DOI:10.1021/jf904583b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Resistant Starch, Fermented Resistant Starch, and Short-Chain Fatty Acids Reduce Intestinal Fat Deposition in *Caenorhabditis elegans*

Jolene Zheng,*,[†] Fred Enright,[†] Michael Keenan,[‡] John Finley,[#] Jun Zhou,[§] Jianping Ye,[⊥] Frank Greenway,[⊥] Reshani N. Senevirathne,[#] Chris R. Gissendanner,[⊗] Rosaly Manaois,[#] Alfredo Prudente,[#] Joan M. King,[#] and Roy Martin*,[‡,⊥]

[†]Veterinary Science Department, [‡]School of Human Ecology, and [#]Food Science Department, LSU AgCenter, Louisiana State University, Baton Rouge, Louisiana 70803, [§]Geriatric Endocrinology and Metabolism Laboratory, Veteran's Administration Medical Center, Washington, D.C. 20422, [⊥]Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808, and [⊗]Department of Biology, University of Louisiana at Monroe, Monroe, Louisiana 71209

Obesity is a growing global public health dilemma. The objective of this project is to develop and validate a screening mechanism for bioactive compounds that may reduce body fat and promote health. Resistant starch (RS) reduces body fat in rodents. Amylose starch that has a high content of RS, endogenous compounds obtained from the ceca of amylose starch fed mice (fermented RS), and individual short-chain fatty acids (SCFA) were tested. The *Caenorhabditis elegans* model and Nile red staining were selected to determine the intestinal fat deposition response to bioactive components. The fluorescence intensity of Nile red was reduced to 76.5% (amylose starch), 78.8% (fermented RS), 63.6% (butyrate), or 28−80% (SCFAs) of controls, respectively ($P < 0.001$). The reduced intestinal fat deposition suggests reduced food intake or increased energy expenditure. *C. elegans* is a practical animal model to screen for bioactive compounds that may prevent or treat obesity.

KEYWORDS: Obesity; resistant starch (RS); *Caenorhabditis elegans*; short-chain fatty acids (SCFA); butyrate; acetate trihydrate; propionate; tributyrin

INTRODUCTION

The prevalence of obesity is rising and is accompanied by an increase in the prevalence of diabetes and other obesity-associated diseases. Diabetes, in particular, is driving an increase in health care costs and is compromising public health. Biological systems have evolved a complex network of physiologic systems to maintain food intake, body weight, and energy homeostasis.

Over the past half century, there has been growing acceptance of obesity treatment interventions. 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. Stimulation of the sympathetic nerves to the upper gastrointestinal tract is a new and promising method that is still under development (*1*, *2*). Safety concerns and limited efficacy have curtailed the use of antiobesity pharmaceuticals. There are only two obesity drugs presently approved in the United States for long-term use. Sibutramine, one of these drugs, causes a rise in blood pressure, and orlistat, the other drug, is associated with embarassing gastrointestinal side effects such as anal leakage and incontinence. Phentermine is approved only for the short-term treatment for obesity, is a stimulant, and is scheduled by the drug enforcement administration, suggesting that it has a potential for addiction. All three of these antiobesity medications give < 5% greater weight loss than a placebo (*3*). Because obesity is a prevalent chronic disease without a cure, there remains a high demand for safe and effective obesity treatments and a pressing need for the new innovative treatment strategies that are emerging (*4*). Many lines of evidence suggest that food such as resistant starch (RS) provides medical benefits beyond its function as a nutrient in rodents and may offer a solution to reducing body fat in humans (*5*). RSs are macronutrients that are divided into four categories, one that is physically inaccessible to enzymes and is stable to heat (RS_1), one that has limited access by enzymes through its special granular (ungelatinized) form (RS_2), one that can form retrograde starch that is fermentable such as amylose starch and which is the most abundant resistant starch (RS_3), and one that does not have a naturally occurring structure of chemically altered starch (RS_4) (*6*).

*Address correspondence to either author [(J.Z.) Veterinary Science Department, Louisiana State University, LSU AgCenter, Baton Rouge, LA 70803 (telephone/fax (225) 578-9227, e-mail zzheng@agcenter.lsu.edu); (R.J.M.) Human Nutrition and Food Division, School of Human Ecology, Louisiana State University, LSU AgCenter, Baton Rouge, LA 70803 (telephone (225) 578-1704, fax (225) 578-4443, e-mail rjmartin@lsu.edu].

 Published on Web 03/30/2010 © 2010 American Chemical Society

In particular, the dietary fiber fraction to which amylose starch (RS_2) belongs is thermally stable, is capable of escaping digestion in the small intestine, and is fermented through the action of anaerobic bacteria in the large intestine to yield short-chain fatty acids (SCFA) (*6*, *7*). Keenan et al. and Zhou et al. have demonstrated that the RS fermentation product, SFCAs, plays a major role in obesity prevention in RS-induced fat reduction in rodents (*8*, *9*).

The present study describes a method of screening for bioactives in an efficient manner. The nematode animal model, *Caenorhabditis elegans* (*C. elegans*), has been increasingly utilized for biological and medical studies, and > 65% of the genes relating to human diseases are conserved in *C. elegans* (*10*, *11*). Three hundred and five genes in *C. elegans* have been shown to be involved in reducing body fat, and 112 genes are involved in increasing fat storage as demonstrated by RNAi and Nile Red staining (*10*). *C. elegans* is well-suited to obesity studies, because deposits of fat for energy storage can be found along its intestinal tract and the bodies of *C. elegans* are transparent (*12*). Thus, lipid-staining dyes such as Nile Red or BODIPY can be visualized directly and quantitated photometrically in the intact animals (*13*). The rapid reproduction cycles and large brood sizes of *C. elegans* allow for a variety of cellular, molecular, genetic, and behavioral analyses. *C. elegans* is well-suited for use in large-scale and high-throughput screening programs to discover drugs and bioactive materials (*14*, *15*). The objective of this study was to optimize the screening of bioactive materials that might reduce body fat in the *C. elegans* model. We tested the effects of amylose starch that has a high content of RS, endogenous compounds obtained from the ceca of amylose starch fed mice (fermented RS), and individual SCFAs.

MATERIALS AND METHODS

C. elegans and the food source, *Escherichia coli* (OP50), of this study were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota).

Culture of Wild-Type *C. elegans* (N2 Strain). One day prior to the experiment, 200 μL of a feeding medium containing *E. coli*, OP50) (see below) was added to the agar dish. Animals were cultured in the agar dish at 20 °C in a cold incubator (Revco Tech., Nashville, NC). Control animals were fed the *E. coli* only. The experimental groups were fed additional amylose starch (Hi-Maize 260 contains 60% RS that is indigestible and fermentable), amylopectin (AMIOCA, highly digestible starch; both from National Starch and Chemical Co., Bridgewater, NJ), wash-out of gut contents from amylose starch-fed lean mice, or four types of SCFA, respectively. Intestinal fat deposition of the experimental groups was compared with their paired control groups.

Culture of Nonpathogenic *E. coli* (OP50, Uracil Auxotroph). *E. coli* was cultured according to the standard method. Briefly, a scoop (10 μL±) of stock *E. coli* was added to feeding medium (see solution composition below) and incubated at 37 °C for 48 h. *E. coli* was then subcultured in Petrifilm (3M Corp., Minneapolis, MN) at 37 °C for 24 h to confirm the colonies. Densities of $5 \times 10^8 - 5 \times 10^{11}$ cfu/mL were selected, and the animals were allowed to eat ad libitum (*16*).

Fluorescence Microscopy. Prior to fixing the animals, an S-basal (see below) solution (1 mL/dish) was used to wash the animals. The solution containing the animals was centrifuged for 20 s at 3000 rpm, and this procedure was repeated twice. Animals were then fixed with 4% paraformaldehyde for > 2 h at 4 °C and washed with PBS for 5 min three times. Nile Red was dissolved in acetone (0.5 mg/mL), and glycerol/water (75:25) was added into the solution. Fifty microliters of the solution was applied to the animals/specimen for 10 min. A drop of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 20 μL of the medium containing Nile Red stained animals. A cover glass was mounted on the glass slide. The slides were viewed with a fluorescence microscope (Nikon Eclipse, *Ti*) equipped with a Texas Red filter. Digital images were taken with the Retiga 4000R. The fluorescence micrographs were analyzed using ImagePro Plus (Media Cybernetics, Inc., Bethesda, MD). Line profiles of the optical densities (arbitrary units, percent of control) of Nile Red labeled intestinal fat deposition were determined for each group. The animals were at mixed growing stages. Adult animals (lavar 4) were selected for the measurement.

Starch Preparation. The amylopectin (AMIOCA) and amylose starch (RS_2, Hi-Maize 260 consists of 60% amylose starch and 40% amylopectin) were gifts from National Starch and Chemical Co. The absence or presence of amylose starch in AMIOCA or in Hi-Maize 260 was confirmed with differential scanning calorimetry (DSC) measurements, respectively. A peak of Hi-Maize VII consisting of 38.2% RS was detected in our Hi-Maize 260 sample. Trace levels of amylose starch without the peak were found in AMIOCA (1.45%). Amylose starch and amylopectin were added in sterile distilled water and sterile filtered before use in the experiments. Endogenous colonic contents were collected after 30 days from mice fed either a high-amylose starch diet (28% RS, wt/wt) or an amylopectin diet containing an equal energy density, respectively (n = 10) (*8*, *9*). The cecal contents in succession were diluted with deionized water and sterile filtered, and a final solution was obtained (diluted 1:4 with deionized water), respectively. The final solution was filtered (∅ 0.20 μm) to eliminate any bacteria from the endogenous compounds and seeded into an agar dish 24 h prior to the experiment. The fermented cecal RS from amylose starch fed mice were previously measured as 243 ± 73, 160 ± 47, 59.0 ± 21, and 17.7 ± 5.2 μmol for total SCFAs, acetate, propionate, and butyrate, respectively.

In Vivo Assays. Control animals were fed freshly cultured *E. coli* only. In addition to feeding the *E. coli*, each experimental group was coadministered one of the following compounds: (1) amylose starch (0.25, 0.5, and 1%); (2) amylopectin (0.25, 0.5, and 1%, served as a control); (3) fermented RS (endogenous fermentation products of RS in high-amylose starch obtained from filtered cecal contents; see Starch Preparation); (4) no fermented RS (endogenous compounds from filtered cecal contents from mice fed amylopectin; see Starch Preparation); (5) sodium butyrate (300 μM); (6) sodium acetate trihydrate (5, 50, or 300 mM); and (7) sodium propionate (0.1, 7, or 100 mM) or tributyrin (50 μM, 500 μM, or 3 mM), respectively.

Standard *C. elegans* (NGM Agar) Plates. Three grams of NaCl, 20.0 g of Bacto-agar (Becton), 2.5 g of Bacto-peptone (Becton), 0.1% cholesterol solution (0.005/mL 95% ethanol), and 975 mL of dH_2O were mixed. The following were then added to the autoclaved solution: $CaCl_2$, 1 mL (1 M); $MgSO_4$, 1 mL (1 M); and KPO_4, 25 mL (1 M) at pH 6.

***E. coli* (OP50) Culture Medium.** Twenty-five grams of LB broth, 1 L of dH_2O (autoclave), one scoop (10 μL±) of *E. coli* (CGC), and 10 μL/mL streptomycin were mixed for 16 h at 37 °C in a shaker−incubator and stored at 4 °C. A final concentration of of $5 \times 10^8 - 5 \times 10^{11}$ cfu/mL (ad libitum) was obtained.

S-Basal Solution. NaCl (5.8 g, 0.1 M), KPO_4 (50 mL, 0.05 M) at pH 6, and cholesterol (1 mL) were made up to 1 L with dH_2O and autoclaved.

Fixative. Four percent paraformaldehyde and 0.4 M PBS were used.

PBS: 115 mM NaCl, 75 mM $Na_2HPO_4 \cdot 7H_2O$, and 7.5 mM KH_2PO_4, pH 7.4.

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

Statistical Analyses. Results are expressed as means ± SEM. Data were analyzed with one-way ANOVA Tukey's test (SigmaStat 3.5), and a P value of ≤0.05 was taken as significant.

RESULTS

All of the study groups (RS, endogenous cecal compounds, and SCFA) tested in the *C. elegans* showed reduction of the Nile Red positive staining for fat deposition.

RS. Concentrations of 0.25, 0.5, and 1% amylose starch containing RS or amylopectin were used (n = 4). After 7 days, the fluorescence intensity of the Nile Red positive intestinal fat deposition was significantly reduced to 76.5% in 1.0% amylose starch treated animals compared to the animals that received 1.0% amylopectin (P < 0.001, **Figure 1**). The lower concentrations of amylose starch had no significant effect on the intestinal fat deposition (data not shown).

Fermented RS from Filtered Cecal Contents from Mice. When filtered cecal contents from mice fed amylose starch that contained

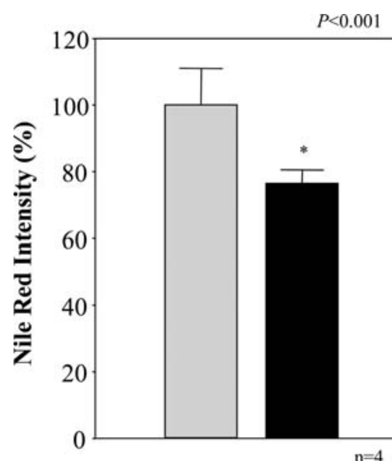

Figure 1. Nile Red staining of the intestinal fat in *C. elegans* (N2) fed with or without unfermented RS (1.0%, 7 days). The fluorescence intensity of the Nile Red positive intestinal fat was significantly reduced to 76.5% in animals fed 1.0% amylose starch (60%, Hi-maize 260, black bar) compared with animals that received amylopectin (AMIOCA, gray bar, $P < 0.001$).

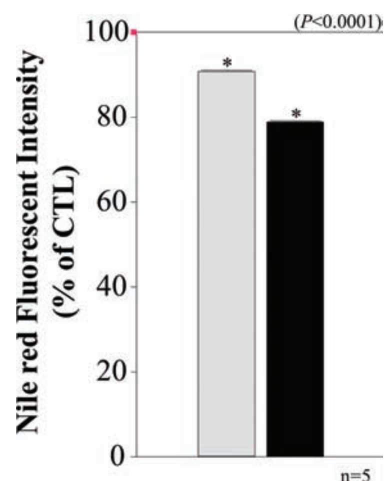

Figure 2. *C. elegans* fed with endogenous compounds from filtered cecal contents from amylose starch fed mice, from amylopectin fed mice. The reduction of Nile Red positive fat deposition showed trends similar in RS fed *C. elegans* (see **Figure 1**). The reduction was significantly decreased to 78.8% in fermented RS group (black bar, $n = 5$, from amylose fed mice,) compared to control group ($n = 5$, fed only *E. coli*) or compared to no fermented starch group (gray bar, $n = 5$, from amylopectin fed mice, 4 days, $P < 0.0001$).

fermented RS were fed to *C. elegans*, the same general results as observed for feeding of amylose starch and SCFAs (see below) were observed. The filtered cecal contents from mice fed amylose starch would contain endogenous compounds from fermentation of the RS component of the amylose starch. The fluorescence intensity of the Nile Red positive intestinal fat deposition in the *C. elegans* fed endogenous compounds from cecal contents of the amylose starch fed mice was significantly reduced (78.8%, 4 days) compared to the *E. coli* only fed *C. elegans* group ($n = 5$, $P < 0.001$) and the *C. elegans* fed filtered cecal contents from amylopectin fed mice ($n = 5$, 90.7%, $P < 0.001$, **Figure 2**).

Individual SCFAs. Feeding of SCFA to *C. elegans* showed similar effects to feeding of amylose starch or fermented RS from filtered cecal contents from amylose starch fed mice. The reduction was found in all treated animals (5 days). The fluorescence intensity of Nile Red positive intestinal fat depositions was significantly reduced in butyrate (300 μM) treated animals compared to control animals that were fed only *E. coli*. The fluorescence intensity in butyrate-fed animals was reduced to 63.6% of the control group ($n = 3$, $P < 0.001$, **Figure 3**). The sodium acetate trihydrate reduction was more potent than the reduction induced by sodium propionate. In the millimolar range, sodium acetate trihydrate induced a reduction from 60.7% (30 mM) to 46.6% (300 mM) ($n = 5$, $P < 0.001$) and sodium propionate induced a reduction to 79.8% ($n = 5$, 100 mM) compared to control. In the micromolar range, tributyrin induced a reduction to 30% (3 μM) compared to control ($n = 5$, $P < 0.001$, **Figure 4**).

DISCUSSION

We utilized the nematode model, wild-type *C. elegans*, to screen the fat deposition effects of amylose starch with a high content of resistant starch, fermented resistant starch (endogenous compounds from filtered cecal contents from mice fed amylose starch), and individual SCFAs. Our results indicate that they all reduce intestinal fat deposition in *C. elegans*. These outcomes are congruent with the findings in rodents. Thus, they are predictive of the results seen in higher animal species.

RS and their fermentation products have increasingly caught the attention of both the scientific and lay public due to its benefits related to the improvement of metabolic syndrome components and other obesity-related disease. Foods high in dietary fiber are known to increase bowel movements, eliminate fat from gastrointestinal tract, reduce body weight, decrease calorie intake, and elevate metabolic rate (*17−19*). We have previously demonstrated that amylose starch (RS_2) and the butyric acid that results from its fermentation reduce body fat, stimulate production of gut satiety hormones, increase insulin sensitivity, elevate energy expenditure, boost mitochondria function, and augment gastrointestinal motility in rodents (*5*, *8*, *9*, *17*, *20*). It needs to be pointed out that there are a variety of other poorly digested carbohydrates that offer the potential to enhance butyrate production in the lower gastrointestinal tract (*21*).

The fermentation products of RS_2 in the fluid of the large intestine are a complex mixture. Food intake containing RS directly alters the colonic microflora and increases formation of SCFAs, which are a major part of the mixture. The bacteria of the colonic microflora in different animal species vary in their effect on RS. Wang et al. found that after feeding amylose (400 g/kg) for ~30 days, butyrate concentration of the fecal slurry was ~30 mmol/mL (gas chromatograph) (*22*). By way of contrast, in the sheep rumen total fasted SCFA concentration is 50 mM, which reaches 160 mM with a ratio of 7:1.5:1.5 for acetic/propionic/butyric acids, respectively, but this ratio is higher in the reticulorumen (*23*). Our previous studies have demonstrated that SCFAs from fermentable RS have beneficial physiological effects in the prevention of overweight and obesity in rodents (*17−19*), and others demonstrated increased motility of the gastrointestinal smooth muscle in vitro (*24*).

The ultimate goal of our research is to optimize the type and source of RS that will prevent and/or reverse metabolic syndrome and obesity-related diseases. We took advantage of the *C. elegans* model when selecting an animal model for screening to detect the effects of bioactive materials on fat reduction. A procedure is carried out in the present study to remove the colonic contents and isolate the endogenous compounds, presumably SCFAs from ceca of the RS_2-fed mice. The material washed out of the ceca was sterile filtered prior to the study to ensure accuracy and purity. We verified the RS_2 content from the commercial product of RS (Hi-Maize 260) by differential scanning calorimetry (DSC). The trace amount of amylose detected in the amylopectin (AMIOCA), the commercial nonfermentable starch product that we used as a control, was insignificant.

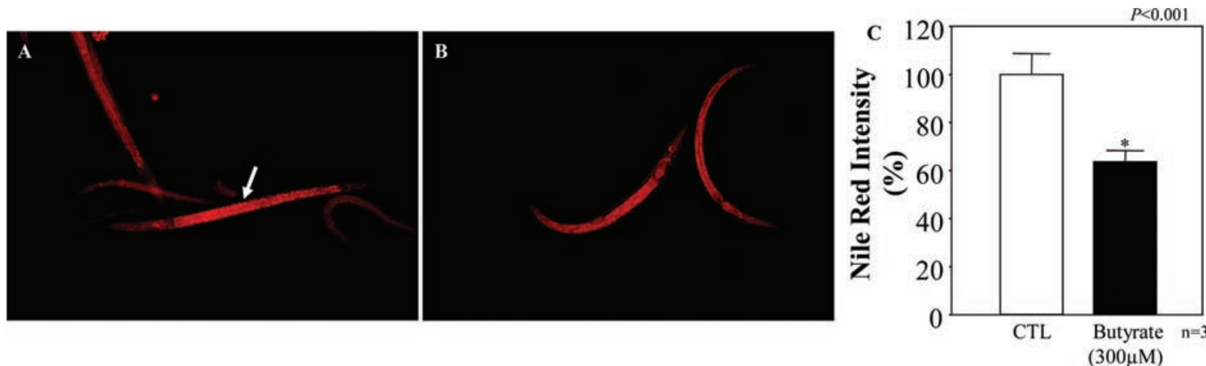

Figure 3. Butyrate treatment reduced Nile Red staining of the intestinal fat deposition in *C. elegans*. (**A**) The arrow points indicates intestinal fat droplets in the control animals that received only *E. coli*. (**B** and **C**) The fluorescence intensity of Nile Red positive intestinal fat deposition in butyrate (300 μM) treated animals was significantly reduced to 63.6% compared to the control group (black bar, $n = 3$, 5 days, $P < 0.001$).

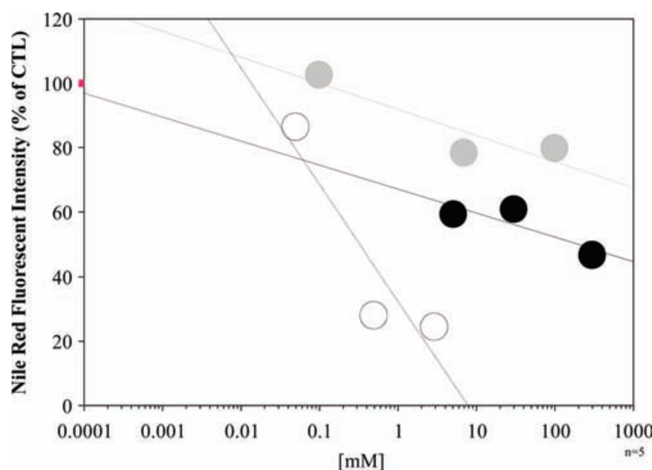

Figure 4. SCFA treatment reduces Nile Red positive intestinal fat deposition in *C. elegans*. The reduction was found in all treated animals. Sodium acetate trihydrate (black circles, $n = 5$), however, is more potent than sodium propionate (gray circles, $n = 5$) induced reduction, which was in the millimolar range. Tributyrin-induced reduction was in the micromolar range and was more potent than sodium acetate trihydrate (white circles, $n = 5$, 5 days, $P < 0.001$, see text).

C. elegans is an ectothermic animal, and a variety of metabolic rate measures have been reported despite its diminutive size (*25*−*27*). Nevertheless, measuring metabolic rate is beyond the scope of this study. One of the strengths of using the *C. elegans* model is the ability to monitor the Nile Red positive staining as a marker of intestinal fat deposition through their transparent bodies. The decreased intestinal fat deposition predicts an elevated energy expenditure, a reduction in food intake, or both. In the present study, reduction of the Nile Red positive staining of the intestinal fat deposition was detected across all three groups with remarkably distinguished efficacies. The reduction in Nile Red staining was dose dependent and required a concentration of at least 1.0% RS. The fermented RS (endogenous compounds in cecal contents from mice fed amylose) were diluted 4-fold to be able to pass through the sterile filter, which is necessary to eliminate bacteria from the compounds that were to be tested. The endogenous compounds are presumably highly bioactive, because only one-fourth of the original dose was used. The individual SCFAs provide more details about the efficacy. In the millimolar range, sodium acetate trihydrate is 20-fold more potent than sodium propionate, which can reduce cholesterol and improve metabolism (*28*). Tributyrin gives the most dramatic reduction in fat deposition with activity down to a micromolar concentration range. The performance of tributyrin, three butyric acids esterified to glycerol, is consistent with studies in rodents showing that butyric acid plays the major role in reducing body fat, preventing diet-induced obesity and averting insulin resistance in muscle, adipose tissue, and liver (*20*). Butyrate is a histone deacetylase (HDAC) inhibitor and regulates the transcription of several genes (*29*). Considering that butyrate contributes only ~7% to the total endogenous SCFA, the high efficacy of butyrate in the current study might be due to epigenetic factors involving histone deacetylation, which is known to contribute to the silencing of critical genes. In *C. elegans*, the *hda-1* gene encodes a histone deacetylase. Mutation of the *hda-1* gene [*hda-1*(*cw2*)] affects the development of the nervous system (*30*). The next step in our series of studies will focus upon developing a sensitive method to detect the concentration of the SCFAs fermented in *C. elegans* and to evaluate the epigenetic impact of the SCFAs in the *C. elegans* model.

Nematodes can survive under a hypoxic condition, and *C. elegans* has both an aerobic and an anaerobic metabolism (*31*). In *C. elegans*, *daf-22* and *dhs-28* are critical for synthesizing SCFAs and are expressed primarily in the intestine, the major organ for fatty acid metabolism (*32*, *33*). In general, oxygen diffuses bidirectionally between the intestinal tract and the external environment (through the cuticle). Vice versa, the intestinal "hypoxia gradient" can also diffuse outward from the interior of the alimentary tract to the exterior of the cuticle (*31*). The ingested *E. coli* tolerate anoxia as well. Fermentation pathways have been described in *C. elegans*, and fermentation products can diffuse across a hypoxic gradient when there is oxygen deprivation (*31*, *34*, *35*). Thus, living in an anaerobic environment or feeding on the products of anaerobic bacterial fermentation, *C. elegans* nutrition would closely resemble the effect of colonic RS fermentation and endogenous SCFAs on the gut in rodents or in humans. For example, one sees similar results from the culture of the *C. elegans* in aerobic conditions, the culture of *C. elegans* with the anaerobic bacteria of the gut microflora in the cecal contents, or the feeding of *C. elegans* with the culture medium in which the anaerobic bacteria are grown containing fermentation products. In the current study we cultured the *C. elegans* in aerobic conditions and fed the *C. elegans* either filtered RS or the cecal contents with fermentation products (SCFAs) of the gut microflora. In the former, the *C. elegans* would ferment the RS first. In the latter, the *C. elegans* would consume fermented products (SCFAs) directly.

In conclusion, RS serves as a functional food that reduces body fat deposition by an apparent inducement of negative energy balance in *C. elegans* through its fermentation products. Thus,

highly fermentable fiber sources can be screened with *C. elegans* to isolate fermentation components and identify the most effective bioactives for body fat reduction. The short lifespan, low cost, ease of cultivation, and predictive value for what will take place in higher species of animals make *C. elegans* a more time-efficient and cost-effective animal model than other preclinical screening methods.

ACKNOWLEDGMENT

We thank Dr. Yuan Luo (Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MD). Her suggestion was critical in the use of the *C. elegans* animal model for research. The nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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Received for review December 30, 2009. Revised manuscript received February 15, 2010. Accepted February 16, 2010. This study was supported by the LSU AgCenter.