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***Prowashonupana* barley dietary fibre reduces body fat and increases insulin sensitivity in *Caenorhabditis elegans* model**

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Abstract

Prowashonupana barley (PWB) is high in β -glucan with moderate content of resistant starch. PWB reduced intestinal fat deposition (IFD) in wild type *Caenorhabditis elegans* (*C. elegans*, N2), and in *sir-2.1* or *daf-16* null mutants, and sustained a surrogate marker of lifespan, pharyngeal pumping rate (PPR), in N2, *sir-2.1*, *daf-16*, or *daf-16/daf-2* mutants. Hyperglycaemia (2% glucose) reversed or reduced the PWB effect on IFD in N2 or *daf-16/daf-2* mutants with a sustained PPR. mRNA expression of *cpt-1*, *cpt-2*, *ckr-1*, and *gcy-8* were dose-dependently reduced in N2 or *daf-16* mutants, elevated in *daf-16/daf-2* mutants with reduction in *cpt-1*, and unchanged in *sir-2.1* mutants. mRNA expressions were increased by hyperglycaemia in N2 or *daf-16/daf-2* mutants, while reduced in *sir-2.1* or *daf-16* mutants. The effects of PWB in the *C. elegans* model appeared to be primarily mediated via *sir-2.1*, *daf-16*, and *daf-16/daf-2*. These data suggest that PWB and β -glucans may benefit hyperglycaemia-impaired lipid metabolism.

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Chemical compounds studied in this article

Beta glucan (PubChem CID: 46173706)

Glucose (PubChem CID: 79025)

Vitamin B1 (Thiamin) (PubChem CID: 1130)

Vitamin B3 (Niacin) (PubChem CID: 938)

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Conflict of Interests

The authors declare that there are no conflicts of interest.

Keywords

obesity; barley; animal models; insulin sensitivity; aging

1. Introduction

Obesity costs the US health care system over \$100 billion a year and predisposes to diabetes which generates even higher costs (Ogden et al., 2006). 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 behaviour 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, Hartzell, Peterson, Williams, & Fagan, 2006; Wadden & Frey, 1997).

Daily consumption of non-digestible fibre may offer a strategy to increase energy expenditure and reduce body fat by stimulation of mitochondrial fat oxidation (Chandrasekara & Shahidi, 2011; John & Shahidi, 2010; Madhujith, Amarowicz, & Shahidi, 2004). Average daily consumption of dietary fibre in the United States is currently 3 to 10 g/day, 4 to 13 fold less than recommended (Anderson & Akanji, 1991; Goldring, 2004). Non-digestible carbohydrate, fermented to short chain fatty acids (SCFAs) in the lower gastrointestinal tract, increases satiety hormone peptides YY (PYY) and glucagon-like peptide-1 (GLP-1). These hormones augment fat oxidation (lower respiratory quotient), elevate energy expenditure (Keenan et al., 2006; Zhou et al., 2006; Zhou et al., 2008), 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 *Caenorhabditis elegans* (*C. elegans*) model organism (Abate & Blackwell, 2009; Gao et al., 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 resistant starch (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 palmitoyltransferase-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 & 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% RS type 3 (RS₃) and β -glucan (Asare et al., 2011; Vasanathan & Bhatty, 1998), a mixture that provides a distinctive amylose-amylopectin interaction (Behall, Scholfield, & Hallfrisch, 2005; Dongowski, Huth, Gebhardt, & Flamme, 2002; Lifschitz, Grusak, & Butte, 2002; Rendell et al., 2005; Tang,

Ando, Watanabe, Takeda, & Mitsunaga, 2001). 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, Flores, & Hicks, 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 glycaemic response (Lifschitz et al., 2002). Dietary fermentable fibre 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 RS type 2 (RS₂) from corn (Ingredion Incorporated, Westchester, IL, 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 β -glucan than most other barleys, which are linked to half of the PWB fibre (Behall et al., 2005; Rendell et al., 2005). Retrograded amylose starch, RS type 3 (RS₃), and β -glucan of PW barley provide distinctive amylose–amylopectin and amylopectin– β -glucan interactions that increase the viscosity and delay energy absorption in the GI tract (Asare et al., 2011; Moreau et al., 2007; Tang et al., 2001; Vasanathan & Bhatta, 1998) reducing the glycaemic peak by 50%, lowering low density lipoprotein (LDL) cholesterol in healthy humans, decreasing fat accumulation in humans and rodents (Wursch & Pi-Sunyer, 1997), augmenting immunity by activating the dectin-1 receptor and activating multiple genetic signalling pathways, including the DAF-2/insulin-like receptor (ILR) pathways. The details of the mechanism by which insulin-resistance is improved, however, are unclear (Engelmann & Pujol, 2011; Tsoni & Brown, 2008).

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 & Greenway, 2012). *C. elegans* dauer formation (*daf*) gene, *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 & Greenway, 2012). Decreased *daf-2* signalling increases *C. elegans* lifespan (Koga, Take-uchi, Tameishi, & Ohshima, 1999; Kramer, Davidge, Lockyer, & Staveley, 2003) 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 (Burks et al., 2000; Hertweck, Gobel, & Baumeister, 2004; Hunt et al., 2011; Tissenbaum & Ruvkun, 1998; Yu et al., 2010). Hyperglycaemia (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 & Blackwell, 2009). *sir-2.1* overexpression prolongs *C. elegans* lifespan (Frojdo et al., 2011). In fact, doubling *C. elegans sir-2.1* gene number induces a 50% increase in the lifespan that is DAF-16/FOXO dependent (Dorman, Albinder, Shroyer, & Kenyon, 1995; Tissenbaum & Guarente, 2001), and the null mutant of *sir-2.1(ok434)IV* has a shorter lifespan than the N2 (Gami & 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, Kim, Raizen, & Avery, 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 & 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 hyperglycaemia-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, Glenn, Johnston, Goldberg, & Wolkow, 2006; Finley et al., 2013), was also monitored to determine effect of PWB on lifespan.

2. Material 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).

2.1 Culture of *E. coli* (OP50)

OP50 were cultured by the standard method described elsewhere (Zheng et al., 2010). Briefly, 10 μ l $\pm$ 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, Minneapolis, MN, USA) at 37°C for 24h, and the colonies of OP50 were confirmed to be at a density of 5 $\times$ 10⁸ – 5 $\times$ 10¹¹cfu/ml at which time the *C. elegans* were allowed to eat *ad libitum*.

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 Nematode Growth Media (NGM) agar plates (Ø35 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 μ l, v/v) and sodium hypochlorite solution (5.25% , 200 μ l, 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.

2.3 Diet composition

Prowashonupana barley (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 (DDH₂O; 5%, w/v). Detailed nutritional data of PWB by Ardent Mills Corporate is listed in Table 1.

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 2). The dietary nutrient composition is listed in Table 3 (Feijo Delgado et al., 2013). *C. elegans* were transferred to fresh dishes every other day receiving treatment (50µl).

2.4 Pharyngeal movement (pumping rate, PPR)

Animals were examined periodically using a stereomicroscope (Nikon SMZ1500, Melville, NY, USA) with transmitted light. The pharyngeal pumping rate (PPR) was recorded manually by independent observers and the animals were returned to the incubators.

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 × 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]).

2.6 Quantitative RT-PCR

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.

2.6.2 RNA isolation—Total RNA was extracted using the Trizol[®] reagent as described elsewhere (Ye, Gao, Yin, & He, 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 were 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).

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. 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) 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). 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). The relative quantification of gene expression (2^{-C_t}) was calculated.

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 0.05.

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. 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.

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 (Fig. 1), by 18 to 28% (0.5, 1.0, or 3.0%, $n=9$, $P<0.01$) in *daf-16(mgDf50)I* (Fig. 2b), or by 18 to 36% (0.5, 1.0, or 3.0%, $n=9$, $P<0.01$) in the *sir-2.1(ok434)IV* (Fig. 2d). The reduction in *daf-16(mgDf50)I* or *sir-2.1(ok434)IV* was reversed in presence of 2% glucose (Fig. 2b and 2d). In *daf-16(mgDf50)I;daf-2(m65)III*, 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 manner ($n=3$, $P<0.05$) (Fig. 2c).

3.2 Pharyngeal pumping rate (PPR)

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

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%). 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% ($P<0.05$, Fig. 4).

3.4 Principal component analyses (PCA)

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*, and *cpt-2* have strong positive relationship with factor 1 but these 4 genes have an indirect relationship with factor 2 (Fig. 5).

4. Discussion

PWB (3%) induced significant reductions of Nile Red fluorescent intensity representing intestinal fat deposition (IFD) in N2 (40%), *sir-2.1(ok434)IV* (30%) and *daf-16(mgDf50)I* (25%) deficient mutants, but not in the *daf-16(mgDf50)I;daf-2(m65)III* deficient mutant. The pharyngeal pumping rate (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* deficient 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 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.

Hyperlipidaemia is often co-existent with hyperglycaemia. Insulin-resistance induces hyperglycaemia in rodent diabetes due to an increased peroxisome proliferator activated receptor- α (PPAR α) (Hostetler, Huang, Kier, & Schroeder, 2008). Deletion of nuclear hormone receptor-49 (*nhr-49*), a homologue of PPAR α in *C. elegans*, prevents hyperlipidaemia and restores insulin-sensitivity comparable to that seen in PPAR α null mice (Atherton, Jones, Malik, Miska, & Griffin, 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 signalling pathways and is the major target of the *daf-2* pathway which negatively regulates *daf-16* (Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993; Lee, Murphy, & Kenyon, 2009), possibly, PWB/ β -glucans indirectly affected insulin-sensitivity by affecting hyperglycaemia 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 & 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 (Keenan et al., 2006; Shen et al., 2009; Zhou et al., 2008), 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 (Keenan et al., 2006; Zhou et al., 2008). 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 *gcy-8* gene

expression which promotes fat storage. This effect is lost in the presence of the *sir-2.1* deficient mutant or with the lack of *daf-2/daf-16*, which suggest that the additional glucose induced insulin resistance is dependent on *daf-2* pathway and is suppressed by *sir-2.1*. The other genes do not seem to play much of a role, but the small increase in *cpt-2* with glucose is reversed in *sir-2.1* deficient mutant and the lack of both *daf-2/daf-16*. Since *cpt-2* gene expression in the *daf-16* deficient mutant did not change, the effect of glucose on *cpt-2* was thought to be through *daf-2* and that *sir-2.1* inhibited that effect.

Extra glucose reduces *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 *daf-16/daf-2* deficient mutant. Like IFD, the effect of PWB on PPR was reversed in the *daf-16/daf-2* deficient mutant with addition of glucose suggesting an independent mechanism which may involve a pathway outside of *daf-2* or *daf-2/daf-16* (Engelmann & Pujol, 2011; Zheng & Greenway, 2012), or that absence of the *daf-2* promoted insulin sensitivity despite high levels of glucose. The PPR was dose-dependently elevated in all but the *daf-2/daf-16* mutant indicating a dependency on *daf-2* or *daf-2/daf-16* as their absence eliminated the elevation of PPR. The *sir-2.1* gene is related to the co-regulator/genes (Kyrylenko, Kyrylenko, Suuronen, & Salminen, 2003) and is partially responsible for aging in *C. elegans* (Houthoofd, Braeckman, Johnson, & Vanfleteren, 2003; Lakowski & Hekimi, 1998). Apparently, the effect of improved healthspan or lifespan was independent of *sir-2.1* in the present study, which was more robust than in the *daf-16* mutant. The *daf-2/daf-16* mutant showed either no change or a minor change in PPR suggested the effect was insulin/IGF-1 like receptor pathway dependent, which was consistently absent in *daf-16* mutant.

5. Conclusions

Our data demonstrated that PWB reduced IFD, decreased insulin-resistance, and improved healthspan or lifespan in the *C. elegans* model system which appeared to be primarily mediated via *sir-2.1*, *daf-16*, and *daf-16/daf-2*. *C. elegans* is an attractive *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, β -glucan, may provide more advantageous weight loss and obesity prevention. This could result in future improvements in public health.

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Abbreviations

IFD	intestinal fat deposition
PWB	<i>Prowashonupana</i> barley
PPR	pharyngeal pumping rate

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Highlights

- *Prowashonupana* barley (PWB) is rich in β -glucan and dietary fermentable fiber.
- PWB consumption reduced intestinal fat deposition in the *C. elegans* model organism.
- PWB consumption improved healthspan in *C. elegans*.
- PWB may help to reduce the world-wide obesity epidemic and the cost of health care.

Fig. 1. Intestinal fat deposition (IFD) was significantly reduced in the *C. elegans* (N2) that received the *Prowashonupana* barley (PWB, 0.5% or 3%) compared to a control group. a) IFD stained by Nile red in control animals. b) IFD stained by Nile red in animals that received the PWB (0.5% or 3%). c) Fluorescence intensity of Nile red was reduced in the PWB treated animals. d) The effect of PWB on fluorescence intensity of Nile red reduction was reversed in the presence of 2% glucose.

Fig. 2.

Intestinal fat deposition (IFD) was reduced in mutant *C. elegans*. a) N2 Fluorescence intensity of Nile red was reduced in the *Prowashonupana* barley (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)I*. 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)I;daf-2(m65)III*. 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)IV*: Fluorescence intensity of IFD stained by Nile red in animals that received the PWB (0.5%, 1%, or 3%).

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Fig. 3. Pharyngeal pumping rate (PPR) of *C. elegans* was reduced in all groups with aging. An elevation of the PPR was observed in animals that received the *Prowashonupana* barley (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* ($P < 0.05$).

Fig. 4.

Prowashonupana barley (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.

Fig. 5. Principal component analyses (PCA) showed the relationships of the lipid metabolic related mRNAs (*cckr-1*, *cpt-1*, *cpt-2*, and *gcy-8*), pharyngeal pumping rate (PPR) slope, and intestinal fat deposition (IFD) with two strong factors.

Table 1

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

Content	Quantity (g)	Content	Quantity (mg)
Calories	390 kCal	Vitamin A	0 IU
Calories from Fat	60 kCal	Vitamin C	0
Fat	6.5	Sodium	12
Saturated Fat	1.8	Calcium	33
Cholesterol	0	Iron	3.6
Carbohydrates	64.3	Vitamin B1 (Thiamin)	0.6
Total Dietary Fiber	30	Vitamin B2 (Riboflavin)	0.3
Beta glucan	15	Vitamin B3 (Niacin)	4.6
Soluble Fiber	12	Potassium	452
Protein	18	Zinc	2.8

¹ http://www.ardentmills.com/uploads/Ardent_Mills_Sustagrain.pdf

Table 2

Protocol of experiment

Treatments (50μl)	Dosage (%)									
	Without glucose					With glucose				
	0	0.5	1	3	0	0.5	1	3	0	1
OP50 (2×10 ⁶ cfu/ml, μl)	10	10	10	10	10	10	10	10	10	10
PWB (5%, μl)	0	5	10	30	0	5	10	30	0	10
Glucose (50%, μl)	0	0	0	0	0	2	2	2	2	2

Table 3

Diet ingredient composition

Nutrients	PWB diet (mg/ml)	OP50 (mg/ml)	PWB treatment (mg/plate)			
			0	0.5%	1%	3%
Beta-glucan	150	-	0.000	0.012	0.023	0.069
Total dietary fiber	300	-	0.000	0.018	0.037	0.110
Carbohydrate	643	7.5	0.375	1.983	3.590	10.020
Protein	180	37.5	1.875	2.325	2.775	4.575
Fat	65	5.0	0.250	0.413	0.575	1.225