FOOD & FUNCTION

Tolerance, fermentation, and cytokine expression in healthy aged male C57BL/6J mice fed resistant starch

June Zhou[1,2], Michael J. Keenan[3], Jeffrey Keller[1], Sun O. Fernandez-Kim[1], Paul J. Pistell[1], Richard T. Tulley[3], Anne M. Raggio[1,3], Li Shen[1,3], Hanjie Zhang[1,3], Roy J. Martin[1,3] and Marc R. Blackman[2,4]

[1] Pennington Biomedical Research Center, Louisiana State University System, Louisiana, USA
[2] Research Service, Veterans Affairs Medical Center, Washington DC, USA
[3] Louisiana State University Agricultural Center, Louisiana, USA
[4] George Washington University School of Medicine, Washington DC, USA

Health benefits of resistant starch (RS), a dietary fermentable fiber, have been well documented in young, but not in old populations. As the essential step of more comprehensive evaluations of RS on healthy aging, we examined the effects of dietary RS on tolerance, colonic fermentation, and cytokine expression in aged mice. Healthy older (18–20 months) C57BL/6J male mice were fed control, 18% RS, or 36% RS diets for 10 weeks. Body weight gain, body composition, and fat pad weights did not differ among the three groups after 10 weeks, indicating good tolerance of the RS diet. Fermentation indicators (cecum weights, and cecal proglucagon and PYY mRNA expression) were enhanced in an RS dose-dependent manner ($p < 0.01$). Serum concentrations of soluble cytokine receptors (sTNF-Rb, sIL-4R, sIL-2Rα, sVEGFR1, and sRAGE) and TNFα expression (gene and protein) in visceral fat did not differ significantly among groups. Adiponectin protein concentrations, but not gene expression, were greater in epididymal fat of the 36% RS versus control groups ($p < 0.05$). As a conclusion in aged mice, dietary RS is well tolerated, fermented in the colon, and stimulates colonic expression of proglucagon and PYY mRNA, and adiponectin protein in visceral fat.

Received: July 28, 2011
Revised: September 19, 2011
Accepted: October 7, 2011

Keywords:
Adiponectin / Aging / GLP-1 / Resistant starch / TNFα

Resistant starch (RS) is a fermentable dietary fiber used as a carbohydrate source in food. Multiple animal and human studies in young populations have shown that dietary RS improves health indicators associated with aging [1–3], including improving gastrointestinal health [4–6], glucose tolerance and insulin sensitivity [7–11], and bone density [12, 13]. However, the benefits of dietary RS in older populations are not clear.

RS resists digestion in the small intestine and is fermented in the large intestine by gut microflora. Beneficial effects of dietary RS are dose related [14]. Increased dietary RS consumption augments RS fermentation in the large intestine, which may cause gastrointestinal discomfort. Importantly, certain benefits of dietary RS are also associated with its colonic fermentation, such as expression of the gut anti-obesity/diabetic hormones GLP-1 and PYY, and body fat loss [10, 15, 16]. At the dosages that show benefits, young rodents and humans tolerate fermentation of dietary RS well [4, 9–12, 16–19]. Given that gut microflora are altered with aging [20], fermentation and toleration of dietary RS may also change with aging. Thus, we investigated tolerance, fermentation, tissue and systemic cytokine expression in healthy aged mice fed two dosages of dietary RS.

Fifty healthy male C57BL/6J mice (18–20 months, analogous to 56–65 years of age in humans (http://research.jax.org/faculty/harrison/ger1vLifespan1.html). [21]) were divided into three dietary treatment groups for a 10-wk study: control diet, 18% RS diet, or 36% RS diet. Amioca® cornstarch

Correspondence: Dr. June Zhou, Research Service (151), Veterans Affairs Medical Center, 50 Irving Street NW, Washington DC 20422, USA
E-mail: june.zhou@va.gov
Fax: +1-202-518-4611

Abbreviations: GI, gastrointestinal; **GLP-1,** glucagon-like peptide-1; **RS,** resistant starch

(100% amylopectin) and Type 2 RS, (Hi-maize 260®) were used as starch sources in control and RS diets (for housing and diet details, see Supporting information). All three diets had the same metabolizable energy density and micro/macro-nutrient contents. The protocol was approved by the Animal Care and Use Committees of the Pennington Biomedical Research Center (#419) and the Washington DC VA Medical Center (01282). Data were analyzed by one-way ANOVA followed by an *F*-protected *t*-test. Data obtained from mice with tumors were not included for statistical analyses.

Colonic fermentation of dietary RS may cause chronic gastrointestinal discomforts and change appetite, food intake and body weight. Thus, we measured body weight changes over a 10-wk study period as indirect indices of chronic tolerance of dietary RS. Body composition and tissue weights were also recorded as general indicators of tolerance to the RS diets. At week 8, total body fat, free body fluid (body fluids not bound in tissues), and total lean body mass measured by NMR were not differ among groups (Supporting information). At the end of the study, total abdominal fat was calculated as the sum of the epididymal, perirenal, and retroperitoneal fat pads. Percentages of body fat were calculated as the total abdominal fat divided by the disemboweled body weights (obtained by subtracting the full gastrointestinal (GI) weight from the whole body weight). Disemboweled body weight was used here because of the significantly greater GI weight in RS fed mice. RS and control diets fed mice gained similar amounts of body weight, and exhibited similar body compositions, and body fat, liver and spleen weights ($p > 0.05$, Table 1). These results suggest that aged mice tolerated the 18 and 36% RS diets well over a 10-wk study period.

Prior reports demonstrated that when control and RS diets had the same metabolizable energy density, it took at least 12 wk for RS fed young rodents to lose body fat [15, 16, 22]. Thus, the study duration of 10 wk allowed us to examine possible changes in body weight and composition that affected by adverse effects of the RS diet, such as chronic GI discomfort. It remains to be determined whether a longer duration of RS ingestion would decrease body fat in old mice.

Fermentation of the RS diet in aged mice was measured at the end of study. Although aging affects certain gut microflora [20], dietary RS is still fermented in the colon/cecum of aged mice. Full GI tract (from proximal stomach to anus) weight, full cecum weight, empty cecum weight and cecal mRNA levels of proglucagon (gene encodes GLP-1) and PYY were used as indicators of cecal fermentation of dietary RS. All these indicators were increased in RS versus control fed mice ($p < 0.01$, Fig. 1). Moreover, GI and cecal weights, and proglucagon and PYY gene expression increases were all greater in mice fed 36 versus 18% RS diets. Thus, similar to young mice, dietary RS can be fermented in a dose responsive manner in aged mice.

Because age-related perturbations in body composition and metabolic outcomes are often associated with alterations in pro-inflammatory cytokine and adipocytokine activity, we measured selected cytokine or cytokine receptor levels in serum, liver and fat pads as biomarkers of systemic and tissue inflammation. Serum concentrations of soluble cytokine receptors (sTNF-Rb, sIL-4R, sIL-2Rα, sVEGFR1, sRAGE) measured by MILLIPLEX Mouse Soluble Cytokine Receptor Panel did not differ among groups (Supporting information). Similarly, expression of adiponectin, TNFα, IL-6, and leptin mRNA, measured by real time RT-PCR (Taqman® method, Applied Biosystems) in epididymal and retroperitoneal fat and did not different among groups (data not shown). Adiponectin and TNFα protein concentrations in epdidymal fat and liver were determined by ELISA (see Supporting information for details). Adiponectin concentrations in epididymal fat were increased in RS fed mice, with values in mice fed 36% RS greater than those of controls ($p < 0.05$). Liver adiponectin and TNFα in epididymal fat or liver were not different among the three treatment groups (Fig. 2). Adiponectin and TNFα protein

Table 1. Body weight, liver, spleen, and fat depot weights in control and RS treatment groups at the end of study

Number of mice (beginning)	Control 17	18% RS 17	36% RS 16	*p*-Value
Initial body weights (g)	34.85 ± 0.69	34.69 ± 0.43	34.56 ± 1.08	> 0.05
Final body weights (g)	36.68 ± 1.18	39.17 ± 0.73	37.58 ± 1.14	> 0.05
Disemboweled body weights (g)	34.42 ± 1.18	36.20 ± 0.73	33.84 ± 1.13	> 0.05
Liver weights (g)	1.59 ± 0.07	1.90 ± 0.16	1.62 ± 0.07	> 0.05
Spleen weights (g)	0.113 ± 0.009	0.168 ± 0.038	0.112 ± 0.011	> 0.05
Epididymal fat (g)	1.851 ± 0.187	1.967 ± 0.163	1.789 ± 0.182	> 0.05
Perirenal fat (g)	0.379 ± 0.040	0.423 ± 0.033	0.328 ± 0.041	> 0.05
Retroperitoneal fat (g)	0.568 ± 0.061	0.589 ± 0.045	0.507 ± 0.053	> 0.05
Total fat (g)	2.798 ± 0.280	2.978 ± 0.236	2.625 ± 0.266	> 0.05
% Fat/body weight	7.84 ± 0.61	8.10 ± 0.53	7.55 ± 0.55	> 0.05
Number of mice that died during the study	1	1	0	
Number of mice with tumor(s) at the end of study	1	2	1	

Data shown are mean ± SE values.

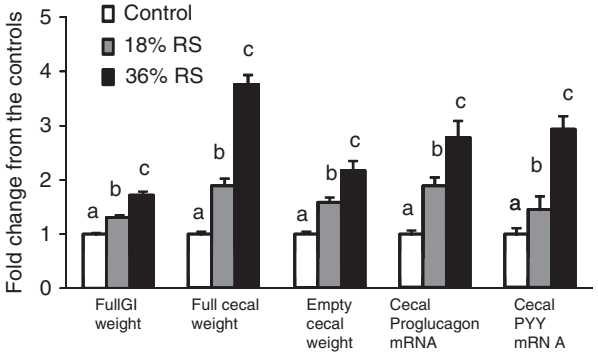

Figure 1. Fermentation indicators (full GI weight, full cecal weight, empty cecal weight, cecal proglucagon and PYY gene expression) of mice fed control, 18% RS, and 36% RS diet for ten weeks. Tissues were collected by dissection. mRNA was measured by quantitative RT-PCR. Data shown are mean ± SE (n = 13–15 per group), and are expressed as fold changes in RS versus control mice. Values with different letters indicate significant differences ($p < 0.01$).

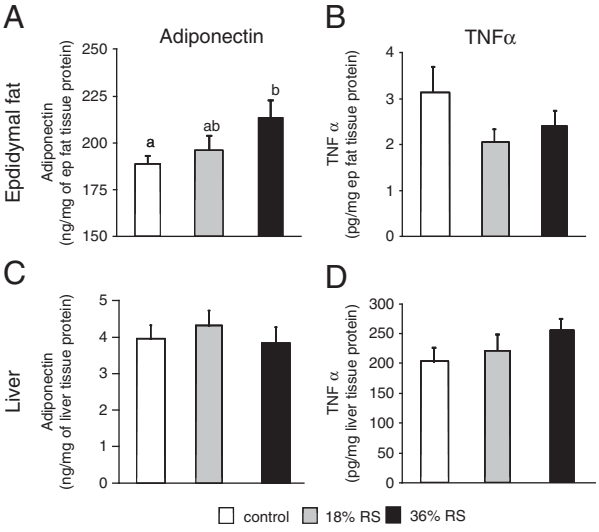

Figure 2. Adiponectin (A, C) and TNFα (B, D) protein levels in epdidymal fat (A, B) and liver (C, D) of aged mice fed control diet, 18% RS, or 36% RS diet for ten weeks. Adiponectin and TNFα concentrations were detected by ELISA. Data shown are mean ± SE (n = 12–15 per group). Values with different letters indicate significant differences ($p < 0.05$).

concentrations were not measured in retroperitoneal fat as there was insufficient tissue for the measurements.

The aims of the current study were to examine the effects of chronic dietary RS ingestion on tolerance, fermentation ability, and systemic and tissue cytokine expression in healthy aged mice. We compared two different doses of RS diets with a control diet. In prior studies, 10–35% RS (w/w) diets produced health benefits in young animals [10, 11, 15–17, 23–28]. Thus, our results provide useful dosage information that is necessary for varies evaluations of dietary RS as a multifaceted nutritional approach to enhance healthy aging.

Additional, two benefits of dietary RS were noted in the aged mice: increased cecal proglucagon expression and increased adiponectin level in visceral fat. Although circulating GLP-1 was not measured due to limited blood volume in current study, the increased cecal proglucagon expression is always associated with increased circulating GLP-1 in young rats [10, 11, 16]. GLP-1 can reverse the age-related decline in glucose tolerance [29] in part by increasing pancreatic insulin, glucose transporter 2 and glucokinase mRNA, suggesting that GLP-1 reverses some of the aging defects that arise in pancreatic beta cells. GLP-1 receptor stimulation also reduces amyloid-β peptide accumulation in animal models of Alzheimer's disease [30]. Finally, GLP-1 preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinson's disease [31]. Further studies are necessary to determine whether aged mice, like their younger counterparts, would benefit from increased expression of proglucagon by dietary RS.

Of note, plasma adiponectin was not changed by dietary RS in young people [9]. Thus, the increased visceral fat adiponectin may only improve insulin sensitive in visceral fat and is not accompanied by an increase in circulating adiponectin improve in aged mice.

In conclusion, our data suggest that in aged male C57BL/6J mice, dietary RS is well tolerated, is fermented in the colon, stimulates gut proglucagon and PYY expression, and increases adiponectin in visceral fat. Further studies are required to determine the metabolic and other physiologic consequences of RS ingestion in young and old rodents and humans.

This research was supported by NIH R21 DK073403 to Dr. Roy J. Martin, Dr. June Zhou and Dr. Michael Keenan; by Pennington Biomedical Research Center intramural funds to Dr. Jeffrey Keller, and by DCVAMC intramural funds to Dr. Marc Blackman. Dr. Roy Martin and Dr. Michael Keenan received research funding from National Starch, LLC. This paper was approved for publication by the Director of Louisiana Agricultural Experiment Station as manuscript no. 2011-239-6324.

The authors have declared no conflict of interest.

References

[1] Haralampu, S., Resistant starch – a review of the physical properties and biological impact of RS. *Carbohydr. Polysacch.* 2000, 285–292.

[2] Higgins, J. A., Resistant starch: metabolic effects and potential health benefits. *J. AOAC Int.* 2004, *87*, 761–768.

[3] Kendall, C. W., Emam, A., Augustin, L. S., Jenkins, D. J., Resistant starches and health. *J. AOAC Int.* 2004, *87*, 769–774.

[4] Birkett, A., Muir, J., Phillips, J., Jones, G., O'Dea, K., Resistant starch lowers fecal concentrations of ammonia and phenols in humans. *Am. J. Clin. Nutr.* 1996, *63*, 766–772.

[5] Bird, A. R., Brown, I. L., Topping, D. L., Starches, resistant starches, the gut microflora and human health. *Curr. Issues Intest. Microbiol.* 2000, *1*, 25–37.

[6] Ahmed, R., Segal, I., Hassan, H., Fermentation of dietary starch in humans. *Am. J. Gastroenterol.* 2000, *95*, 1017–1020.

[7] Behall, K. M., Howe, J. C., Effect of long-term consumption of amylose vs amylopectin starch on metabolic variables in human subjects. *Am. J. Clin. Nutr.* 1995, *61*, 334–340.

[8] Brennan, C. S., Dietary fibre, glycaemic response, and diabetes. *Mol. Nutr. Food Res.* 2005, *49*, 560–570.

[9] Johnston, K. L., Thomas, E. L., Bell, J. D., Frost, G. S., Robertson, M. D., Resistant starch improves insulin sensitivity in metabolic syndrome. *Diabet. Med.* 2010, *27*, 391–397.

[10] Zhou, J., Martin, R. J., Tulley, R. T., Raggio, A. M. et al., Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am. J. Physiol. Endocrinol. Metab.* 2008, E1160–E1166.

[11] Shen, L., Keenan, M. J., Raggio, A., Williams, C., Martin, R. J., Dietary-resistant starch improves maternal glycemic control in Goto-Kakizaki rat. *Mol. Nutr. Food Res,* 2011, *55*, 1499–1508.

[12] Tousen, Y., Abe, F., Ishida, T., Uehara, M., Ishimi, Y., Resistant starch promotes equol production and inhibits tibial bone loss in ovariectomized mice treated with daidzein. *Metabolism* 2011, *60*, 1425–1432.

[13] Bogden, J. D., Kemp, F. W., Huang, A. E., Shapses, S. A. et al., Bone mineral density and content during weight cycling in female rats: effects of dietary amylase-resistant starch. *Nutr. Metab. (London)* 2008, 5, 34.

[14] Penn-Marshall, M., Holtzman, G. I., Barbeau, W. E., African americans may have to consume more than 12 grams a day of resistant starch to lower their risk for type 2 diabetes. *J. Med. Food* 2010, 13, 999–1004.

[15] Zhou, J., Martin, R. J., Tulley, R. T., Raggio, A. M. et al., Failure to ferment dietary resistant starch in specific mouse models of obesity results in no body fat loss. *J. Agric. Food Chem.* 2009, *57*, 8844–8851.

[16] Keenan, M. J., Zhou, J., McCutcheon, K. L., Raggio, A. M. et al., Effects of resistant starch, a non-digestible fermentable fiber, on reducing body fat. *Obesity (Silver Spring)* 2006, *14*, 1523–1534.

[17] Shen, L., Keenan, M. J., Martin, R. J., Tulley, R. T. et al., Dietary resistant starch increases hypothalamic POMC expression in rats. *Obesity (Silver Spring)* 2009, *17*, 40–45.

[18] Hylla, S., Gostner, A., Dusel, G., Anger, H. et al., Effects of resistant starch on the colon in healthy volunteers: Possible implications for cancer prevention. *Am. J. Clin. Nutr.* 1998, *67*, 136–142.

[19] Cheng, H. H., Lai, M. H., Fermentation of resistant rice starch produces propionate reducing serum and hepatic cholesterol in rats. *J. Nutr.* 2000, *130*, 1991–1995.

[20] Hebuterne, X., Gut changes attributed to ageing: Effects on intestinal microflora. *Curr. Opin. Clin. Nutr. Metab. Care* 2003, *6*, 49–54.

[21] Harrison, D. E., How we define the life history phases of mice; how they compare with humans. *Life Span Biomarker* 2011.

[22] Pawlak, D. B., Kushner, J. A., Ludwig, D. S., Effects of dietary glycaemic index on adiposity, glucose homoeostasis, and plasma lipids in animals. *Lancet* 2004, *364*, 778–785.

[23] Bajka, B. H., Clarke, J. M., Cobiac, L., Topping, D. L., Butyrylated starch protects colonocyte DNA against dietary protein-induced damage in rats. *Carcinogenesis* 2008, *29*, 2169–2174.

[24] de Deckere, E. A., Kloots, W. J., van Amelsvoort, J. M., Resistant starch decreases serum total cholesterol and triacylglycerol concentrations in rats. *J. Nutr.* 1993, *123*, 2142–2151.

[25] Higgins, J. A., Brown, M. A., Storlien, L. H., Consumption of resistant starch decreases postprandial lipogenesis in white adipose tissue of the rat. *Nutr. J.* 2006, *5*, 25.

[26] Le Leu, R. K., Brown, I. L., Hu, Y., Young, G. P., Effect of resistant starch on genotoxin-induced apoptosis, colonic epithelium, and lumenal contents in rats. *Carcinogenesis* 2003, *24*, 1347–1352.

[27] Lopez, H. W., Levrat-Verny, M. A., Coudray, C., Besson, C. et al., Class 2 resistant starches lower plasma and liver lipids and improve mineral retention in rats. *J. Nutr.* 2001, *131*, 1283–1289.

[28] Morita, T., Kasaoka, S., Ohhashi, A., Ikai, M., Numasaki, Y., Kiriyama, S., Resistant proteins alter cecal short-chain fatty acid profiles in rats fed high amylose cornstarch. *J. Nutr.* 1998, *128*, 1156–1164.

[29] Wang, Y., Perfetti, R., Greig, N. H., Holloway, H. W. et al., Glucagon-like peptide-1 can reverse the age-related decline in glucose tolerance in rats. *J. Clin. Invest.* 1997, *99*, 2883–2889.

[30] Li, Y., Duffy, K. B., Ottinger, M. A., Ray, B. et al., GLP-1 receptor stimulation reduces amyloid-beta peptide accumulation and cytotoxicity in cellular and animal models of Alzheimer's disease. *J. Alzheimers Dis.* 2010, *19*, 1205–1219.

[31] Li, Y., Perry, T., Kindy, M. S., Harvey, B. K. et al., GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. *Proc. Natl. Acad. Sci. USA* 2009, *106*, 1285–1290.

