amylose starch containing 60% RS (Hi-Maize® 260, Ingredion Incorporated, Bridgewater, NJ). The effect of amylose starch on the pharyngeal pumping rate of C. elegans was tested with the NGM agar culture method. The control animals were fed with E. coli (OP50) only. One experimental group was fed with additional amylose starch (0.5%, 1.0%, & 3.0% w/v) in addition to E. coli (OP50). Other experimental groups were fed amylose starch (0.5%, 1.0% and 3.0%), and an additional 2% glucose to mimic the hyperglycemia effect. The animals were examined periodically using a stereomicroscope (Nikon SMZ1500, Melville, NY) with transmitted light. The pharyngeal pumping rate (PPR) was recorded manually by independent observers and the animals were returned to the incubators.

3.2.4 Nile red staining for fluorescence microscopy

Lipophilic dye, Nile red, was selected to stain the intestinal fat deposition in C. elegans and Fluorescent intensity was evaluated. To prepare the samples for taking digital images, the animals grown in liquid culture were harvested and washed with S-Basal buffer. The sample containing the animals was centrifuged at 2000 ×G for 20 seconds and the procedure was washed with S-Basel buffer 2 times. The animals were fixed with paraformaldehyde (4%) solution for 30 minutes at 4°C and washed with PBS 3 times. Nile red solution (50µl) was added to the tube containing the animals and allowed to sit for 30 minutes (Zheng et al., 2010). Ten microliters of fluoromount-G solution (Southern Biotechnology Associates, Birmingham, AL) was applied on the glass slide. Ten microliters of the specimen containing Nile Red stained C. elegans was applied on to the glass slide. A cover glass was mounted on the glass slide. The slides were
viewed with an epifluorescence microscope (Nikon Eclipse Ti) equipped with a Texas Red filter. Fluorescent micrographs were taken with a digital camera (Andor, DU-885K). The micrographs were analyzed using ImagePro Plus (Media Cybernetics, Inc., MD) and analyzed with NIS-elements AR3.22.11 software (Nikon Instrument, Melville, NY). The micrographs were Line profiles of the optical densities of Nile Red labeled intestinal fat deposition (Arbitrary units, percent of control) was determined for each treatment group.

3.2.5 Statistical analyses

All results were expressed as mean±s.e.m. The data for the pharyngeal pumping rate were analyzed with ANCOVA method by SAS 9.4. Survival curves of lifespan were displayed by binomial probabilities as surrogates for survival probabilities and the mean survival time (mean lifespan) was estimated by Kaplan-Meier using SAS (SAS Institute Inc., USA). The data for fluorescence intensity and quantitative real-time PCR analyzed by (SAS 9.4). Statistical significance was set as P≤0.05.

3.3 Results

3.3.1 Effect of sodium butyrate on the lifespan of *C. elegans*

The sodium butyrate at doses of 0.3 and 0.6 mM increased the lifespan of the wild type *C. elegans* N2 (P<1×10⁻⁵ and P<1×10⁻⁶, respectively) compared with the *E. coli* OP50 only control (Figure 9 {A}). The mean survival time was increased by 0.3 and 0.6mM sodium butyrate to 33.8 days and 36.9 days, respectively, compared to 24.9 days of control (Figure 9 {A} and Table 5). Strain *sir-2.1(ok434)IV* lacks the *sir-2.1* gene which has previously been implicated in aging. For this strain, sodium butyrate at concentrations of 0.3 and 0.6mM mildly decreased the lifespan compared with the *E. coli* OP50 fed control (Figure 9 {B} and Table 6). Sodium butyrate at concentrations of 0.3 and 0.6mM increased the lifespan of the *daf-
16(mgDf50) I (GR1307) C. elegans strain (P=7×10^{-5} and P=5×10^{-5}, respectively) compared with the E. coli OP50 fed control (Figure 9 {C}). The mean survival time was increased to 25.9 days and 26.1 days by the treatment of 0.3 and 0.6mM sodium butyrate, respectively, compared with the 22.0 days for the E. coli OP50 fed control (Figure 9 {C} and Table 7). The daf-16(mgDf50);daf-2(m65) III C. elegans lacks the insulin receptor and longevity pathway. In this strain, sodium butyrate at concentrations of 0.3 and 0.6mM slightly decreased the lifespan compared with the E. coli OP50 fed control (Figure 9 {D}). The mean survival times were decreased to 20.9 days and 20.5 days by the 0.3 and 0.6mM sodium butyrate, respectively, compared to the 21.7 days for the E. coli OP50 fed control (Figure 9 {D} and Table 8).

Figure 9 Sodium butyrate modified the lifespan of C. elegans.
A) Lifespan survival curve, mean survival time, and IFD results of *C. elegans* N2; B) Lifespan survival curve, mean survival time, and IFD results of *sir*-2.1(ok434) IV *C. elegans*; C) Lifespan survival curve, mean survival time, and IFD results of *daf-16*(mgDf50) I (GR1307) *C. elegans*; D) Lifespan survival curve, mean survival time, and IFD results of *daf-16*(mgDf50) I;*daf-2*(m65) III *C. elegans*.

Table 5 The effect of SCFAs on the mean survival time of Bristol wild type *C. elegans* N2.

<table>
<thead>
<tr>
<th>#</th>
<th>Samples</th>
<th>(mM)</th>
<th>Mean survival days</th>
<th>SEM</th>
<th>(%)</th>
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<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>24.9</td>
<td>0.914</td>
<td>100.0</td>
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<td>2</td>
<td>Sodium butyrate</td>
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<td>33.8</td>
<td>1.056</td>
<td>135.3</td>
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<tr>
<td>3</td>
<td>Sodium butyrate</td>
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<td>36.9</td>
<td>1.173</td>
<td>148.0</td>
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<tr>
<td>4</td>
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<td>5</td>
<td>31.7</td>
<td>1.627</td>
<td>127.1</td>
</tr>
<tr>
<td>5</td>
<td>Sodium acetate</td>
<td>50</td>
<td>16.8</td>
<td>0.607</td>
<td>67.5</td>
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<td>Sodium propionate</td>
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<td>1.112</td>
<td>101.4</td>
</tr>
<tr>
<td>7</td>
<td>Tributyrin</td>
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<td>31.1</td>
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<td>124.7</td>
</tr>
<tr>
<td>8</td>
<td>Tributyrin</td>
<td>3</td>
<td>25.4</td>
<td>0.735</td>
<td>102.0</td>
</tr>
</tbody>
</table>

3.3.2 Effect of sodium acetate on the lifespan of *C. elegans*

Sodium acetate (5mM) also significantly increased the lifespan of *C. elegans* N2 (P<1×10^{-5}, Figure 10 {A}). The mean survival time was also increased to 31.7 days (Figure 10 {A} and Table 5). However, 50mM sodium acetate significantly decreased the lifespan of *C. elegans* (P<1×10^{-5}, Figure 10 {A}). The mean survival time of the animals in this treatment was decreased to 16.8 days (Figure 10 {A} and Table 5). This means that low dose of sodium acetate can extend the lifespan of *C. elegans* but high dose of sodium acetate is toxic to the *C. elegans*. Sodium acetate at 5mM and 50mM significantly decreased the lifespan of *C. elegans* strain *sir*-2.1(ok434) IV (P=0.004 and P<0.001, Figure 10 {B}). The mean survival times were decreased to 20.0 days and 12.9 days, respectively, compared to the 28 days of control group (Figure 10 {B} and Table 6). Sodium acetate at 5 and 50mM significantly (P=3.9×10^{-3} and p=1.1×10^{-4}, respectively) increased the lifespan of the *daf-16*(mgDf50) I (GR1307) *C. elegans* strain (Figure 10 {C}). The mean survival time was increased to 26.1 days and 24.9 days, respectively for the two concentrations used while the mean survival time for control is 22.0 days (Figure 10 {C})
and Table 7). Sodium acetate at 5mM mildly decreased the lifespan of *daf-16(mgDf50)I;daf-2(m65) III C. elegans* (Figure 10 {D}). Sodium acetate at 50mM significantly decreased (*P*=0.0008) the lifespan of this strain (Figure 10 {D}). The mean survival times were decreased to 19.8 days and 15.1 days, respectively, for the sodium acetate treatments (Figure 10 {D} and Table 8), compared to the control of 21.7 days.

Figure 10 Sodium Acetate modified the lifespan of *C. elegans.*
Experimental group received additional sodium acetate (5, 50mM). The control group obtained standard laboratory food OP50 only (*n*=6). A) Lifespan survival curve, mean survival time, and IFD results of *C. elegans* N2; B) Lifespan survival curve, mean survival time, and IFD results of *sir-2.1(ok434)IV C. elegans*; C) Lifespan survival curve, mean survival time, and IFD results of *daf-16(mgDf50)I;daf-2(m65) III C. elegans*.
daf-16(mgDf50) I (GR1307) C. elegans; D) Lifespan survival curve, mean survival time, and IFD results of daf-16(mgDf50)I;daf-2(m65) III C. elegans.

Table 6 The mean survival times of different SCFAs on sir-2.1(ok434)IV C. elegans.

<table>
<thead>
<tr>
<th>#</th>
<th>Treatment</th>
<th>(mM)</th>
<th>Mean survival days</th>
<th>SEM</th>
<th>(%)</th>
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<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>28.1</td>
<td>1.5</td>
<td>100.0</td>
</tr>
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<td>2</td>
<td>Sodium butyrate</td>
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<td>26.2</td>
<td>1.4</td>
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<td>71.5</td>
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<td>Sodium acetate</td>
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<td>12.9</td>
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<td>Tributyrin</td>
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<td>17.7</td>
<td>1.4</td>
<td>63.0</td>
</tr>
<tr>
<td>8</td>
<td>Tributyrin</td>
<td>3</td>
<td>28.2</td>
<td>1.3</td>
<td>100.5</td>
</tr>
</tbody>
</table>

3.3.3 Effect of sodium propionate on the lifespan of C. elegans

The lifespan of C. elegans N2 treated with 7mM sodium propionate did not have significant difference compared with the control (P=0.7794, Figure 11 {A}). The C. elegans strain sir-2.1(ok434)IV lifespan was significantly decreased by the treatment of 7mM sodium propionate compared to the E.coli OP50 fed control (P<0.001, Figure 11 {B}). However, the lifespan of the strain daf-16(mgDf50) I (GR1307) C. elegans with the treatment of 7mM sodium propionate was 24.4 days (Figure 11 {C} and Table 7), not significantly different from that of the control (P=0.08775, Figure 11 {C}). The C. elegans strain daf-16(mgDf50)I;daf-2(m65) III lifespan was mildly increased by the treatment of 7 mM sodium propionate compared to the E.coli OP50 fed control (Figure 11 {D}).

Table 7 The mean survival times of different SCFAs on daf-16(mgDf50) I (GR1307) C. elegans.

<table>
<thead>
<tr>
<th>#</th>
<th>Treatment</th>
<th>(mM)</th>
<th>Mean survival days</th>
<th>SEM</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>22.0</td>
<td>0.829</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>Sodium butyrate</td>
<td>0.3</td>
<td>25.9</td>
<td>0.827</td>
<td>117.4</td>
</tr>
<tr>
<td>3</td>
<td>Sodium butyrate</td>
<td>0.6</td>
<td>26.1</td>
<td>0.707</td>
<td>118.4</td>
</tr>
<tr>
<td>4</td>
<td>Sodium acetate</td>
<td>5</td>
<td>26.1</td>
<td>0.494</td>
<td>118.5</td>
</tr>
</tbody>
</table>
Figure 11 Sodium propionate modified the lifespan of *C. elegans*. The control group obtained standard laboratory food OP50 only and experimental group received additional sodium propionate (7mM) (n=6). A) Lifespan survival curve, mean survival time, and IFD results of *C. elegans* N2; B) Lifespan survival curve, mean survival time, and IFD results of *sir-2.1(ok434)* IV *C. elegans*; C) Lifespan survival curve, mean survival time, and IFD results of *daf-16(mgDf50)* I (GR1307) *C. elegans*; D) Lifespan survival curve, mean survival time, and IFD results of *daf-16(mgDf50)* I; *daf-2(m65)* III *C. elegans*.

3.3.4 Effect of tributyrin on the lifespan of *C. elegans*
The lower dose of tributyrin (0.5mM) significantly increased (P=0.0046, Figure 12 {A}) the lifespan but higher dose of tributyrin (3mM) did not significantly change the lifespan of *C. elegans* N2 (P =1.00, Figure 12 {A}). The mean survival time of *C. elegans* N2 in the treatments with 0.5 and 3 mM tributyrin were 31.1 and 25.4 days compared to 25.7 days of control (Figure 12 {A} and Table 5). Tributyrin at 0.5mM significantly decreased the lifespan of *sir-2.1* mutant *C. elegans* (P=0.0005, Figure 12 {B}). The mean survival time of the animals was 13.0 days while it is 28 days in the control group (Figure 12 {B} and Table 6). In the *daf-16*(mgDf50) I (GR1307) *C. elegans*, tributyrin (3mM) had no significant difference with the *E. coli* OP50 fed control (Figure 12 {B}). The lower dosage of tributyrin (0.5mM) significantly increased the lifespan of *daf-16*(mgDf50) I (GR1307) *C. elegans*. The mean survival time was 25.0 days. However, the high dose of tributyrin (3mM) did not significantly affect the lifespan of *C. elegans* (P=0.398, Figure 12 {C}) or the mean survival time (24.3 days, Figure 12 {C} and Table 7). The tributyrin at 0.5 and 3mM also mildly increased the lifespan of *C. elegans* strain *daf-16*(mgDf50)I;*daf-2*(m65) III (Figure 12 {D}). The mean survival time of the animals for the sodium propionate was 24.0 days (Figure 12 {D} and Table 3.4). The mean survival time for the tributyrin (0.5 and 3mM) were 22.4 and 21.7 days, respectively (Figure 12 {D} and Table 8).

Table 8 The mean survival times of different SCFAs on *daf-16*(mgDf50)I;*daf-2*(m65) III *C. elegans*.

<table>
<thead>
<tr>
<th>#</th>
<th>Treatment</th>
<th>(mM)</th>
<th>Mean survival days</th>
<th>SEM</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>21.7</td>
<td>0.608</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>Sodium butyrate</td>
<td>0.3</td>
<td>20.9</td>
<td>0.791</td>
<td>96.3</td>
</tr>
<tr>
<td>3</td>
<td>Sodium butyrate</td>
<td>0.6</td>
<td>20.5</td>
<td>1.155</td>
<td>94.2</td>
</tr>
<tr>
<td>4</td>
<td>Sodium acetate</td>
<td>5</td>
<td>19.8</td>
<td>0.792</td>
<td>91.2</td>
</tr>
<tr>
<td>5</td>
<td>Sodium acetate</td>
<td>50</td>
<td>15.1</td>
<td>0.727</td>
<td>69.3</td>
</tr>
<tr>
<td>6</td>
<td>Sodium propionate</td>
<td>7</td>
<td>24.0</td>
<td>0.633</td>
<td>110.6</td>
</tr>
<tr>
<td>7</td>
<td>Tributyrin</td>
<td>0.5</td>
<td>22.4</td>
<td>0.815</td>
<td>103.0</td>
</tr>
<tr>
<td>8</td>
<td>Tributyrin</td>
<td>3</td>
<td>21.7</td>
<td>0.671</td>
<td>99.7</td>
</tr>
</tbody>
</table>
3.3.5 Feeding of SCFAs reduced the fluorescence intensity of Nile red positive intestinal fat deposition of *C. elegans*

In this study, the SCFAs reduced the fluorescence intensity of Nile Red positive Intestinal Fat Deposition (IFD) in *C. elegans* N2 (Figure 9 {A}, 10 {A}, 11 {A}, and 12 {A}). The reduction was found in all treated animals as observed on day 5. As shown in Figure 9 {A}, the IFD level of the wild type *C. elegans* N2 was significantly decreased to 75.7% and 74.9% of the control group that were fed with only *E. coli* OP50 by 0.3 and 3mM sodium butyrate, respectively, (P=0.0016 and P=0.0011, n=5). The application of 100mM sodium acetate reduced the IFD to 80.7% (Figure 10 {A}, P < 0.05, n = 5) of the control.

Figure 12 Tributyrin modified the lifespan of *C. elegans*.
The control group obtained standard laboratory food OP50 only and experimental group received additional tributyrin (0.5, 3mM) (n=6). A) Lifespan survival curve, mean survival time, and IFD results of *C. elegans* N2; B) Lifespan survival curve, mean survival time, and IFD results of sir-2.1(ok434)IV *C. elegans*; C) Lifespan survival curve, mean survival time, and IFD results of daf-16(mgDf50) I (GR1307) *C. elegans*; D) Lifespan survival curve, mean survival time, and IFD results of daf-16(mgDf50)I;daf-2(m65) III *C. elegans*.

The IFD of 0.3mM sodium propionate resulted in a reduction to 83% of the control (Figure 11 {A}, P=0.0394, n=5). Tributyrin at concentrations of 0.5 and 3mM also resulted in a reduction to 77% and 66.3% of the control (Figure 12 {A}, P<0.05 and P<0.0001, n=5).

For the *daf-16(mgDf50)* I (GR1307) *C. elegans* strain, the effect of SCFAs on the IFD is different from that observed on the wild type *C. elegans* N2. The treatment with sodium butyrate did not significantly reduce the IFD at the concentrations of 0.3 and 0.6mM (Figure 9 {C}) in contrast to the significant IFD reduction in wild type *C. elegans* N2 (Figure 9 {A}). The IFD was slightly increased by the application of sodium acetate at the concentrations of 5 and 50mM (Figure 10 {C}). Similar trend was observed in the treatments with sodium propionate and tributyrin. The application of 7mM sodium propionate significantly (Figure 11 {C}, P<0.05, n=5) increased the IFD in the *daf-16* mutant. The IFD was also increased by 0.5 (P<0.05, n=5) and 3mM (P=0.122, n=5) tributyrin (Figure 12 {C}). These results demonstrated that sodium butyrate, sodium acetate, and tributyrin regulate the *C. elegans* fat metabolism in a *daf-16* gene dependent way, but they modify the lifespan in a *daf-16* gene in an independent way.

Sodium butyrate, sodium acetate, and tributyrin treatment did not significantly modify the IFD in the *daf-16(mgDf50)*I;daf-2(m65) III double null mutants (Figure 9 {D}, 10 {D}, 11 {D}, and 12 {D}). The IFD in the treatment with 0.3 and 0.6mM sodium butyrate was 96.6% (P=1, n=5) and 89.1% (P=0.95, n=5) (Figure 9 {D}), respectively, compared with the *E. coli* OP50 fed control; 10mM sodium acetate slightly increased the IFD content to 109% (P=0.96, n=5) compared with the *E. coli* OP50 fed control (Figure 10 {D}). The IFD for the treatment
with 100mM sodium acetate was decreased to 90% of the control but the difference was not
significant (Figure 10 {D}, P=0.97, n=5). Sodium propionate at the concentration of 0.3mM
slightly increased IFD (Figure 11 {D}, P=0.0636, n=5). Tributyrin at the concentrations of 0.5
and 3mM did not significantly change the IFD (Figure 12 {D}, P=1 and P=0.88, respectively).

3.3.6 Amylose starch sustained the PPR

Amylose starch consists of 60% amylose starch and 40% amylopectin (Zheng et al.,
2010). Concentrations of 0.5, 1, and 3% amylose starch were used to feed wild type C. elegans
N2 and daf-16(mgDf50)I mutant. In the wild type C. elegans N2, the 0.5, 1, and 3% amylose
starch treatments sustained the PPR (P<0.05, Figure 13) compared with the control treated with
E. coli OP50 only. The 0.5, 1, and 3% amylose starch treatments did not significantly sustain the
PPR in the presence of 2% glucose (Figure 13). In the daf-16(mgDf50)I strain, the 0.5, 1, and 3%
of amylose starch treatments increased the PPR in this strain (Figure 13). In the presence of 2%
glucose, the 0.5,1 and 3% PWB treatments also sustained the PPR in the presence of glucose
(Figure 13).
Figure 13  Amylose starch sustained the PPR

3.4 Discussion

The mean survival times of *E. coli* OP50 fed control for the wild type N2 and *daf-16(mgDf50)I;daf-2(m65) III C. elegans* were 24.9 and 21.7 days, respectively. Sodium butyrate significantly increased the lifespan of the wild type *C. elegan*, but it did not significantly increase the lifespan of the *daf-16(mgDf50)I;daf-2(m65) III C. elegans*. The low dose of sodium acetate and tributyrin significantly increased the lifespan of the wild type and *daf-16(mgDf50)I;daf-2(m65) III C. elegans*, but the high dose of sodium acetate and tributyrin did not significantly increase the lifespan, in fact, they slightly reduced the lifespan. Sodium propionate at 7 mM did not significantly increase the lifespan for both the wild type and *daf-16(mgDf50)I;daf-2(m65) III C. elegans*. The effect of SCFAs on the lifespan is dependent on the insulin signaling gene *daf-2*, but not dependent on *daf-16*.
The results for the sir-2.1(ok434)IV C. elegans strain demonstrated that sodium butyrate increased the lifespan in a sir-2.1 gene dependent way. Low dose of sodium acetate also increased the lifespan in a sir-2.1 gene dependent way, but the high dose of sodium acetate decreased the lifespan in both strains. Tributyrin increased the lifespan also in a sir-2.1 gene dependent way. Sodium propionate at 7mM did not significantly increase the lifespan in both the wild type N2 and sir-2.1(ok434)IV C. elegans strains.

Sodium butyrate can increase the lifespan and the daf-2 and sir-2.1 genes are necessary to adjust the lifespan of C. elegans. Sodium acetate can increase the lifespan of C. elegans but the dosage is important. High dosage of sodium acetate is harmful to the healthspan of C. elegans. Sodium acetate adjusts the lifespan of C. elegans in daf-2 and sir-2.1 dependent way. Tributyrin also increases the lifespan of C. elegans in a daf-2 and sir-2.1 genes dependent way.

The lifespan of C. elegans as well as other organisms such as flies, yeast, and possibly mammals is regulated by the insulin signaling pathway gene daf-16 as daf-16 protein plays an important role in the regulation of insulin signaling pathway in C. elegans (Lin et al., 2001). How the SCFAs regulate the lifespan through insulin signaling pathway is still not fully understood. Therefore, C. elegans daf-16(mgDf50) I (GR1307) which is a strain that has a complete elimination of daf-16 coding region was used in this study. The results from this study indicate that the effect of SCFAs on the lifespan of C. elegans is independent of the insulin signaling gene daf-16.

The results from this study demonstrate that sodium butyrate, low dose sodium acetate and low dose tributyrin are beneficial in extending the lifespan of C. elegans and in the reduction of intestinal fat deposition. Sodium butyrate has the best effect among all the SCFAs tested. Previous studies by other researchers have also shown similar effects (Zheng et al., 2010, Zheng
et al., 2008, Keenan et al., 2013). High dose of sodium acetate negatively affected the lifespan. The $daf$-$16$ gene is a key factor in the insulin signaling pathway of $C. elegans$ to regulate the longevity gene expression, but our results demonstrated that the regulation of longevity by SCFAs is independent on the $daf$-$16$ gene.
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CHAPTER 4: PROWASHONUPANA BARLEY DIETARY FIBER REDUCES BODY FAT AND INCREASES INSULIN SENSITIVITY IN CAENORHABDITIS ELEGANS MODEL

4.1 Introduction

Obesity costs the US health care system over $147 billion to nearly $210 billion a year and predisposes to diabetes which generates even higher costs (Ogden et al., 2006, Cawley J, 2012). Only limited medications are available to treat obesity, and surgical treatments are second-line obesity treatments. Diet intervention, exercise, and lifestyle modification have been promoted by the National Institutes of Health (NIH), the United States Department of Agriculture (USDA), and other government institutions, but sustainable weight loss has been elusive. In an intensive diet and behavior modification program with meal replacements, adults lost 22.6-25.5±1% of their body weight temporarily, but maintained only a 5% weight loss after 3 years (Kress et al., 2006, Wadden and Frey, 1997).

Daily consumption of non-digestible fiber may offer a strategy to increase energy expenditure and reduce body fat by stimulation of mitochondrial fat oxidation (Madhujith et al., 2004, Chandrasekara and Shahidi, 2011, John and Shahidi, 2010). Average daily consumption of dietary fiber in the United States is currently 3 to 10 g/day, 4 to 13 fold less than recommended (Goldring, 2004, Anderson and Akanji, 1991). Non-digestible carbohydrate, fermented to SCFAs in the lower gastrointestinal tract, increases satiety hormone PYY and GLP-1. These hormones augment fat oxidation (lower respiratory quotient), elevate energy expenditure (Zhou et al., 2006, Zhou et al., 2008, Keenan et al., 2006), and inhibit body fat accretion in rodents (Keenan et al., 2006, Shen et al., 2009). The most efficient SCFA, butyrate, stimulates the production of PYY, GLP-1 (Zhou et al., 2006) and prevents diet-induced insulin resistance which is directly related to a shorter lifespan in mice, a reduced cell survival, and a
decreased lifespan in the C. elegans model organism (Gao et al., 2009, Abate and Blackwell, 2009). A diet supplemented with β-glucans at 4g/day for 14 weeks in 7 healthy overweight and obese subjects increased fasting PYY, GLP-1, and increased post-meal satiety in humans (Greenway et al., 2007). We found that fermentable carbohydrate or RS, endogenous gut fermentation products of rodents fed RS, or the exogenous SCFA, butyrate, all reduce intestinal fat deposition (IFD) in the wild type C. elegans (N2) (Zheng et al., 2010). The lipid oxidation pathway including cluster differentiation transport protein (CD36), carnitine palmitoyltranferase-1 (CPT-1), acetyl co-enzyme A carboxylase (ACC), and acetyl CoA synthetase ACS have been identified using genetically manipulated C. elegans (see review (Zheng and Greenway, 2012)).

Barley is the fourth most important cultivated foodstuff, and contains 62-77% starch (w/w) which is composed of 25-35% atypical amylose starch with 3–5% RS₃ and β-glucan (Asare et al., 2011, Vasanthan and Bhatt, 1998), a mixture that provides a distinctive amylose–amylopectin interaction (Tang et al., 2001, Lifschitz et al., 2002, Behall et al., 2005, Rendell et al., 2005, Dongowski et al., 2002). Barley also contains high levels of functional lipids, such as, total phytosterols (1.2-9.6% of barley oil) and total tocotrienols (0.3-0.6% of barley oil) which are 6-12 fold higher than in palm oil (0.05%) and 4-8 fold higher than in rice bran oil (0.08%) (Moreau et al., 2007). The barley variety Prowashonupana (PWB) contains 17% β-glucan and 15% fermentable starch (Rendell et al., 2005). These carbohydrates delay or decrease the absorption of dietary fats and lower the postprandial glycemic response (Lifschitz et al., 2002). Dietary fermentable fiber such as RS is present in a variety of cereals and offers the opportunity to reduce body fat and control body weight. We demonstrated that RS₂ from corn (Ingredion Incorporated, Westchester, Illinois, USA) induced a 23% reduction of intestinal fat deposition in wild type C. elegans (Zheng et al., 2010). The specific PWB used in the present study has a
lower RS content compared to high amylose corn starch, but has significantly more soluble \(\beta\)-glucan than most other barleys, which are linked to half of the PWB fiber (Behall et al., 2005, Rendell et al., 2005). Retrograded amylose starch, RS\(_3\), and \(\beta\)-glucan of PW barley provide distinctive amylose–amylopectin and amylopectin-\(\beta\)-glucan interactions that increase the viscosity and delay energy absorption in the GI tract (Asare et al., 2011, Vasanthan and Bhatt, 1998, Tang et al., 2001, Moreau et al., 2007) reducing the glycemic peak by 50%, lowering low density lipoprotein (LDL) cholesterol in healthy humans, decreasing fat accumulation in humans and rodents (Wursch and Pi-Sunyer, 1997), augmenting immunity by activating the dectin-1 receptor and activating multiple genetic signaling pathways, including the DAF-2/insulin-like receptor (ILR) pathways. The details of the mechanism by which insulin-resistance is improved, however, are unclear (Tsoni and Brown, 2008, Engelmann and Pujol, 2011).

*C. elegans* is a small, free-living soil nematode, a multicellular eukaryotic organism, distributed widely around the world. *C. elegans* is the first animal to have its genome completely sequenced and conserves 65% of the genes associated with human disease (Zheng and Greenway, 2012). *C. elegans daf-2*, is a homolog of the insulin and insulin-like growth factor (IGF) receptor in humans and is the only gene encoding the insulin/IGF-1 like receptor in *C. elegans* (Zheng and Greenway, 2012). Decreased daf-2 signaling increases *C. elegans* lifespan (Kramer et al., 2003, Koga et al., 1999) which fully depends upon daf-16 (Patel et al., 2008), since daf-16 null mutations completely suppress the extension of lifespan by daf-2 (e1370)III null mutant (Hertweck et al., 2004, Tissenbaum and Ruvkun, 1998, Burks et al., 2000, Yu et al., 2010, Hunt et al., 2011). Hyperglycemia (2% glucose) causes insulin resistance in N2 and daf-2(e1370)III null mutants, and reduces pharyngeal movement, a surrogate marker directly related to lifespan (Abate and Blackwell, 2009). *sir-2.1* overexpression prolongs *C. elegans* lifespan
In fact, doubling *C. elegans sir-2.1* gene number induces a 50% increase in the lifespan that is DAF-16/FOXO dependent (Tissenbaum and Guarente, 2001, Dorman et al., 1995), and the null mutant of *sir-2.1(ok434)IV* has a shorter lifespan than the N2 (Gami and Wolkow, 2006), which recently became controversial, because RNA interference (RNAi) did not suppress the longevity (Burnett et al., 2011). The *gcy-8* gene (thermotaxis) is a subtype of guanylyl cyclase (GCYs) which has been proven to promote fat storage and mediate satiety, as does *cck*.

Two thirds of the genes associated with human diseases have homologs in *C. elegans*, and the *C. elegans* intestine has similarities to the human gastrointestinal track (You et al., 2008). Over 300 genes have been shown to cause a reduction in body fat when inactivated, and inactivation of more than 100 genes has shown an increased fat storage (Ashrafi et al., 2003). The genes governing lipid metabolism do not necessarily overlap with genes governing the aging process (Zheng and Greenway, 2012). Not only does the *C. elegans* model organism offer reduced costs when screening for bioactive substances, the translucent bodies of the worms are convenient for analysis of intestinal fat deposition (IFD) by lipid-staining dyes. Nile red fluorescent dye has been validated through lipid-extraction, separation with thin layer chromatography, and quantification with gas chromatography (Ashrafi et al., 2003). Recently, Nile red staining *in vitro* has been recommended as being superior to coherent anti-Stokes Raman scattering (CARS) microscopy (Yen et al., 2010).

The goal of the current study was to determine the effects of PWB on IFD, insulin-resistance, and hyperglycemia-impaired lipid metabolism using the *C. elegans* N2, and null mutants, *sir-2.1(ok434)IV, daf-16(mgDf50)I*, or *daf-16(mgDf50)I;daf-2(m65) III*. Pharyngeal pumping rate (PPR), a surrogate marker of *C. elegans* lifespan (Chow et al., 2006b, Finley et al.,
2013b), was also monitored to determine effect of PWB on healthspan.

4.2 Materials and methods

*C. elegans* strains and their standard lab food source, *Escherichia coli* (*E. coli*, OP50, *Uracil auxotroph*), were provided by the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The *C. elegans* model is not regulated by the Institutional Animal Care and Use Committees (IACUC) nor does it require IACUC approval (Zheng et al., 2013).

4.2.1 Culture of *E. coli* (OP50)

OP50 were cultured by the standard method described elsewhere (Zheng et al., 2010). Briefly, 10µl± of stock *E. coli* solution was added to Luria Broth (LB) medium and the medium was incubated at 37°C for 48h. The OP50 were then sub-cultured in Petrifilm™ *E.coli/Coliform Count Plates* (3M Corporate, MN, USA) at 37°C for 24h, and the colonies of OP50 were confirmed to be at a density of 5x10⁸ – 5x10¹¹ cfu/ml at which time the *C. elegans* were allowed to eat *ad libitum*.

4.2.2 Culture of *C. elegans*

*C. elegans* N2, *sir-2.1(ok434)IV, daf-16(mgDf50)I*, and *daf-16(mgDf50)I;daf-2(m65)III* null mutants were used in this study. Each strain was age synchronized, grown on NGM agar plates (⌀35 mm), transferred to a new dish every other day, and stored in a low temperature incubator (Revco Tech., Nashville, NC, USA) at 20°C or 15°C (*daf-16(mgDf50)I;daf-2(m65)III*) throughout the experiments. Mature gravid *C. elegans* were transferred individually onto the agar plates and treated with a lysis solution consisting of NaOH (1M, 500 µl, v/v) and sodium hypochlorite solution (5.25%, 200 µl, v/v) to dissolve the *C. elegans* body and to release the viable eggs. Stock solutions have been previously described in detail. One day prior to the
experiment, 200µl of a feeding media containing *E. coli* was added to the agar dish.

4.2.3 Diet composition

*Prowashonupana* barley (PWB, Sustagrain®, Ardent Mills Corporate, Denver, CO, USA) were powdered using a centrifugal mill with a 0.75mm sieve (Retsch ZM 200; Haan, Germany), autoclaved at 121°C, and suspended in distilled deionized water (DDH,O, 5% w/v). Detailed nutritional data of PWB by Ardent Mills Corporate is listed in Table 9.

Table 9 Nutritional data of *Prowashonupana* barley (Sustagrain® Barley¹), reported on a 100g basis.

<table>
<thead>
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<th>Content</th>
<th>Quantity (mg)</th>
</tr>
</thead>
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<td>Vitamin A</td>
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</tr>
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<td>Calories from Fat</td>
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</tr>
<tr>
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</tr>
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<td>Saturated Fat</td>
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</tr>
<tr>
<td>Cholesterol</td>
<td>0</td>
<td>Iron</td>
<td>3.6</td>
</tr>
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<td>Carbohydrates</td>
<td>64.3</td>
<td>Vitamin B1 (Thiamin)</td>
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</tr>
<tr>
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<td>30</td>
<td>Vitamin B2</td>
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</tr>
<tr>
<td>Beta glucan</td>
<td>15</td>
<td>(Riboflavin)</td>
<td></td>
</tr>
<tr>
<td>Soluble Fiber</td>
<td>12</td>
<td>Vitamin B3 (Niacin)</td>
<td>4.6</td>
</tr>
<tr>
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<td>452</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zinc</td>
<td>2.8</td>
</tr>
</tbody>
</table>


Control animals were fed with OP50 only. The experimental groups larval stage 2 (L2) were fed supplementary *Prowashonupana* barley (PWB, 0.5, 1.0, or 3.0%) that had no additives. To create insulin resistance, two percent glucose was added to an additional group of each strain, with and without PWB (Table 10). The dietary nutrient composition is listed in Table 11 (Feijo Delgado et al., 2013). *C. elegans* were transferred to fresh dishes every other day receiving treatment (50µl).
Table 10 PWB diet composition

<table>
<thead>
<tr>
<th>Treatments (50µl)</th>
<th>Without glucose</th>
<th>With glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>OP50 (2×10⁹ cfu/ml, µl)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PWB (5%, µl)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Glucose (50%, µl)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 11 Diet ingredient composition

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>PWB diet (mg/ml)</th>
<th>OP50 (mg/ml)</th>
<th>PWB treatment (mg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.5%</td>
<td>1%</td>
</tr>
<tr>
<td>Beta-glucan</td>
<td>150</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total dietary fiber</td>
<td>300</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>643</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>180</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>65</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

4.2.4 Pharyngeal movement (pumping rate, PPR)

Pharyngeal pumping rate (PPR) as a surrogate marker of C. elegans lifespan (Chow et al., 2006a, Finley et al., 2013a, Kang et al., 2007) was also monitored for the effect of these compounds. Animals were examined periodically using a stereomicroscope (Nikon SMZ1500, Melville, NY) with transmitted light. The pharyngeal pumping rate (PPR) was recorded manually by independent observers and the animals were returned to the incubators.

4.2.5 Fluorescence microscopy

The lipophilic dye, Nile red, was selected to stain the intestinal fat deposition. Prior to fixing the animals, an S-basal (see below) solution was added to the dish to wash the animals. The solution containing the animals was centrifuged for 20 seconds at 805G and this procedure was repeated twice. Animals were then fixed with 4% paraformaldehyde over 2h at 4°C and washed with phosphate buffered saline (PBS) for 5 min x 3. Nile red (50 µl) was applied to the
specimens for 10 min. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, AL, USA) was applied to a glass slide followed by 20µl of the medium containing Nile red stained animals. A cover glass was mounted on the glass slide. The slides were viewed with an epifluorescence microscope (Nikon Eclipse, Ti) equipped with a Texas Red filter. Fluorescent micrographs were taken with a digital camera (Andor, DU-885k, Concord, MA, USA). The micrographs were analyzed using NIS elements (Nikon). Line profiles of the optical densities (arbitrary units, % of control) of Nile red labelled IFD were determined for adult animals (larval stage 4 [L4]).

4.2.6 Quantitative RT-PCR

4.2.6.1 Reagents

The Trizol® reagent (T9424), chloroform (C2432), and isopropanol (I9516) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Taqman PCR core kit (N8080228), MuLV Reverse Transcriptase (N8080018), and RNase Inhibitor (N8080119) were obtained from Life Technologies (Grand Island, NY, USA). RNase-free micro tubes and pipette tips were used in isolating RNA and in quantitative RT-PCR.

4.2.6.2 RNA isolation

Total RNA was extracted using the Trizol® reagent as described elsewhere (Ye et al., 2007). C. elegans samples were homogenized in 1ml Trizol® Reagent (Sigma-Aldrich, St. Louis, MO, USA) per 50-100 mg of tissue. The samples went through 5 freeze-thaw cycles in which they were frozen in liquid nitrogen and thawed in a 37°C water bath. The homogenized samples were vortexed at room temperature to allow the complete dissociation of nucleoprotein complexes. Two hundred microliters of cold pure chloroform per 1ml of Trizol® reagent were added and the samples were vortexed for 15s before being incubated at room temperature for
5min. The samples were centrifuged at 12,000g for 10 min at 4°C, and the top aqueous phase was transferred to a fresh tube. A 600µl aliquot of cold isopropanol per 1ml of Trizol® reagent was used for the initial homogenization. The samples were then incubated at room temperature for 10min, vortexed for 15s, and centrifuged at 12,000g for 10min at 4°C. The supernatant was removed and the RNA pellet was washed once with 1ml cold 75% ethanol. The sample was vortexed and centrifuged at 7,500g for 5min at 4°C. The supernatant was removed completely and the RNA pellet was briefly air-dried. The RNA pellet was re-suspended in 0.1% diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C. The RNA concentration was analysed with a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE, USA).

4.2.6.3 Quantitative RT-PCR

The mRNA levels of *ckr-1*, *gcy-8*, *cpt-1*, and *cpt-2* were determined using Taqman® quantitative RT-PCR (Figure 14). The following components were added in one-step RT-PCR: 2.2µl H₂O, 1µl 10× Taqman® buffer A, 2.2µl 25mM MgCl₂ solution, 0.3µl 10mM dATP, 0.3µl 10mM dCTP, 0.3µl 10mM dGTP, 0.3µl 10mM dUTP, 0.05µl 20U/µl RNase inhibitor, 0.05µl 50U/µl MuLV reverse transcriptase, 0.05µl 5U/µl Amplitaq gold®, 0.25µl Taqman® probe and primers, and 3µl 5ng/µl RNA. The PCR reaction was conducted in triplicate using a Taqman® probe and primers set (Life Technologies, Grand Island, NY) for *ckr-1* (Ce02408606_m1), *gcy-8* (Ce02456184_g1), *cpt-1* (Ce02440434_m1), and *cpt-2* (Ce02459919_g1). The mRNA signal was normalized over a eukaryotic 18S rRNA (Hs99999901-s1) internal control. The reaction was conducted using a 7900 HT Fast real-time PCR system (Life Technologies, Grand Island, NY, USA). Reverse transcription was done at 48°C for 30min, and AmpliTaq gold® activation (denaturation) was done at 95°C for 10min. Amplification of the DNA involved 40 cycles of 15s at 95°C and 1min at 60°C. Data was analyzed using the Sequence Detector Software (Life
Technologies, Grand Island, NY, USA). The relative quantification of gene expression \(2^{-\Delta \Delta C_t}\) was calculated.

Figure 14 The procedures of animal culture, DNA or RNA extraction, and qRT-PCR in C. elegans model organism (http://www.gene-quantification.de/chemistry.html).

4.2.7 Statistical analysis

All data were presented as mean ± sem. Student \(t\)-test, analysis of variance (ANOVA) was used for IFD, analysis of co-variance (ANCOVA) was used to compare the slopes of PPR data, and principal component analyses (PCA) was conducted using SAS 9.4 (Cary, NC, USA). Statistical significance was set at \(P \leq 0.05\).

4.3 Results

Changes of IFD indicated by fluorescent intensity of Nile red and alterations in lifespan suggested by reduced PPR indicating aging, with or without 2% glucose, were quantified in this study. The IFD was reduced in N2, and null mutants of *sir-2.1(ok434)IV* and *daf-16(mgDf50)I*. 81
These reductions were reversed in the presence of 2% glucose. The PPRs declined in all groups as the animals aged. PWB treatment, however, increased the PPR in all groups compared to their control that did not receive PWB treatment. These responses were reversed in the presence of 2% glucose.

Figure 15 Intestinal fat deposition was significantly reduced in the *C. elegans* (N2) that received the PWB (0.5% or 3%) compared to a control group.

4.3.1 Intestinal fat deposition (IFD)

Post PWB treatment, the fluorescence intensity of Nile Red was dose-dependently reduced by 19 to 44% (0.5 or 3.0%, n=6, P<0.05) in N2 (Figure 15), by 18 to 28% (0.5, 1.0, or 3.0%, n=9, P<0.01) in *daf-16(mgDf50)*I (Figure 16b), or by 18 to 36% (0.5, 1.0, or 3.0%, n=9, P<0.01) in the *sir-2.1(ok434)*IV (Figure 16d). The reduction in N2 or *daf-16(mgDf50)*I was reversed in presence of 2% glucose (Figure 16a and 16b). In *daf-16(mgDf50)*I;*daf-2(m65) III*, PWB did not have statistically significant effect on IFD (Figure 16c). In *sir-2.1(ok434)*IV, only in presence of 2% glucose, PWB (0.5 or 3.0%) induced a 40-50% reduction of the fluorescence intensity in a dose-dependent manor (n=3, P<0.05) (Figure 16d).
Figure 16 IFD was reduced in mutant *C. elegans*.

a) N2 Fluorescence intensity of Nile red was reduced in the PWB (0.5%, 1%, or 3%) treated animals. The fluorescence intensity of Nile red reduced by PWB treated animals was reversed in the presence of 2% glucose.  
b) *daf-16(mgDf50)*: The fluorescence intensity of Nile red reduced by PWB treated animals was no different compared with control animals with or without glucose.  
c) *daf-16(mgDf50)*;*daf-2(m65)*: Fluorescence intensity of Nile red was not reduced in the PWB (0.5% or 3%) treated animals. PWB treatment reduced Nile red fluorescence intensity in the presence of 2% glucose.  
d) *sir-2.1(ok434)*: Fluorescence intensity of IFD stained by Nile red in animals that received the PWB (0.5%, 1%, or 3%).

4.3.2 Pharyngeal pumping rate (PPR)

The PWB treatment dose-dependently elevated the PPR in N2 (1.0 or 3.0%, *P*<0.05) (Figure 17a), *daf-16(mgDf50)* (0.5, 1.0, or 3.0%, Fig. 17b), *daf-16(mgDf50)*;*daf-2(m65)* (0.5,
1.0, or 3.0%, Fig. 17c), or *sir-2.1(ok434)IV* (3.0%, Fig. 17d) (n = 20 animals/3 dishes, *P*<0.05) without additional 2% glucose. In the presence of additional 2% glucose, the PWB treatment dose-dependently elevated the PPR in N2 (0.5, 1.0, or 3.0%, *P*<0.05) (Figure 17e), *daf-16(mgDf50)I* (0.5 or 1.0%, Figure 17f), *daf-16(mgDf50)I;daf-2(m65)III* (0.5, 1.0, or 3.0%, Figure 17g), or *sir-2.1(ok434)IV* (3.0%, Figure 17h) (n = 20 animals/3 dishes, *P*<0.05).

Figure 17 PPR of *C. elegans* was reduced in all groups with aging. An elevation of the PPR was observed in animals that received the PWB in a) N2 (*P*<0.05), b) *daf-16(mgDf50)I* groups (*P*<0.05), c) *daf-16(mgDf50)I;daf-2(m65) III* (*P*<0.05) or d) *sir-2.1(ok434)IV* (*P*<0.05). Similar results were observed in the presence of glucose in e) N2 (*P*<0.05), f) *daf-16(mgDf50)I* groups (*P*<0.05), g) *daf-16(mgDf50)I;daf-2(m65) III* (*P*<0.05) or h) *sir-2.1(ok434)IV* (*P*<0.05).
4.3.3 Lipid metabolism gene expression

PWB feeding (0.5, 1, and 3%) dose-dependently reduced mRNA expression of *cpt-1* (20-30%), *cpt-2* (30-40%), *ckr-1* (30-40%), and *gcy-8* (30-50%) in N2. The reduction was observed in *daf-16* deficient mutant as *cpt-1* (30%), *cpt-2* 20-30%, *ckr-1* (40%), and *gcy-8* (40%).

Figure 18 PWB dose-dependently altered mRNA expressions of *cpt-1*, *cpt-2*, *ckr-1*, and *gcy-8* in the *C. elegans* model.

a) Reduced in N2 and was reversed by additional glucose; b) Elevated in *daf-16* mutant with reduction on *cpt-1* with further augmentation by glucose; and c) Decreased in *daf-16/daf-2* mutant and further reduced by glucose with an initial increase except *cpt-1*; d) Did not change in *sir-2.1* mutant while further reduced in presence of glucose.
In *daf-16*/*daf-2* mutant, *cpt-1* was reduced by 30% while other three mRNA expressions were elevated by *cpt-2* (50%), *ckr-1* (70-60%), and *gcy-8* (80%). Although there was a 20% reduction in *gcy-8*, feeding did not change the other three mRNA expressions in the *sir-2.1* mutant. In presence of glucose, however, these mRNA expressions were increased in N2 by 10% and in the mutant *daf-16* by 40%, while mRNA expression was reduced in the mutant *sir-2.1* by 50% after an initial increase of 30% and in the *daf-16*/*daf-2* by 10-50% after an initial reduction of 40-60% (P<0.05, Figure 18).

4.3.4 Principal component analyses (PCA)

Figure 19 Principal component analyses (PCA) showed the relationships of the lipid metabolic related mRNAs (*ckr-1*, *cpt-1*, *cpt-2*, and *gcy-8*), PPR slope, and IFD with two strong factors.
Two factors explained the results of PCA. IFD has a strong negative effect with factor 1 in N2, while it has weak relationship with factor 1 in other strains. IFD has a strong positive relationship with factor 2 in daf-16, daf-16/daf-2, and sir-2.1 null mutants. PPR slope has a strong negative relationship with factor 2 in daf-16/daf-2, and sir-2.1 null mutants, but there was a strong positive relationship with factor 1 in daf-16 mutant. IFD and PPR have an inverse relationship in all strains. cpt-1, ckr-1, gcy-8, andcpt-2 have strong positive relationship with factor 1 but these 4 genes have an indirect relationship with factor 2 (Figure 19).

4.4 Discussion

PWB induced significant reductions of IFD in N2 (40%) and daf-16(mgDf50)I (25%) deficient mutants, but not in the daf-16(mgDf50)I;daf-2(m65)III deficient mutant. The PPR was elevated with all varieties of animals fed PWB. On the contrary, reduced fluorescence intensity and elevated PPR were detected in daf-16/daf-2(m65)III deficient mutant in the presence of an additional 2% glucose. PWB reduced cpt-1, cpt-2, ckr-1, and gcy-8, which was reversed in sir-2.1 deficient mutant suggesting that the major pathway of this effect is mediated by sir-2.1. It appeared that glucose reversed the effect of PWB in the N2. Dissimilarly, daf-16 deficiency had only a reduction of cpt-1 with elevations on cpt-2, ckr-1, and gcy-8, all of which had an initial reduction. Additional glucose augmented the PWB effects to a greater degree than in N2. Thus, daf-16 seemed to be playing an inhibitory role on lipid and glucose metabolism. PWB had similar effects in daf-16/daf-2 mutant as in N2. Unlike N2, the effect of additional glucose in daf-16/daf-2 mutant was eliminated by an initial increase in cpt-2, ckr-1, and gcy-8 followed by further reduction. These findings indicated that daf-2 mediated the PWB effect but to a lesser degree than sir-2, and facilitated glucose metabolism.
Hyperlipidemia is often co-existent with hyperglycemia. Insulin-resistance induces hyperglycemia in rodent diabetes due to an increased peroxisome proliferator activated receptor-α (PPARα) (Hostetler et al., 2008). Deletion of nuclear hormone receptor-49 (nhr-49), a homologue of PPARα in C. elegans, prevents hyperlipidemia and restores insulin-sensitivity comparable to that seen in PPARα null mice (Atherton et al., 2008). In the current study, the PWB (3.0%) induced more than a 40% reduction in the IFD, which was daf-16 or sir-2.1 dependent since the effect was not seen in the daf-16/daf-2 mutant. The effect of reducing or not reducing insulin sensitivity in the treatment groups described above was reversed when decreased insulin-sensitivity was created by excessive glucose, and produced IFD reduction in daf-16/daf-2 mutant insulin-resistance model. Since daf-16 has a central role in mediating the downstream insulin signaling pathways and is the major target of the daf-2 pathway which negatively regulates daf-16 (Lee et al., 2009, Kenyon et al., 1993), possibly, PWB/β-glucans indirectly affected insulin-sensitivity by affecting hyperglycemia impaired lipid metabolism and served as a unique functional food in addition to providing a source of RS. daf-2 is the only gene that encodes the insulin/IGF-1 like receptor in C. elegans and the genes governing lipid metabolism do not necessarily overlap with genes governing the aging process (Zheng and Greenway, 2012). We found that neither sir-2.1 nor daf-16 was required for the PWB diet to reduce IFD. Due to increased fat oxidation (with decreased RQ) and increased energy expenditure (Zhou et al., 2008, Keenan et al., 2006, Shen et al., 2009), rodents that received RS had significantly greater body fat reduction with continually elevated serum levels of the hormones PYY and GLP-1 rather than peaks of these hormones in serum only after food consumption to signal satiety (Zhou et al., 2008, Keenan et al., 2006). In agreement with the rodent studies, the reduced IFD suggested an increased fat oxidation and increased energy
expenditure rather than an augmented satiety or reduced food intake. Glucose appeared to increase \textit{gcy}-8 gene expression which promotes fat storage. This effect is lost in the presence of the \textit{sir}-2.1 deficient mutant or with the lack of \textit{daf}-2/\textit{daf}-16, which suggest that the additional glucose induced insulin resistance is dependent on \textit{daf}-2 pathway and is suppressed by \textit{sir}-2.1. The other genes do not seem to play much of a role, but the small increase in \textit{cpt}-2 with glucose is reversed in \textit{sir}-2.1 deficient mutant and the lack of both \textit{daf}-2/\textit{daf}-16. Since \textit{cpt}-2 gene expression in the \textit{daf}-16 deficient mutant did not change, the effect of glucose on \textit{cpt}-2 was thought to be through \textit{daf}-2 and that \textit{sir}-2.1 inhibited that effect.

Extra glucose reduces \textit{C. elegans} lifespan which may be independent of insulin-sensitivity and changes in body fat. As indicated by a surrogate marker of aging PPR, PWB sustained healthspan or lifespan. However, the PPR was not increased and was reduced with aging when an additional 2\% glucose was added to all the strains tested except the \textit{daf}-16/\textit{daf}-2 deficient mutant. Like IFD, the effect of PWB on PPR was reversed in the \textit{daf}-16/\textit{daf}-2 deficient mutant with addition of glucose suggesting an independent mechanism which may involve a pathway outside of \textit{daf}-2 or \textit{daf}-2/\textit{daf}-16 (Zheng and Greenway, 2012, Engelmann and Pujol, 2011), or that absence of the \textit{daf}-2 promoted insulin sensitivity despite high levels of glucose. The PPR was dose-dependently elevated in the N2, or \textit{daf}-16, and \textit{daf}-2/\textit{daf}-16 null mutants indicating an independency on \textit{daf}-2 or \textit{daf}-2/\textit{daf}-16 as their absence eliminated the elevation of PPR. The \textit{sir}-2.1 gene is related to the co-regulator/genes (Kyrylenko et al., 2003) and is partially responsible for aging in \textit{C. elegans} (Lakowski and Hekimi, 1998, Houthoofd et al., 2003). Apparently, the effect of improved healthspan or lifespan was partially dependent of \textit{sir}-2.1 in the present study, which was more robust than in the \textit{daf}-16 mutant. The \textit{daf}-2/\textit{daf}-16 mutant showed increase in PPR suggested the effect was insulin/IGF-1 like receptor pathway
dependent, which was consistently absent in \textit{daf-16} mutant.

4.5 Conclusions

Our data demonstrated that PWB reduced IFD, decreased insulin-resistance, and improved healthspan or lifespan in the \textit{C. elegans} model system which appeared to be primarily mediated via \textit{sir-2.1, daf-16}, and \textit{daf-16/daf-2}. \textit{C. elegans} is an attractive \textit{in vivo} animal model for initial studies of nutrition interventions prior to confirmation in higher animal species. The identified optimal functional food components may be incorporated in various ways into daily diet, because these are already known to have acceptability as food, and have been tested in clinical trials. A broader test of other sources of the functional components of PWB, such as, \(\beta\)-glucan, may provide more advantageous weight loss and obesity prevention. This could result in future improvements in public health.
4.6 References


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CHAPTER 5: THE EFFECT OF OATS ON LIPID METABOLISM AND INSULIN SENSITIVITY IN THE CAENORHABDITIS ELEGANS MODEL

5.1 Introduction

Modern lifestyle-related chronic diseases, such as obesity, insulin resistance, and type II diabetes, are major health challenges of the 21st century (Cordain et al., 2005). Changes in dietary habits and lifestyle are among the primary causes for this undesirable development, which is already being seen in the younger population.

An inverse connection of longevity and fat mass is characteristic of humans above 25±5% body fat when accompanied by obesity related disease (Padwal et al., 2011). Both obesity and aging decrease insulin sensitivity, impair the immune response, increase inflammation, weaken the gut-bloodstream barrier, and decrease physical mobility (Padwal et al., 2011). Promotion of dietary intervention, exercise, and lifestyle modification, including second-line obesity treatments, to sustain weight loss has been elusive (Kress et al., 2006, Ogden et al., 2006). Even though a direct relationship between calorie restriction and healthspan has been observed in animal models (Ingram et al., 2002, Lee et al., 2006), controlling food intake in humans is difficult, mainly transient, and often unsustainable as it involves multiple factors including psychological factors.

C. elegans is well-suited to obesity studies. Lipid oxidation pathways are present in the Caenorhabditis elegans (C. elegans) model, such as, cluster differentiation transport protein (CD36), carnitine palmitoyltranferase-1 (CPT-1), acetyl co-enzyme A carboxylase (ACC) and acetyl CoA synthetase (ACS). Resistant starch and short chain fatty acids reduce intestinal fat deposition (IFD) in C. elegans demonstrating that the model can be used to evaluate bioactive components from gut fermentation (Zheng et al., 2010, Zheng and Greenway, 2012, Finley et al., 2012).
2013). *C. elegans* is a small, free-living soil nematode, a multicellular eukaryotic organism, distributed widely around the world. *C. elegans* is the first animal to have its genome completely sequenced and conserves 65% of the genes associated with human disease (Zheng and Greenway, 2012). *C. elegans* deposits fat for energy storage along its intestinal tract of its transparent body (Hostetler et al., 2008). Thus, lipid-staining dyes such as Nile Red can be visualized directly and quantitated photometrically in the intact *C. elegans* (Fei et al., 2004). Food intake and transport in *C. elegans* is regulated by pharyngeal movements and relaxation in the terminal bulb of the pharynx. The PPR declines with age due to sarcopenia, and PPR is a surrogate marker of aging (Chow et al., 2006).

Many dietary interventions with enriched non-digestible but fermentable carbohydrates may reinforce optimal nutrition, enhance physiological function, reduce obesity, improve metabolic dysfunction, limit disease (Ogden et al., 2006), and produce anticancer effects in humans (Cassidy et al., 1994, Higgins, 2004). The mechanisms of these interventions have been reported to be due to increased mRNA expression of the GLP-1 precursor, proglucagon and PYY in rodents (Zhou et al., 2006), improved glucose tolerance (Keenan et al., 2012, Zhou et al., 2008), increased phase I xenobiotic metabolizing enzymes which are a hallmark of long-lived mice (Steinbaugh et al., 2012), promotion of mitochondrial fatty acid oxidation, and increased energy expenditure in *Drosophila* or adult rodents at ages analogous to 56–65 years in humans (Keenan et al., 2006, Shen et al., 2011, Zhou et al., 2008). Consequently, fermentable dietary fiber reduces body fat accumulation (Keenan et al., 2006, Shen et al., 2009), plasma cholesterol and triglycerides (de Deckere et al., 1993), and insulin resistance (Gao et al., 2009) in rodents and humans.

In addition to RS, oats (*Avena sativa*, Table 12) contain the soluble fermentable dietary β-
glucan fiber and the unique phytoalexins avenanthramides (avns). Avns provide safe, anti-
oxidant, anti-inflammatory, and anti-angiogenic properties (Chen et al., 2007, Meydani, 2009,
Chu et al., 2013). Consuming oats introduces a hypocaloric intervention through caloric dilution
(Greenway et al., 2007, Saltzman et al., 2001); reduces low density lipoprotein (LDL) cholesterol
(Davy et al., 2002), body fat (Weickert et al., 2006), and coronary heart disease risk factors in
human clinical trials (Chang et al., 2013, Zhang et al., 2012); increases fasting PYY, GLP-1,
post-meal satiety, and insulin sensitivity (Huang et al., 2011); and elevates endothelium nitric
oxide production (Andersson and Hellstrand, 2012, Juvonen et al., 2009). Like resistant starch,
β-glucans are fermentable dietary fibers resistant to digestion and high-temperature cooking. An
association was detected between total fermentable phenolics, a reduction in triacylglycerols and
fewer aorta inflammatory lesions in mice (Brito et al., 2012).

Table 12 Composition of oats (Quaker® Oats)*

<table>
<thead>
<tr>
<th>Content</th>
<th>Quantity (%)</th>
<th>Content</th>
<th>Quantity (ppm)</th>
</tr>
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<tbody>
<tr>
<td>Beta-glucan</td>
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<td>5.4</td>
</tr>
<tr>
<td>Insoluble dietary fiber</td>
<td>7.3</td>
<td>Ave 2f</td>
<td>8.8</td>
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</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Decreased daf-2 signaling, the only homolog of the human insulin and insulin-like
growth factor (IGF) receptor gene in *C. elegans*, increases *C. elegans*’ lifespan in a DAF-
16/FOXO-dependent manner (Hertweck et al., 2004, Patel et al., 2008, Tissenbaum and Ruvkun,
1998), which is reversed by hyperglycemia (Abate and Blackwell, 2009). Furthermore, doubling
the *C. elegans sir-2.1* gene number increases lifespan by 50% in a daf-16-dependent manner
(Burnett et al., 2011, Tissenbaum and Guarente, 2001). We hypothesized that oat consumption
would reduce IFD, improve insulin resistance created by added glucose (2%) to the C. elegans feeding media, improve hyperglycemia-impaired lipid metabolism, and increase healthspan through daf-16 and/or sir-2.1 pathway(s). A number of genes in C. elegans have been proven to promote fat storage and mediate satiety, for example, guanylyl cyclases (GCYs) and the CCK receptor. The objectives of this study were to quantify IFD by the fluorescence intensity of Nile red staining, to evaluate healthspan by counting PPR, and to determine the genetic pathway(s) using daf-16 and/or sir-2.1 deficient mutants.

5.2 Methods and materials

C. elegans strains and their standard lab food source Escherichia coli (E. coli, OP50, Uracil auxotroph) were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). The C. elegans model does not require Institutional Animal Care and Use Committees (IACUC) regulation or approval (Zheng et al., 2013).

5.2.1 Culture of E. coli (OP50)

OP50 were cultured by the standard method described elsewhere (Zheng et al., 2010). Briefly, approximately 10µl of stock E. coli solution was added to media and incubated at 37°C for 24h. The OP50 were then sub-cultured in Petrifilm™ (3M Corporate, St. Paul, MN) at 37°C for 24h, and the colonies were confirmed to be at a density of 5×10⁸ – 5×10¹¹ cfu/ml and the C. elegans were allowed to eat ad libitum.

5.2.2 Culture of C. elegans

C. elegans wild type (N2), and null mutants sir-2.1(ok434)IV, daf-16(mgDf50)I, and daf-16(mgDf50)I;daf-2(m65)III were used in this study (Table 1). They were grown on Nematode Growth Media (NGM) agar plates (⌀35mm), and stored in a low-temperature incubator (Revco Tech., Nashville, NC) at 20°C or 15°C (daf-16(mgDf50)I;daf-2(m65)III). C. elegans were age-
synchronized. Mature gravid *C. elegans* were transferred individually onto the agar plates and treated with a NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) solution to dissolve the body and release viable eggs (Zheng et al., 2010). One day prior to the experiment, 200µl of a feeding media containing *E. coli* was added to the agar dish.

5.2.3 Diet composition

Oats (Quaker® Oats instant flakes, PepsiCo Inc., Chicago, IL, Table 12) were powdered using a centrifugal mill with a 0.75mm sieve (Retsch ZM 200; Haan, Germany), autoclaved at 121°C, and suspended in distilled deionized water (DDH2O, 5% w/v).

Control *C. elegans* were fed with OP50 only. The experimental groups were fed OP50 supplemented with 0.5%, 1.0%, or 3.0% oats at the larval stage L2 (Table 13). Two percent glucose was added to an additional group of each strain with the oats. The dietary nutrient composition is listed in Table 14 (Feijo Delgado et al., 2013). *C. elegans* were transferred to fresh dishes every other day receiving treatment (50µl).

Table 13 Oats treatment composition

<table>
<thead>
<tr>
<th>Volume (50µl)</th>
<th>Dosage (%)</th>
<th>Without glucose</th>
<th>With glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Dose (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>OP50 (2×10⁹ cfu/ml, µl)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Oats (5%, µl)</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Glucose (50%, µl)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 14 Diet ingredient composition

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Oat diet (mg/ml)</th>
<th>OP50 (mg/ml)</th>
<th>Oat treatment (mg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.5%</td>
</tr>
<tr>
<td>Beta-glucan</td>
<td>0.046</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>Insoluble dietary fiber</td>
<td>0.073</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>0.609</td>
<td>7.5</td>
<td>0.375</td>
</tr>
<tr>
<td>Protein</td>
<td>0.130</td>
<td>37.5</td>
<td>1.875</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.066</td>
<td>5.0</td>
<td>0.250</td>
</tr>
</tbody>
</table>
5.2.4 Pharyngeal movement (pumping rate, PPR)

*Caenorhabditis elegans* were examined periodically using a stereomicroscope (Nikon SMZ1500, Melville, NY) with transmitted light. The PPR was recorded manually by independent observers then were returned to the incubators (Finley et al., 2013, Zheng et al., 2014).

5.2.5 Fluorescence microscopy

Lipophilic dye, Nile red, was used to stain for intestinal fat deposition (IFD) (Zheng et al., 2010). S-basal solution was added to the dish to wash the *C. elegans*. The solution containing the *C. elegans* was centrifuged for 20s at 805g and this procedure was repeated twice. *C. elegans* were then fixed with 4% paraformaldehyde over 2h at 4°C and washed with PBS for 5min x 3. Nile red (50µl) was applied to the specimens for 10min. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 20µl of the medium containing Nile red stained *C. elegans*. A cover glass was mounted on the glass slide, and the slides were viewed with an epifluorescence microscope (Nikon Eclipse, Ti) equipped with a Texas Red filter. Fluorescent micrographs were taken with a digital camera (Andor, DU-885k) and were analyzed using Nikon-Elements (version 3.22.11). Optical densities (arbitrary units, % of control) of Nile red labeled IFD were determined for adult *C. elegans* (larvae stage 4).

5.2.6 Reagents

Trizol® reagent (T9424), chloroform (C2432), and isopropanol (I9516) were purchased from Sigma-Aldrich (St. Louis, MO). Taqman PCR core kit (N8080228), MuLV Reverse
Transcriptase (N8080018), and Ribonuclease (RNase) Inhibitor (N8080119) were obtained from Life Technologies (Grand Island, NY). RNase free micro tubes and pipette tips were used in RNA isolation and quantitative RT-PCR.

5.2.7 RNA isolation

Total RNA was extracted using the Trizol® reagent as described elsewhere [66]. *C.elegans* samples were homogenized in 1ml Trizol® Reagent per 50-100 mg of tissue. Samples went through 5 freeze-thaw cycles in which they were frozen in liquid nitrogen and thawed in a 37°C water bath. The homogenized samples were vortexed at room temperature to allow the complete dissociation of nucleoprotein complexes. Two hundred microliters of cold pure chloroform was added and the samples were vortexed for 15s and incubated at room temperature for 5min. The samples were centrifuged at 15,616G for 10 min at 4°C, and the top aqueous phase was transferred to a fresh tube. A 600µl cold isopropanol was used for the initial homogenization, and the samples were incubated at room temperature for 10min, vortexed for 15s, and centrifuged at 15,616G for 10min at 4°C. The supernatant was removed and the RNA pellet was washed once with 1ml cold 75% ethanol. The sample was vortexed and centrifuged at 6,100G for 5min at 4°C. The supernatant was removed completely and the RNA pellet was briefly air-dried. The RNA pellet was re-suspended in 0.1% diethyl pyrococarbonate (DEPC)-treated water and stored at -80°C. The RNA concentration was analyzed with a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE).

5.2.8 Quantitative RT-PCR

The mRNA levels of *ckr-1*, *gcy-8*, *cpt-1*, and *cpt-2* were determined using Taqman® quantitative RT-PCR. The following components were added in one-step RT-PCR: 2.2µl H₂O, 1µl 10× Taqman® buffer A, 2.2µl 25mM MgCl₂ solution, 0.3µl 10mM dATP, 0.3µl 10mM
dCTP, 0.3µl 10mM dGTP, 0.3µl 10mM dUTP, 0.05µl 20U/µl RNase inhibitor, 0.05µl 50U/µl MuLV reverse transcriptase, 0.05µl 5U/µl AmpliTaq gold®, 0.25µl Taqman® probe and primers, and 3µl, 5ng/µl RNA. The PCR reaction was conducted in triplicate using a Taqman® probe and primer set (Life Technologies, Grand Island, NY) for Cholecystokinin receptor homolog (ckr-1, Ce02408606_m1), guanylyl cyclase-8 (gcy-8, Ce02456184_g1), carnitine palmitoyltranferase-1 (cpt-1, Ce02440434_m1), and carnitine palmitoyltranferase-2 (cpt-2, Ce02459919_g1). The mRNA signal was normalized over a eukaryotic 18S rRNA (Hs99999901_s1) internal control. The reaction was conducted using a 7900 HT Fast real-time PCR system (Life Technologies). Reverse transcription was added at 48°C for 30min, and AmpliTaq gold® activation (denaturation) was performed at 95°C for 10min. Amplification of the DNA involved 40 cycles of 15s at 95°C and 1min at 60°C. Data were analyzed using the Sequence Detector Software (Life Technologies, Carlsbad, CA, USA). The relative quantification of gene expression (2^{-ΔΔCt}) was calculated.

5.2.9 Statistics

All data were presented as means ± SEM. Student t-tests and ANOVA were used for IFD, and ANCOVA was used to compare the slopes of PPR data (SAS 9.4). Statistical significance was set at P≤0.05. Principal component analyses (PCA) was performed to cluster the factors of the oats results (SAS 9.4). Power analyses were performed to predict the sample size to achieve 80% power for PPR (minimum n=7) and IFD (n=5) with statistical significance as 0.05.

5.3 Results

5.3.1 Intestinal fat deposition (IFD)
Oat feeding reduced IFD by 30% (0.5% and 1.0%) in the N2 strain (P<0.05), and the addition of glucose to the oats increased IFD. IFD decreased by 17% in response to 0.5% oats and 37% in response to 1.0% oats in the *daf-16(mgDf50)I*, and increased with glucose treatment compared to the group that did not receive glucose (P<0.05). The *daf-16(mgDf50)I;daf-2(m65) III* showed a similar dose-dependent reduction in IFD in response to oat feeding as well as in the presence of glucose (P<0.05). IFD was mildly lower following oat (0.5% and 1.0%) consumption in the *sir-2.1(OK434)IV* (P>0.05) (Figure 20).

Figure 20  Nile Red staining of the intestinal fat deposition in *C. elegans* post Oat feeding in the absence or presence of 2% glucose.
5.3.2 Pharyngeal pumping rate (PPR)

The PPRs declined in all groups as the *C. elegans* aged. The oat treatment (0.5%, 1.0%, or 3%) increased the PPR in N2, *daf-16*, and *sir-2.1* mutants (n=24 *C. elegans*/3 dishes, P<0.001-0.05). The PPR in N2 was increased in oat group (0.5%, 1.0%, or 3%) in presence of glucose (P<0.005-0.03). This increase persisted in the presence of glucose at a low dose in *daf-16* or *daf-16/daf-2* mutant (P<0.05) (Figure 21).

Figure 21  Oat feeding affected pharyngeal pumping rate, a surrogate marker of lifespan in *C. elegans*. 

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5.3.3 Lipid metabolism gene expression

Oat feeding (0.5% and 1%) increased the mRNA expression of ckr-1, gcy-8, cpt-1, and cpt-2 in the *C. elegans* N2, and sir-2.1 and daf-16 deficient strains (P<0.01). Additional glucose further increased these mRNA expressions in N2 and sir-2.1. In the daf-16 deficient strain, ckr-1 and gcy-8 were increased after oat feeding (P<0.01). All four genes tested were elevated in response to glucose treatment alone, and oat consumption in the presence of glucose significantly reduced mRNA expression of the four genes (P<0.01). Oat feeding with glucose reduced mRNA expression of all four genes in daf-16/daf-2 deficient strains (P<0.01). Oats (0.5%) plus glucose increased cpt-1 and cpt-2 (P<0.01, Figure 22).
5.3.4 Principal component analysis (PCA)

PCA analyses revealed two theoretical strong factors that played important roles in the relationship of the components that were tested in this study (Figure 23, Table 15). Inverse relationship between the IFD and the PPR was detected in N2, daf-16/daf-2 and sir-2.1 mutants. The PPRs were strongly and positively related to the two factors in the three strains. By contrast,
the IFDs were strongly and positively related to the factor 1 in the N2 and the daf-16/daf-2 double mutant, which was negatively associated in the sir-2.1 mutant. The IFD or PPR seemed weakly related to the ckr-1, cpt-1, cpt-2, and gcy-8. The ckr-1, cpt-1, cpt-2, and gcy-8 had strong positive relationships with factor 1 N2, daf-16/daf-2 and sir-2.1 mutants. A loose but positive relationship of the 4 genes with the factor 2 was seen in the daf-16 mutant, while the IFD was negatively related to the factor 1 and the PPR did not have any association with either the factors or the 4 genes.

Table 15 PCA description: factor 1 and factor 2

<table>
<thead>
<tr>
<th>Relation to factors</th>
<th>Factor 1</th>
<th>( \text{Factor 2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPR</td>
<td>IFD</td>
</tr>
<tr>
<td>N2</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>daf-16(mgDf50)I</td>
<td>-20</td>
<td>-15</td>
</tr>
<tr>
<td>daf-16(mgDf50)I;</td>
<td>-24</td>
<td>64</td>
</tr>
<tr>
<td>sir-2.1(ok434)IV</td>
<td>-17</td>
<td>-34</td>
</tr>
</tbody>
</table>

5.4 Discussion

The effect of oat consumption paired with or without 2% glucose was evaluated in the C. elegans model by observing changes in IFD determined by the fluorescent intensity of Nile red, alterations in healthspan indicated by PPR, and variations in selected mRNA expression indicated by PCR in this study. The IFD was reduced in N2, and the daf-16 and daf-16/daf-2 deficient mutants. Apart from natural age-related decline of the PPR in all groups, oat treatment sustained the PPR in N2 with or without glucose. Oat treatment also sustained the PPR in daf-16 and sir-2.1 mutants without glucose. Low dose oat treatment (0.5%) increased the PPR in the
presence of glucose. Oat consumption increased mRNA expression of ckr-1, gcy-8, cpt-1, and cpt-2 genes, which were greater in N2 than in the sir-2.1 mutant, and additional glucose further increased the expression by 1.5-fold in both strains. The effects of oats on healthspan and IFD in N2 was mediated by daf-2 and partially depended on daf-16, with and without the presence of hyperglycemia.

Figure 23 Scatterplot of principle component analysis (PCA) showing the two strong factors in relationships with ckr-1, cpt-1, cpt-2, gcy-8, PPR slope, and IFD.

Hyperglycemia often coexists with hyperlipidemia. In C. elegans, inactivation of over
300 genes has been shown to reduce IFD, and inactivation of more than 100 genes to increase fat storage (Ashrafi et al., 2003). Oat consumption significantly reduced IFD in the N2, daf-16, and daf-16/daf-2 deficient mutants, whereas the presence of glucose increased IFD. However, oats reduced IFD even when there was insulin resistance, as seen in the daf-16/daf-2 deficient group and other groups with additional glucose. Glucose did not attenuate the oat-induced IFD reduction in the daf-16/daf-2 deficient mutant suggesting an indirect improvement in insulin sensitivity through lipid metabolism activating other pathway(s), such as the serotonin pathway. In rodent diabetes, hyperglycemia is associated with an increase in peroxisome proliferator activator alpha (PPARα) activity associated with insulin-resistance (Hostetler et al., 2008). In a C. elegans model, deletion of the nuclear hormone receptor-49 (nhr-49), a homologue of PPARα, prevents hyperlipidemia and restores insulin-sensitivity in a manner similar to PPARα null mice (Atherton et al., 2008). The reduced IFD following oat consumption in the daf-16/daf-2 deficient mutant suggests that oats may improve hyperglycemia-impaired lipid metabolism.

Reduced food intake, reduced lipid metabolism, and a reduction in metabolic turn over in N2 are associated with an extension of lifespan (Burnett et al., 2011). Unlike the N2, or daf-16/daf-2 and daf-16 deficient mutants, the IFD was only mildly reduced by the same amount of oat consumption in the sir-2.1 deficient mutant with increased mRNA expression of the four genes similar to N2 suggesting increased lipid metabolism. The ability of both the wild type and mutant nematodes to sustain the PPR indicates that oat consumption improved healthspan independent of the sir-2.1 pathway which relates to the co-regulator/genes in the aging process in C. elegans (Houthoofd et al., 2003, Kyrylenko et al., 2003). Our data is in agreement with the literature that shows involvement of sir-2.1 in lipid metabolism has multiple factors similar to lifespan (Burnett et al., 2011), and either a higher dose is required in the absence of the sir-2.1
gene pathway or the \textit{sir-2.1} pathway was not critically involved in the IFD reduction.

Hyperglycemia reduced the \textit{C. elegans} lifespan (Lee et al., 2009) and completely suppressed the long lifespan of \textit{daf-2(-)} insulin/IGF-1 receptor mutants in \textit{C. elegans} (Ogden et al., 2006). The \textit{daf-16} gene plays a central role in activating the downstream insulin signaling pathways and is the major target of the \textit{daf-2} which negatively regulates \textit{daf-16} (Kenyon et al., 1993, Lee et al., 2009). In the present study, oat feeding increased PPR and decreased IFD which were mediated by the \textit{daf-16/daf-2} pathways. The benefit from oats’ may be attributed to its being high in fibers, \(\beta\)-glucan, proteins, and unique avens.

Although activation of the CCK receptor does not change circulating triacylglycerol or adipose tissue, it increases glucose tolerance and insulin sensitivity, and decreases liver triacylglycerol in mice (Irwin et al., 2013, Kissileff et al., 1981). Similarly in humans, activation of CCK pathway does not appear to play a central role in long-term energy balance but signals postprandial satiety (Jordan et al., 2008, Kissileff et al., 1981). However, \textit{ckr-1}, the analogous gene in the \textit{C. elegans} species, increased in response to oat consumption which decreased IFD in the presence of glucose and preserved PPR, suggesting \textit{ckr-1} has potential role in the lipid metabolism. The \textit{gcy-8} is exclusively expressed in AFD neurons and contributes to thermotaxic behavior (Inada et al., 2006). Increased expression of \textit{gcy-8} may indicate an increase in metabolic rate. In the \textit{daf-16} deficient mutant, the mRNA expression of \textit{ckr-1} and \textit{gcy-8} were increased in response to oat feeding. This effect was reversed in presence of glucose that initially elevated \textit{cpt-1} and \textit{cpt-2} followed by a reduction when oats were added.

An elevated \textit{cpt-1} or \textit{cpt-2} suggests an augmented lipid beta-oxidation and turn-over. Expression of all four genes was reduced in the \textit{daf-16/daf-2} deficient mutant when treated with oats, and \textit{cpt-1} and \textit{cpt-2} expression increased nearly 2-fold with additional glucose. The
reduced \textit{cpt-1} or \textit{cpt-2} in \textit{daf-16/daf-2} deficient mutant suggests that the \textit{C. elegans} were either unable to utilize/metabolize the nutrients from the food as seen in \textit{daf-16} deficient mutant, or were unable to adequately down-regulate metabolic turnover as observed in the \textit{daf-16/daf-2} deficient mutant, thus reducing IFD. In the three strains N2, \textit{daf-16/daf-2} and \textit{sir-2.1} mutants, the factor 2 of the PCA was closely related to healthspan, which is inversely related to IFD, and the factor 1 had strong positive relationships with \textit{cpt-2}, and \textit{gcy-8}. The PCA data showed an inverse relationship of the \textit{daf-16} to the IFD, \textit{cpt-1}, and \textit{cpt-2}.

The exclusive and unique avns in oats are fermentable polyphenols that also resist digestion and provide health benefits. A high β-glucan concentration increases viscosity in the GI tract, delays nutrient absorption, decreases the glycemic peak by 50%, lowers LDL cholesterol, reduces body fat in humans and rodents (Wursch and Pi-Sunyer, 1997), counteracts obesity-related inflammation (Meydani, 2009), and enhances immunity in \textit{C. elegans} by activating the dectin-1 receptor and DAF-2/insulin-like receptor (ILR) pathways (Engelmann and Pujol, 2011, Tsoni and Brown, 2008). These avns, along with the β-glucan content of oats make oats a unique food that may aid in lowering body fat and controlling body weight. Future studies will investigate possible synergistic effects of avns and β-glucan, as well as the role of the serotonin pathway in improving hyperglycemia-induced hyperlipidemia and increasing healthspan.

This study had limitations as well. \textit{C. elegans} have 2/3 of genes that are related human diseases. Thus, \textit{C. elegans} is utilized as a stage for use in high-through put screening to search for nutritional interventions for health prior to confirmatory studies in higher animal models and human clinical trials. Compensatory pathways that exist in higher animals may be not discovered by the present study using \textit{C. elegans}. Active components of the oats extract will be
further tested in follow up studies using higher animal species.

In conclusion, our data indicates that oats have unique properties which induce an improvement in insulin sensitivity, reduce intestinal fat deposition, and extend healthspan in the *C. elegans* model organism giving support to our study hypothesis. As part of a healthy diet oats are an appropriate food to improve public health. Avns and β-glucan are functional food components of oats that can be incorporated into the daily diet in various ways. *C. elegans* is an attractive *in vivo* animal model for initial studies of nutrition interventions prior to confirmation in higher animal species.
5.5 References


28. IRWIN, N., FRIZELLE, P., O'HARTE, F. P. & FLATT, P. R. 2013. Metabolic effects of activation of CCK receptor signaling pathways by twice-daily administration of the


6.1 Introduction

Obesity are affecting more than one-third of the adults and 17% of the youth in the U.S. (Popkin et al., 2012). Obesity are related with chronic diseases including type II diabetes, cardiovascular diseases, high blood pressure, high cholesterol, fatty liver disease, atherosclerosis, hypertension, sleep apneas, and certain types of cancers (Hotamisligil et al., 1994). Obesity related chronic diseases increasingly endanger the public health and costs more than $140 billion to the U.S health care system.

Obesity is still a non-cure epidemic disease. Although anti-obesity drugs have been approved by FDA and exist in the market, the side effects of these drugs and the limited weight loss have narrowed their usage. For example, sibutramine resulted in a weight loss of 4.5 kg after 1 year treatment compared with placebo (Arterburn et al., 2004). Sibutramine may cause blood pressure increase, dizziness or blurred vision; back pain, dry mouth, headache, nausea, and nervousness (Snow et al., 2005, Bray and Greenway, 2007, Greenway and Bray, 2010). Surgical treatments are used for excessive obese patients but high cost, side effects, and weight regain have restricted the utilization of surgery. Surgery also has potential dangers to cause mortality. For example, early mortality rates for Roux-en-Y gastric bypass (RYGB) range from 0.3% to 1% (Snow et al., 2005). Safe and effective obesity treatments are in a growing demand. Fermentable dietary fiber, RS, presents an emerging solution to obesity.

The consumption of cereals decreased dramatically in the “Western” style diet in the past decades (Adam et al., 2001). The decreased dietary fiber intake is related with the decreasing consumption of cereals which may be one of the reasons contributing to the development of
health disorders (Delzenne and Cani, 2005). The dietary guidelines claim that at least half of recommended total grain intake should be whole grains (USDA, 2005). However, less than 5 percent of Americans consume the minimum recommended amount of whole grains, which for many is about 3 ounce-equivalents per day. On average, Americans eat less than 1 ounce-equivalent of whole grains per day (USDA, 2010). The consumption of higher amounts of dietary fiber such as whole-grain foods lower the prevalence of obesity, coronary heart disease, type II diabetes, and certain cancers (Anderson, 2004, Hauner et al., 2012).

Wheat bran by-product (WBP) is the byproduct from milled refined grains production. Although refined grains has extended shelf life and a finer texture, refined grain foods contain higher solid fats, added sugars, and sodium. WBP is high in non-digestible form of carbohydrates. WBP is also high in vitamins, minerals, and dietary fiber while the refining grains are low in these essential nutrients (USDA, 2010) but low in saturated fat, trans-fat, mono- and oligo- saccharides, calories. WBP can be used to make many food products, such as cookies, bread, cereal, pasta, and cereal bar. Bran, though not a whole grain, is an excellent source of dietary fiber. Therefore, WBP can supply a good source of essential nutrient, especially dietary fiber.

The purpose of this study is to investigate the effect of WBP on health or lifespan and fat metabolism in *C. elegans* model. Pharyngeal pumping rate (PPR) as a surrogate marker of *C. elegans* lifespan (Finley et al., 2013, Kang et al., 2007, Chow et al., 2006) was also monitored for the effect of WBP. Nile red, a lipophilic dye, stained intestinal fat deposition (IFD) was also performed by fluorescence microscope. *C. elegans* is a small, free-living soil nematode, multicellular eukaryotic organism distributed widely around the world. *C. elegans* is the first animal to have its genome completely sequenced and conserves 65% of the genes associated
with human disease (Shen et al., 2009). In *C. elegans*, 305 genes are responsible for reducing body fat and 112 genes are responsible for increasing fat storage (Ashrafi et al., 2003). Compared with a rodent model, small size, short lifespan, quick turn over, complete genetic information and their easy maintenance in the laboratory make *C. elegans* a valuable animal model while greatly reducing the time and costs required to obtain answers to research questions (Zheng and Greenway, 2011). The nematode *C. elegans* has been used extensively as a model organism for biological, neurobiology and behavioral biology research (Kuriyama and Makita, 1982). We can learn the effect of WBP on lifespan and fat deposition in *C. elegans* and directly apply to more complex organisms.

6.2 Materials and methods

*C. elegans* strains and the standard laboratory food source *Escherichia coli* (*E. coli*, OP50, *Uracil auxotroph*) were obtained from *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, MN).

6.2.1 Culture of *E. coli*, OP50

*E. coli* OP50 were cultured by the standard method described elsewhere (Zheng et al., 2010). Briefly, 10 µl of stock *E. coli* solution was added into LB broth solution containing 0.2% streptomycin and incubate at 37°C shake incubator overnight and was stored at 4 °C. The *E. coli* OP50 was then sub-cultured in Petrifilm™ *E.coli*/*Coliform Count Plates* (3M Corp., St. Paul, MN) at 37°C for 24h to confirm the concentration of *E. coli* OP50. Densities of 5 × 10⁸ – 5 × 10¹¹ cfu/mL were selected. The animals were allowed to eat *ad libitum*.

6.2.2 Culture of *C. elegans*

*C. elegans* wild type (*N2*), and null mutants *sir-2.1(ok434)IV, daf-16(mgDf50)I* and *daf-16(mgDf50)I;daf-2(m65) III*, were used in this study. *C. elegans* was cultured on Standard
Nematode Growth Medium (NGM) Plates (Ø35mm) at 20°C or 15°C low temperature incubators. *C. elegans* was chunked to new plates seeded with OP50 each week. In order to obtain uniform aged *C. elegans* to reduce the variation in the experimental results, the mature gravid *C. elegans* was collected from a NGM agar plate with 1ml deionized water into a 1.5ml micro centrifuge tube. *C. elegans* was treated with NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) lysis solution to dissolve the *C. elegans* body and release viable eggs. The eggs were allowed to hatch overnight with gentle rocking (Fisher scientific, Waltham, MA) at room temperature. The growth of the larvae was halted at the L1 stage and spread onto OP50 pre-seeded plates.

6.2.3 Wheat bran (WBP) Preparation

WBP was supplied by Ardent Milles Corporate (Denver, CO). WBP was milled by Retsch® ZM200 (Newtown, PA) and was sieved through a 80μm sieve, and then was incorporated to the standard feeding medium as described by Zheng et al. (Zheng et al., 2010). Control animals were fed with *E. coli* OP50 only. The experimental groups were fed with additional WBP (containing 0.5%, 1.0%, or 3.0% WBP) in absence or presence of 2% glucose. *C. elegans* was transferred to a new agar plate pre-seeded with OP50 and treatments with a worm picker every other day under stereo microscope (Nikon, Melville, NY).

6.2.4 Pharyngeal pumping rate (PPR)

Digital images were taken with Retiga 4000R. The locomotion was monitored daily with ImagePro Plus (Media Cybernetics, Inc., Bethesda, MD). The pharyngeal pumping rate (PPR), a surrogate marker of lifespan, was counted every other day under a stereo microscope Nikon SMZ-1500 (Melville, NY).

6.2.5 Nile red staining for intestinal fat deposition (IFD)
Lipophilic dye, Nile red (Ashrafi et al., 2003, Yen et al., 2010), was used to stain for intestinal fat deposition (IFD). Prior to fixing the animals, the animals grown on the NGM agar plate was washed with S-Basal buffer (1ml/plate). The solution containing the animals was centrifuged at 2200×g for 20 seconds and this procedure was repeated twice. The animals were then fixed with 4% paraformaldehyde solution for 30 minutes at 4°C and washed with PBS for 5min ×3. Nile red solution (50µl) was added to the specimens for 30 minutes. Ten microliters of fluoromount-G solution (Southern Biotechnology Associates, Birmingham, AL) was applied on a glass slide. Ten microliters of the specimen containing Nile Red stained C. elegans was applied on to the glass slide. Then, a cover glass was mounted on the glass slide. The sample slides were viewed with a Nikon Eclipse Ti fluorescence microscope (Nikon Instrument, Melville, NY) equipped with a Texas Red filter. Fluorescent micrographs were taken with a digital camera (Andor, DU-885K). The micrographs were analyzed using NIS-elements AR3.22.11 software (Nikon Instrument, Melville, NY). Line profiles of the optical densities of Nile Red labeled IFD (arbitrary units, % of control) were determined for each group.

6.2.6 Reagents for qRT-PCR

The Trizol® reagent (T9424), chloroform (C2432), and isopropanol (I9516) were purchased from Sigma-Aldrich (St. Louis, MO). Taqman PCR core kit (N8080228), MuLV Reverse Transcriptase (N8080018), and RNase Inhibitor (N8080119) were obtained from Life Technologies (Grand Island, NY). RNase free micro tubes and pipette tips were used in RNA extraction and quantitative RT-PCR.

6.2.7 RNA extraction

The total RNA was extracted using the Trizol® reagent as described elsewhere (Ye et al., 2007). Briefly, 50-100 mg of C.elegans tissue was homogenized in 1ml Trizol® Reagent. The
samples went through 5 freeze-thaw cycles repeating frozen in liquid nitrogen and thawed in a 37°C water bath. The homogenized samples were vortexed at room temperature to allow the complete dissociation of nucleoprotein complexes. Cold pure chloroform (200µl) per 1ml of Trizol® reagent was added and the samples were vortexed for 15s and incubated at room temperature for 5min. The samples were centrifuged at 12,000×g for 10 min at 4°C, and the top aqueous phase was transferred to a fresh tube. A 600µl aliquot of cold isopropanol per 1ml of Trizol® reagent was used for the initial homogenization, and the samples were incubated at room temperature for 10min, vortexed for 15s, and centrifuged at 12,000×g for 10min at 4°C. The supernatant was removed and the RNA pellet was washed once with 1ml cold 75% ethanol. The sample was vortexed and centrifuged at 7,500×g for 5min at 4°C. The supernatant was removed completely and the RNA pellet was briefly air-dried. The RNA pellet was re-suspended in 0.1% diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C. The RNA concentration was analyzed with a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE).

6.2.8 Quantitative RT-PCR

The total RNA levels of ckr-1, gcy-8, cpt-1, and cpt-2 were determined using Taqman quantitative RT-PCR. All experiments were conducted at least three times. The PCR reaction was conducted in triplicates using Taqman probe and primers set for ckr-1(Ce02408606_m1), gcy-8(Ce02456184_g1), cpt-1(Ce02440434_m1), and cpt-2(Ce02459919_g1) from Life Technologies (Grand Island, NY). The mRNA signal was normalized over eukaryotic 18S rRNA (4331182) internal control from Life Technologies (Grand Island, NY). The reaction was conducted with a 7900 HT Fast real-time PCR system (Life Technologies, Grand Island, NY). Reverse transcription was done at 48°C for 30 minutes; AmpliTaq gold activate was carried out at 95°C for 10 minutes; Amplification conditions are: 40 cycles of 15 s at 95°C and 1 minutes at
60°C. Post-PCR data analysis was performed using the Sequence Detector Software (Applied Biosystems). A mean value of the duplicates was used for the analysis of relative mRNA level of ckr-1, gcy-8, cpt-1, and cpt-2.

6.2.9 Statistical Analyses

All results were expressed as mean±sem. The data for the pharyngeal pumping rate were analyzed with ANCOVA method by SAS 9.4. The data for IFD and qRT-PCR were analyzed by ANOVA with Dunnett’s test to compare the effects of the treatments (SAS 9.4). Statistical significance was set as P≤0.05. Principle component analyses (PCA) was performed to cluster the factors of the oats results (SAS 9.4).

6.3 Results

6.3.1 Intestinal fat deposition (IFD)

WBP feeding at 0.5%, 1% and 3% reduced IFD by 27%, 17.5%, and 28%, respectively in the N2 strain (P<0.05), and in the presence of additional 2% glucose to the WBP also decreased IFD by 8.7%, 20%, and 22.8%. IFD decreased by 30%, 26%, and 30% in response to 0.5%, 1%, and 3% WBP feeding in the daf-16(mgDf50)I in the absence of glucose (P<0.05). The daf-16(mgDf50)I;daf-2(m65)III strain showed a similar dose-dependent reduction in IFD in response to WBP treatment. In the presence of glucose, WBP feeding did not significantly affect the IFD in the daf-16(mgDf50)I and daf-16(mgDf50)I;daf-2(m65) III mutants. In the sir-2.1(ok434)IV mutant, the IFD was mildly lowered by WBP feeding (0.5%, 1%, and 3%, P>0.05) and increased by 8% and 26% by WBP (1% and 3%, P<0.05) in the presence of glucose (Figure 24).

6.3.2 Pharyngeal pumping rate (PPR)
PPR is a surrogate maker for lifespan and is declined in all groups as the animal aged. The WBP treatment (0.5%, 1%, or 3%) increased the PPR in N2, daf-16(mgDf50)I, and sir-2.1(ok434)IV mutants (n = 18 animals/3 dishes, P<0.05). In the daf-16(mgDf50)I; daf-2(m65) III mutant, the PPR was not significantly mediated by WBP treatment (n = 18 animals/3 dishes). In the presence of glucose, the PPR was also persisted by 3% WBP feeding in the daf-16(mgDf50)I, by 0.5%, 1%, and 3% PWB in sir-2.1(ok434)IV mutants (n = 18 animals/3 dishes, P<0.05) (Figure 25).
Figure 24 The Nile Red stained fluorescence intensity of the *C. elegans* intestinal fat deposition.
Figure 25 Pharyngeal movement was sustained by the WBP treatment in *C. elegans*.

6.3.3 Lipid metabolism gene expression

WBP feeding at 0.5%, 1% and 3% did not significantly affect the mRNA expression of *ckr-1, gcy-8, cpt-1*, and *cpt-2* in the wild type *C. elegans* (N2) in the absence of glucose (P>0.05).
WBP feeding (3%) increased the mRNA expression of ckr-1, gcy-8, cpt-1, and cpt-2 in the daf-16(mgDf50)I mutant (P<0.05). The four mRNA expression was increased by 1% and 3% WBP treatments in the daf-16(mgDf50)I;daf-2(m65) III mutant (P<0.05). In the sir-2.1(ok434)IV mutant, 1% WBP feeding slightly increased these four mRNA expression in the absence of glucose (P>0.05). In the presence of glucose, 0.5% and 1% WBP increased these four mRNA expression in N2 (P<0.05). In the daf-16(mgDf50)I mutant, 1% WBP feeding increased mRNA ckr-1, gcy-8, and cpt-2 expression (P<0.05). The WBP treatment did not have significantly effect on the four mRNA expression in the daf-16(mgDf50)I;daf-2(m65) III and sir-2.1(ok434)IV mutants (Figure 26).

6.3.4 Principal component analysis (PCA)

Two factors were utilized in PCA test to determine the relationship of IFD, PPR, ckr-1, gcy-8, cpt-1, and cpt-2 (Figure 27). Indirect relationship between the IFD and the PPR was detected in N2, daf-16, and daf-16/daf-2 deficient mutants. The PPRs were strongly and positively related to the factor 1 in the three strains. By contrast, the IFDs were strongly and negatively related to the factor 1 in the N2 and the daf-16/daf-2 double mutant, while it was weakly associated with factor 1 in the daf-16 and sir-2.1 mutant. The cpt-1 and cpt-2 have strong positive relation with PPR and strong negative relation with IFD while ckr-1 and gcy-8 are weakly associated with PPR and IFD. The ckr-1, cpt-1, cpt-2, and gcy-8 had strong positive relationships with factor 1 in daf-16 deficient mutants. In daf-16/daf-2 double mutant, gcy-8 has strong positive relation with PPR and strong negative relation with IFD, but another three, ckr-1, cpt-1, and cpt-2 are weakly related to PPR and IFD. In sir-2.1 deficient mutant, the IFD was weakly related to PPR while ckr-1, gcy-8, and cpt-1 strongly and negatively related with PPR.
6.4 Discussion

The effect of WBP consumption was indicated by the fluorescence intensity of Nile Red staining intestinal fat deposition (IFD), alterations in healthspan indicated by PPR, and variations in selected mRNA expression determined by qRT-PCR in four strains of *C. elegans* model with or without 2% glucose. The IFD was reduced in N2, and the *daf-16* and *daf-16/daf-2* deficient mutants. The PPR declined with the age process of *C. elegans*, but the WBP treatment sustained the PPR in N2, *daf-16*, and *sir-2.1* deficient mutants without glucose. The PPR was also persisted by 3% WBP in *daf-16* deficient mutant and WBP (0.5, 1, and 3%) in *sir-2.1* mutant.
WBP feeding slightly increased the mRNA expression of ckr-1, gcy-8, cpt-1, and cpt-2 genes in N2 in the absence of glucose, but it significantly increased these four genes expression with additional glucose. In the daf-16 and daf-16/daf-2 deficient mutants, these four mRNA expressions were up-regulated by WBP consumption.

Figure 27 Principal component analysis

Wheat bran as a byproduct of the milling industry has been demonstrated as an excellent source of dietary fiber (Pruckler et al., 2015). Wheat bran rich diet that has a relatively low glycemic-index (GI) and high fiber content has been associated with low appetite, high satiety response, and reduced the risk of developing type 2 diabetes (Silva et al., 2015). Our data indicate that WBP consumption has positive effect on the healthspan or lifespan in N2 but this
effect was not mediated by *daf-16* and partially mediated by *daf-2*. In the presence of glucose, the effect of WBP consumption on IFD has direct relationship with *daf-16* which is one of the key factors in the insulin signaling pathway. The effects of WBP consumption on IFD were mediated by *sir-2.1* gene which is a critical factor in the aging of *C. elegans* and other animal models.

*C. elegans* is the first animal to have its genome completely sequenced and more than 65% of the genes relating to human disease are conserved in *C. elegans* (Shen et al., 2009). In *C. elegans*, 305 genes are responsible for reducing body fat and 112 genes are responsible for increasing fat storage (Ashrafi et al., 2003). Hyperglycemia often coexists with hyperlipidemia. *daf-16*, *daf-2*, and *age-1* are important factors in the insulin signaling pathway in *C. elegans* (Zheng and Greenway, 2012). The decrease in the intestinal fat deposition of *C. elegans* is directly indicated by the reduction of fluorescence intensity of Nile red staining which linearly correlated with the amount of body fat with a correlation coefficient of 0.998 in a previous publication (Chen et al., 2009). The reduction of fluorescence intensity of intestinal fat deposition was likely a result of a decrease in energy expenditure and muscular activity. The current study focused on the ability of fermentable fiber and RS rich WBP to reduce IFD. WBP consumption significantly reduced IFD in N2, *daf-16*, and *daf-16/daf-2* deficient mutants, whereas the presence of glucose slightly increased IFD in *daf-16* and *daf-16/daf-2* deficient mutants. WBP reduced IFD even when there was insulin resistant as seen in N2 with additional glucose. However, glucose attenuated the WBP-induced IFD reduction in the *daf-16* and *daf-16/daf-2* deficient mutants that demonstrated that insulin signaling pathway is directly related to the lipid metabolism. Hyperglycemia is responsible for an increase in peroxisome proliferator activator alpha (PPARα) activity associated with insulin-resistance (Hostetler et al., 2008).
reduced IFD induced by WBP consumption in the *daf-16/daf-2* deficient mutant suggests that WBP may improve hyperglycemia-impaired lipid metabolism.

*daf-16, age-1, daf-2*, and serotonin receptors are related in regulating lifespan in *C. elegans* and lipid metabolism plays an important role in longevity. The PPR as a surrogate lifespan is directly proportional to the lifespan of *C. elegans*. A previous study showed that legumes that are rich in resistant starch and dietary fibers decreased IFD and increased PPR (Finley et al., 2013).

Resistant starch or Short-chain fatty acids reduce IFD in *C. elegans* (Zheng et al., 2010), increased satiety hormones GLP-1 and PYY in rodents and humans (Zhou et al., 2008, Greenway et al., 2007). In the presence of glucose, *ckr-1, gcy-8, cpt-1, and cpt-2* are endorsed by the WBP consumption while they are slightly increased by WBP in N2. In *daf-16 and daf-16/daf-2* deficient mutants, *ckr-1, gcy-8, cpt-1, and cpt-2* were increased by the WBP feeding, but the presence of glucose attenuate the effect. In the *sir-2.1* null mutant, *ckr-1, gcy-8, cpt-1, and cpt-2* were least affected by the WBP consumption with or without additional glucose.
6.5 References


CHAPTER 7: GUT MICROBIOTA IN WILD TYPE CAENORHABDITIS ELEGANS: STIMULATION BY MEDIA CONTAINING DIETARY FIBER FROM PROWASHONUPANA BARLEY

7.1 Introduction

Obesity is a rapidly increasing epidemic disease affecting a large population in developed countries without an effective cure. Safe and effective anti-obesity diets and new innovative treatment strategies are a pressing need and in high demand (Bray and Greenway, 2007). A present emerging solution affecting metabolism and providing health benefits includes the use of the dietary carbohydrate prebiotic resistant starch (RS) and its fermented products that include short chain fatty acids (SCFAs). Dietary fermentable fibers reduce energy intake by dilution of the energy density, increase satiety, elevate fat oxidation, reduce body fat, stimulate the growth of beneficial bacterial microorganisms in the colon, maintain bowel health (Tachon et al., 2013), increase gene expression of PYY and proglucagon in primary colon and cecal epithelial cells (Zhou et al., 2008), and lower plasma lipid levels, especially cholesterol and triglyceride concentrations in rodent studies (Dethlefsen et al., 2007).

Previous studies support the interaction of host and host microbiota being critical for the fitness of host organisms including development, reproduction, metabolism, immunity, and lifespan in C. elegans, mammals, and humans (Cabreiro and Gems, 2013). In addition, altering the microbiota through dietary changes or antibiotic usage could affect the health of the host (Maurice et al., 2013, Ikeda et al., 2007). Foods with prebiotics, including resistant starch (RS), stimulate the growth of beneficial bacterial microorganisms in the colon (Tachon et al., 2013). The colon microbiota utilizes fermentable carbohydrates as an energy source and produce SCFAs, predominantly acetate, propionate, and butyrate, among others. Increasing lines of
evidence support the concept that the gastrointestinal (GI) tract microbiota have the capacity to impact nutrition-related syndromes like type II diabetes, obesity, and aging (Turnbaugh et al., 2009).

The *C. elegans* model organism is a practical animal model for study of human diseases. We developed a platform using *C. elegans* to screen different sources of prebiotics and pharmaceutical compounds (Zheng et al., 2010, Finley et al., 2013, Zheng et al., 2014, Zheng and Greenway, 2012). The intestine is the largest organ in *C. elegans* and harbors a vast population of symbiotic microbes (Cabreiro and Gems, 2013). These nematodes have both an aerobic and an anaerobic metabolism and can survive under a hypoxic condition (Burnell et al., 2005). Oxygen or “hypoxia gradient”-oxygen deprivation diffuses bidirectionally between the intestinal tract and the external environment (Burnell et al., 2005). Zheng *et al.* in 2010 have demonstrated that high amylose maize RS2, its endogenous fermentation products from cecal contents of rodents fed, and their exogenous SCFA added to the media reduced *in vivo* intestinal fat deposition (IFD) and promoted healthspan in *C. elegans* (Zheng et al., 2010, Zheng and Greenway, 2012). This was also demonstrated as well with a legume (Finley et al., 2013) and *Prowashonupana* barley (PWB) which consists of 17% β-glucan and 15% fermentable starch with high amounts of complex carbohydrate (Finley et al., 2013, Rendell et al., 2005, Lifschitz et al., 2002, O'Donnell et al., 2013). These data suggested the possibility of anaerobic bacteria in the *C. elegans* intestinal tract fermenting dietary fibers.

Colonic anaerobic microbiota *Bifidobacterium* genus, *Bacteroides-Prevotella* group, *Lactobacilli*, and *Clostridia* spp. are necessary for fermentation of dietary fibers in humans or rodents (Keenan et al., 2013). *Clostridium* Cluster XIVa and XIVb comprise more than 20 clostridia species and their total amounts were quantified using reported primers (Rajilic-
Stojanovic et al., 2007). *Bacteroides-Prevotella group* primers were used to enumerate the *Bacteroides-Prevotella group* including *Prevotella* and *Porphyromonas* spp. *Bacteroides* spp. that are anaerobic Gram-negative bacillus bacteria (Duncan et al., 2003). *Bifidobacterium* genus primers were used to quantify *Bifidobacterium* spp. that are classified under phylum Actinobacteria (Bartosch et al., 2004), a strictly Gram-positive anaerobic branched rod producing lactic and acetic acid but not CO$_2$ (Van der Meulen et al., 2006). *Lactobacillus* spp. primers were quantified from the genus *Lactobacillus* under phylum *Firmicutes*, which consume wide varieties of carbohydrate as a fermentation energy source which grow under aerobic conditions or intestinal anaerobic conditions (Van Dyke and McCarthy, 2002).

Although direct identification of *C. elegans* intestinal microbiota has not been reported, we and others (Cabreiro and Gems, 2013) hypothesized that feeding resistant starch to *C. elegans* should closely resemble the effect of colonic fermentation of prebiotics and produce fermentation compounds in the large intestine as in rodents or in humans, by the intestinal anaerobic microbiota. This pilot study tested whether PWB, higher in fermentable fiber and protein and lower in starch than other barley, would increase *C. elegans* intestinal anaerobic microbiota. The alterations of the population of the four predominant intestinal bacterial DNA were quantified by qRT-PCR including *Bifidobacterium* genus, *Bacteroides-Prevotella group*, *Lactobacillus* spp., and *Clostridium* Cluster XIVa and XIVb in wild type *C. elegans* treated by PWB.

**7.2 Materials and methods**

7.2.1 *C. elegans* preparation

Using the *C. elegans* model organism does not require approval by Institutional Animal Care & Use Committees (IACUC). Wild type *C. elegans* (N2) and the standard laboratory food
source *Escherichia coli* (*E. coli*, OP50) were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, MN). Sterile techniques were used to avoid contamination with undesired bacteria. The detailed procedures were previously published (Zheng et al., 2010, Gao et al., Finley et al., 2013). An *E. coli* pellet was inoculated into a LB (Luria-Bertani) broth solution containing 0.2% streptomycin, incubated at 37°C overnight, and stored at 4°C, then plated on petrifilm (3M™ Petrifilm™ *E. coli*/Coliform Count Plates 6404, 3M Corp., Minneapolis, MN) at 37°C for 24h. *E. coli* OP50 was heat treated in a 70°C water bath (Thermo Fisher, Waltham, MA) for 15 minutes (Houthoofd et al., 2003). Densities of 5×10^8 to 5×10^11 colony forming units (cfu/ml) were selected and fed to the *C. elegans ad libitum* (Zheng et al., 2010, Yuan et al., 2012). The mixed stages of life cycled *C. elegans* were cultured in nematode growth medium (NGM) agar dish (⌀100mm, n=5 dishes/group) in a low temperature incubator (20°C, Fisher Scientific, Pittsburgh, PA) for one week. Milled PWB (Ardent Milles, Denver, CO) was sieved (80μm) to a fine powder and incorporated to the standard feeding medium as previously described (Zheng et al., 2010). Control animals were fed the *E. coli* (OP50) only, the experimental group received additional PWB (1% w/v) for one week, followed by removal of OP50 for 3 days for both groups to removal of unwanted bacteria when nematodes were harvested for measuring intestinal bacteria. The animals then were harvested and washed with sterilized M9 buffer x5 to remove any external bacteria (Montalvo-Katz et al., 2013), and total material of 30±5mg/dish (∼7×10^3 ~8×10^3 *C. elegans*) was recovered. To differentiate the intestinal anaerobic bacterial DNA from extra-intestinal *C. elegans’* DNA, the supernatant of the last wash was also saved for DNA extraction. Both negative DNA results of the supernatant and the aerobic experimental condition would support the DNA extracted from the nematodes as being of intestinal origin.
7.2.2 Anaerobic bacteria culture

Five bacteria were cultured individually overnight and serial dilutions were made for analysis of four genera of bacteria: *Lactobacillus plantarum* strain ATCC 4163, *Clostridium cocooides* strain ATCC 29236, *Bacteroides fragilis* strain ATCC 23745, *Bifidobacterium longum* strain ATCC 15708, *E. coli* strain ATCC 25922, and all were used to construct standard curves for four genera and the *E.coli* strain was used for the total bacterial (16S universal) domain standard curve (Keenan et al., 2013, Senevirathne, 2010).

Five culture and quantification conditions were used to determine the total cfu/ml. Luria Broth (Difco Laboratories, Detroit, Michigan, USA) was used to grow total *E. coli* aerobically at 37°C for 24 hrs. de *Man-Rogosa*-Sharpe Agar (MRS agar, Difco Laboratories, Detroit, Michigan, USA) was used to culture *Lactobacillus plantarum*. Brewer Modified Thioglycollate medium (TMBM plates, BD Diagnostic Systems, Sparks, Maryland) was used to culture *Bifidobacterium longum* and *Bacteroides fragilis* and determine bacterial counts. A reinforced Clostridial agar (Oxoid, Basingstoke, UK) was used for *Clostridium cocooides* counts. The MRS agar plates were anaerobically incubated at 30°C for 48 hr, while the TMBM agar plates and the reinforced *Clostridial* agar plates were anaerobically incubated at 37°C for 3 to 4 days in a chemically generated anaerobic system using an *anaerobic box* GasPak™ EZ (Mitsubishi Gas Chemical America, Inc., New York, NY).

Serial dilutions of each bacterial extraction were prepared for generating standard curves and DNA quantification (ng/μl). Ct values from the *C. elegans* were quantified from the standard curves of high efficiency \(E=10^{-1/slope} \geq 90\%\) to determine the amount of DNA extraction (ng/μl). The intestinal bacterial DNA (cfu/ml) was determined (slope/efficiency) with the following criteria: *Bacteroides-Prevotella group* (-3.38/97.6%), *Bifidobacteriumgenus* (-
3.60/90%), and Clostridium Cluster XIVa and XIVb (-3.43/96%), Lactobacillus spp. (-3.39/97%), and 16S universal (-3.48/94%).

7.2.3 Genomic DNA extraction

The method of modified rapid isolation of genomic DNA from human and animal stool samples by Keenan et al has been described elsewhere (Keenan et al., 2013). Briefly, in this study the DNA of each bacterial serial dilution or the intestinal bacteria from the C. elegans was isolated using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) and quantified (Nanodrop® ND-1000 spectrophotometer, Wilmington, DE).

7.2.4 Quantitative real-time PCR (qRT-PCR) assay

DNA extracted from the C. elegans was used for qRT-PCR assay (2ng/µl). The primers were obtained from Integrated DNA Technologies (Coralville, IA): Bacteroides Prevotella group (Bartosch et al., 2004), Bifidobacterium genus (Bartosch et al., 2004), Clostridium Cluster XIVa and XIVab (Matsuki et al., 2002) and Lactobacillus spp. (Haarman and Knol, 2006) (Table 16). Primers targeting universal hypovariable regions of the16S ribosomal DNA gene (16SrDNA) were used to estimate the total bacterial counts (Belenguer et al., 2006). The amplification thresholds (Ct) of all samples were analyzed in duplicate (SYBR® Green PCR master mix, ABI PRISM 7900 Sequence Detection System, and SDS 2.4, Life Technologies, Grand Island, NY). The amount of bacterial DNA extraction from the anaerobic culture was quantified by qRT-PCR (Ct), which was correlated with the bacteria populations (cfu/ml), and standard curves were generated (Figure 28). The amount of unknown DNA of intestinal bacteria extracted from the C. elegans was quantified using the standard curves.

7.2.5 Statistical analysis
Data were presented as mean±sem. A factorial arrangement of the treatments (two-way ANOVA) with Dunnett’s test was used to compare the effects of the treatments (SAS 9.4). Statistical significance is set as $P \leq 0.05$.

Table 16  Quantitative real-time PCR primers used to profile intestinal samples

<table>
<thead>
<tr>
<th>Targeted bacteria</th>
<th>Sequence of oligonucleotide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S Universal Primers</td>
<td>F – GTGSTGCA YGGYYGTCA R- ACGTCRTCCMCNCCCTTCCTC</td>
<td>(Belenguer et al., 2006)</td>
</tr>
<tr>
<td>Bacteroidetes group including Prevotella and Porphyromonas</td>
<td>F – GAAGGTCCCCCACATTG R- GGGTGGTAATGCCGGATG</td>
<td>(Bartosch et al., 2004)</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>F – GGGTGGTAATGCCGGATG R- TAAGCCATGGACTTTCACACC</td>
<td>(Bartosch et al., 2004)</td>
</tr>
<tr>
<td>Clostridium Cluster IV (Clostridium leptum subgroup, includes Faecalibacterium prausnitzii)</td>
<td>F – TTACTGGGTGTAAAGGG R- TAGAGTGCTTTTGCGTA</td>
<td>(Van Dyke and McCarthy, 2002)</td>
</tr>
<tr>
<td>Clostridium Cluster XIVa and XIVb (Clostridium coccoides – Eubacterium rectale subgroup)</td>
<td>F – AAATGACGGTACCTGA CTAA R- CTTTGAGTTTCATTCTTGCGAA</td>
<td>(Matsuki et al., 2002)</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>F – TGGATGCCTTTGGCACTAGGA R- AAATCTCCGGATCAAAGCTTTACTTAT</td>
<td>(Haarman and Knol, 2006)</td>
</tr>
</tbody>
</table>

7.3 Results

The anaerobic bacteria (cfu/ml) were detected in the extracted total genomic DNA by specific primer sets that represented several genera, which indicated that it was of intestinal origin (Table 16) because no bacterial DNA was detected in the last wash supernatant of the nematodes done before DNA extraction of the nematodes.

The amount of unknown DNA of intestinal bacteria extracted from the C. elegans was quantified by qRT-PCR, and the populations of the target bacteria were calculated using the
standard curves. The bacterial counts in control group were: *Bacteroides-Prevotella group* (0.56 cfu/ml), *Bifidobacterium genus* (1.4 cfu/ml), or *Clostridium Cluster XIVa and XIVb* (6.26x10^4 cfu/ml), or *Lactobacillus spp.* (4.01x10^8 cfu/ml). *Lactobacillus spp.* had the highest population which was 7.16x10^8-fold higher than *Bacteroides-Prevotella group*, or 2.86x10^8-fold more than *Bifidobacterium genus*, or 6.4x10^3-fold greater than *Clostridium Cluster XIVa and XIVb*, while the population of *Lactobacillus spp.* did not change in PWB treated group (2.21x10^8 cfu/ml, P=0.2251, n=5). PWB treatment increased the bacterial counts in *Bacteroides-Prevotella group* (2.64 cfu/ml, P=0.007), *Bifidobacterium genus* (3.51 cfu/ml, P<0.001), and *Clostridium Cluster XIVa and XIVb* (3.96x10^5 cfu/ml, P=0.0353, Figure 29). Post PWB treatment, the *Lactobacillus spp.* was reduced (50%) while other anaerobic bacterial species was increased in the following order: *Clostridium Cluster XIVa and XIVb* (633%), *Bacteroides-Prevotella group* (472%), or *Bifidobacterium genus* (250%, Figure 30).
Figure 28  Standard curves for Lactobacillus spp., Clostridium Cluster XIVa and XIVb, Bacteroides-Prevotella group, Bifidobacterium genus and E. coli (ATCC 25922).
Figure 29  Total population by colony-forming units (cfu/ml) of the tested bacteria in the wild type *C. elegans*.

![Figure 29](image)

Figure 30  PWB (1%) treatment increased the total DNA of the tested bacteria (P<0.05). The increase of the anaerobic bacteria was in the following order: *Clostridium* Cluster *XIVa* and *XIVb* (633%) > *Bacteroides-Prevotella group* (472%) > *Bifidobacterium genus* (250%).

### 7.4 Discussion

We previously demonstrated that dietary fibers reduce IFD and promote healthspan in the *C. elegans* model organism (Zheng et al., 2010, Finley et al., 2013), as has been shown by others in rodents (Tachon et al., 2013) and humans (Rendell et al., 2005). The fact that “Oxygen or ‘hypoxia gradient’-oxygen deprivation diffuses bidirectionally between the intestinal tract and the external environment” (Burnell et al., 2005) supports the hypothesis that *C. elegans* intestinal tract contain anaerobic bacteria and prebiotics can alter the bacteria microbiota composition to improve the health of the host (Burnell et al., 2005). This pilot study tested whether anaerobic bacteria inhabited the intestine of the *C. elegans* and if a prebiotic, PWB can increase specific genera of anaerobic bacteria. Four predominant intestinal bacteria normally found in humans
and rodents were detected and quantified by qRT-PCR in wild type *C. elegans* by specific DNA primer sets representing at the genus level of taxonomy. These anaerobic bacteria, except four *Lactobacillus spp.*, were increased in the PWB (1%) treated group. The majority of species in the human GI tract belong to two phyla, the *f Firmicutes* and *Bacteroides-Prevotella group* (Turnbaugh et al., 2006). *Lactobacillus spp.*, and *Clostridium Cluster XIVa and XIVb* are members of phylum *Firmicutes* (Bell, 1969). *Bacteroides spp*, *Bifidobacterium spp*, and *Lactobacillus spp* ferment RS to produce acetate, propionate, succinate, and lactate, but not butyrate (Sekirov et al., 2010, Haarman and Knol, 2006, Duncan et al., 2003, Louis et al., 2007, Dethlefsen et al., 2007). *Clostridium Cluster IV* and *Clostridium Cluster XIV* are two major bacterial genera that have species that produce butyrate (Belenguer et al., 2006, Louis et al., 2007). The high population of *Lactobacillus spp* in the present study is likely due to the survival characteristics in both aerobic and anaerobic conditions based on the oxygen gradient mentioned earlier.

Prebiotic intake, like type 2 RS from high-amylose maize, is directly associated with an increase in the total bacterial population in fecal samples and their production of fermentation products like SCFAs. SCFA’s stimulate production of gut satiety hormones, augment food digestion and GI motility, elevate energy expenditure, promote insulin sensitivity, boost mitochondrial function, augment drug metabolism, inhibit pathogens, produce vitamins, and enhance the health of the host in species ranging from *C. elegans*, rodents, to human (Zheng et al., 2010, Cabreiro and Gems, 2013, Keenan et al., 2013, Turnbaugh et al., 2006, Dethlefsen et al., 2007, Wang et al., 1996, Flint, 2012). In addition, prebiotics alter the composition and population of intestinal microbiota by enhancing the growth of beneficial bacteria which extract
energy from polysaccharides including RS (Turnbaugh et al., 2006) and regulate host metabolism by the xenobiotic cues perceived in the intestine (Cabreiro and Gems, 2013, Shen et al., 2009).

Freshly isolated *C. elegans* from the wild often harbor diverse bacteria in their gut or associated with their bodies (Bumbarger et al., 2013, Foll et al., 1999). To avoid extra-intestinal bacteria contamination, we washed the animals a total of five times and performed bacteria DNA extraction from the supernatant of the last wash from which bacterial DNA was not detected. The control animals had reduced numbers in their anaerobic bacterial populations of CC14a & CC14b, *Bacteroides-Prevotella group*, and *Bifidobacterium genus*. The animals receiving the PWB had increases in the intestinal anaerobic bacterial number attributed to the carbohydrate rich in RS of PWB, and to other fibers that reduced dietary energy density. Detection of the elevated levels of DNA from the anaerobic bacteria in the PWB treated animals demonstrated that dietary changes can alter the host-microbiota and increase subgroups of anaerobic bacterial populations. Fermentable dietary fibers in PWB, such as RS or their fermentation products SCFAs, can extend lifespan and reduce intestinal fat deposition in the *C. elegans* model organism (Zheng et al., 2010), and the microbiota appears to be associated with these improved health effects of fermentable fiber in *C. elegans*.

### 7.5 Conclusions

This preliminary study demonstrated that four predominant anaerobic bacteria genera were present in the intestine of *C. elegans* and there was an increase in the total bacterial population with PWB (1%) treatment. The detected anaerobic bacterial DNA expression proved the general concept for the first time that the *C. elegans* intestinal tract is inhabited by anaerobic
bacteria capable of fermenting dietary fibers. PWB altered the intestinal microbiota of the *C. elegans* presumably by intestinal fermentation of dietary fibers. The results will be pursued with further genomic DNA sequencing to determine the full range of different bacterial species in the *C. elegans* intestine. Further mechanistic studies will also be conducted to identify genetic and functional pathways like genetic manipulations of aging and insulin signaling pathways, the use of various fermentable fibers and the fermentation products of those fibers. These studies will expand research on the potential health benefits of dietary carbohydrate consumption in humans.
7.6 References


CHAPTER 8: CONCLUSIONS

This study indicated that SCFAs including butyrate, low dose acetate and tributyrin increased the lifespan of N2 and \textit{daf-16(mgDf50)I} \textit{C. elegans} while reduced the lifespan in the \textit{daf-16/daf-2} deficient and \textit{sir-2.1(ok434)IV} mutants. The IFD was reduced by butyrate (0.3, 0.6mM), acetate (100mM), propionate (0.3mM), and tributyrin (0.1, 1mM) in N2; and was increased in \textit{sir-2.1} null mutant. High dose of sodium acetate or tributyrin negatively affected the lifespan that indicate that SCFAs alter the lifespan of \textit{C. elegans} is in a dose dependent way. The insulin signaling pathway is a key factor in the regulation the lifespan of \textit{C. elegans} model. Our results demonstrated that the regulation of longevity by SCFAs is partially dependent on the \textit{daf-16} gene and dependent on \textit{daf-2} gene. The SCFAs increased the lifespan of \textit{C. elegans} was dependent on another longevity gene, \textit{sir-2.1}.

Our data demonstrated that PWB sustained the healthspan in N2, \textit{daf-16(mgDf50)I}, and \textit{daf-16(mgDf50)I;daf-2(m65)III} with or without hyperglycemia. High dose of PWB also sustained healthspan of \textit{C. elegans} in \textit{sir-2.1(ok434)IV}. Additionally, the health benefits of PWB also was indicated by the reduction of IFD and the deceleration of insulin-resistance in the \textit{C. elegans} model system which appeared to be primarily mediated via \textit{sir-2.1}, \textit{daf-16}, and \textit{daf-16/daf-2}. These effects were attenuated by hyperglycaemia on IFD in N2 or \textit{daf-16/daf-2} mutant. The functional components of PWB including β-glucan and phenolics, may provide more advantages in weight loss and obesity prevention.

The N2, \textit{daf-16}, or \textit{sir-2.1} mutant increased the PPR following oat consumption. Oat-feeding decreased IFD in N2, and \textit{daf-16} or \textit{daf-16/daf-2} mutant with or without hyperglycaemia. Oats also induced an improvement in insulin sensitivity. Unique component, Avns and β-glucan,
indicate oats as a functional food can be incorporated into the daily diet in various ways including improve the public health.

Wheat bran also sustained the healthspan in N2, *sir-2.1(ok434)IV, daf-16(mgDf50)I*, and *daf-16(mgDf50)I;daf-2(m65)III*. This increase persisted in the presence of glucose at a low dose in *daf-16* or *daf-16/daf-2* mutant. Wheat bran reduced IFD in N2, *sir-2.1* or *daf-16* null mutants. Wheat bran reduced IFD in N2, and in *daf-16* or *daf-16/daf-2* mutants without hyperglycemia, while hyperglycemia increased IFD in *sir-2.1(ok434)IV*.

The pilot study of gut microbiota in *C. elegans* indicated that four predominant anaerobic bacteria genera were present in the intestine of *C. elegans*. The detected anaerobic bacterial DNA expression provided evidence to indicate that the *C. elegans* intestinal tract is inhabited by anaerobic bacteria capable of fermenting dietary fibers. Intestinal fermentation of dietary fibers altered the intestinal microbiota divisions presumably.

In summary, PWB, oats, wheat bran, and SCFAs reduced the IFD and improved the healthspan in *C. elegans*, and these effects were mediated by the *sir-2.1, daf-2*, or *daf-2/daf-16* pathways. This could result in future improvements in public health. The identified optimal functional food components may be incorporated in various ways into daily diet, because these are already known to have acceptability as food, and have been tested in clinical trials. A broader test of other sources of the functional components of PWB, such as, β-glucan, may provide more advantageous weight loss and obesity prevention. *C. elegans* is an attractive *in vivo* animal model for initial studies of nutrition interventions prior to confirmation in higher animal species.
APPENDIX:

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VITA

Chenfei Gao, a native of Hebei, China, received his bachelor’s degree at the Hebei University of Science and Technology majoring in Biotechnology in 2006. Thereafter, he worked in local agricultural institution. As his interest in food science and nutrition, he made the decision to enter graduate school in the Department of Food Science at Louisiana State University. He entered Louisiana State University in 2009 for his Graduate studies and graduated in August 2011 with a master degree from Department of Food Science. As he is highly passionate in Food Science and nutrition, he continued to study dietary resistant starch and other bioactive compounds that improve health using C. elegans model and pursued the Doctor of Philosophy degree in the School of Nutrition & Food Sciences under the supervision of Dr. Jolene Zheng and Dr. Finley. During these years’ study, he has gained solid theoretical knowledge in food science and got rich practical experience in conducting research and lab management. He expects to receive his Doctoral degree in Dec 2015 and plans to begin work in food industry upon graduation.