A microarray study indicates high-amylose resistant starch increases hormones and improves structure and function of the GI tract

Michael J Keenan[1], **Roy J Martin**[1,2], **Anne M Raggio**[1], **Kathleen L McCutcheon**[1], **Ian L Brown**[3], **Anne Birkett**[4], **Susan S Newman**[2], **Jihad Skaf**[5], **Maren Hegsted**[1], **Richard T Tulley**[1], **Eric Blair**[5], and **June Zhou**[6]

[1]Louisiana State University Agricultural Center, Baton Rouge, LA

[2]Pennington Biomedical Research Center, Baton Rouge, LA

[3]Clover Corporation, Gymea NSW 2227, Australia

[4]Corn Products International, Chicago, IL

[5]Life Technologies, Carlsbad, CA

[6]Laboratory of Geriatric Endocrinology and Metabolism, Veterans Affairs Medical Center, Washington, D.C.

Abstract

Background/Aims—Type 2 resistant starch from high-amylose maize (HAM-RS2) is associated with increased fermentation, increased expression of proglucagon (gene for GLP-1) and peptide YY (PYY) genes in the large intestine, and improved health. To determine what other genes are up- or down-regulated with feeding of HAM-RS2 a microarray was performed.

Methods—Adult, male SD rats were fed one of the following three diets for a four week study period: cornstarch control (CC, 3.74 kcal/g), dietary energy density control (EC, 3.27kcal/g), and 30% HAM-RS2 (RS, 3.27 kcal/g). Rat microarray with ~27,000 genes and validation of 94 representative genes with multiple qPCR were used to determine gene expression in total RNA extracts of cecal cells from rats. The RS vs. EC comparison tested effects of fermentation as energy density of the diet was controlled.

Results—For the RS vs. EC comparison, 86% of the genes were validated from the microarray and the expression indicates promotion of cell growth, proliferation, differentiation, and apoptosis. Gut hormones GLP-1 and PYY were increased.

Conclusions—Gene expression results predict improved **structure and function of the GI tract** and production of gut hormones **may** promote healthy functions beyond the GI tract.

Keywords

high-amylose maize; resistant starch; microarray; validation; cecal cells; GI tract

Corresponding Author: Michael J Keenan, LSU AgCenter, 209 Knapp Hall, Corner of Highland and South Stadium, Baton Rouge, LA 70803; phone: 225-578-1708; fax: 225-578-4443, mkeenan@agctr.lsu.edu.

Introduction

Although the mechanism remains unclear, dietary resistant starches have been shown to decrease plasma cholesterol and triglycerides, increase insulin sensitivity, decrease fat storage, and produce anticancer effects in humans [1-13]. The current research is to gather gene expression data that will help identify mechanisms by which resistant starch improves health. Resistant starches (RS) are one type of non-digestible, fermentable fiber. Generally, RS is categorized into 4 categories [14]: RS1 is a result of the structured food matrix in unprocessed whole grains, seeds or legumes; RS2 is generally high amylose, but is primarily the result of an organized granular structure tightly packed in a radial pattern as in high amylose cornstarch products; RS3 is retrograded starch as in cooked then cooled potatoes, and RS4 is considered chemically modified starch with either constituent groups attached or linkages between starch molecules. RS potentially will have three major effects when included in the diet: 1) dilution of dietary energy as the metabolizable energy of one type of RS2, HAM-RS2, is 2.8 kcal/g compared to the 3.6 to 4 kcal/g of typical starches [10], 2) a bulking effect similar to non-fermentable fiber, and 3) fermentation to short chain fatty acids and an increase in expression of genes in the gut and peptide hormones in plasma [5, 8, 9, 11, 12].

RS is fermented to short chain fatty acids by microflora in the large intestine [5, 8]. The colonic fermentation of one specific form of RS from high-amylose maize, HAM-RS2, has been shown to elicit several beneficial changes such as increased cecal and large intestine gene expression of peptide YY (PYY) and proglucagon (gene for GLP-1), increased plasma levels of PYY and glucagon-like peptide-1 (GLP-1) and decreased abdominal fat [5, 8, 9]. Thus, it is important to understand the full scale of gene expression changes by HAM-RS2 in the cecum, where the fermentation occurs. However, our previous studies have been limited to a handful of genes using real time reverse transcriptase polymerase chain reaction (qPCR) and peptide hormone assays. The current study is a microarray with ~27,000 genes of the rat genome followed by a validation or focusing of the microarray that was performed on 94 genes. We compared gene expression profiles in cecal cells of rats fed a HAM-RS2 diet with that of rats fed two types of control diets. One, a standard AIN-93G diet with added cornstarch (CC = control cornstarch) that is greater in metabolizable energy than the RS diet [15]. The second with equal metabolizable energy density due to the addition of cellulose (EC = energy density control). The inclusion of the EC diet allowed for testing of fermentation separate from energy density differences.

Methods

Animals and Diets

Forty-five eight week old male Sprague Dawley (Harlan, Indianapolis, IN) rats (n=15/ treatment; three separate orders from Harlan Co., n=5/treatment) were fed treatment diets for 4 weeks: a rapidly-digestible cornstarch control (CC, 3.74 kcal/g), an energy control with a rapidly-digestible cornstarch and purified, non-fermentable cellulose added to dilute metabolizable energy (EC, 3.27 kcal/g), and the resistant starch treatment (RS, 3.27 kcal/g) using a fermentable, high amylose cornstarch resistant starch category 2, HAM-RS2 (Table

1). Note that the staggering of orders equalized for the three treatments was to control for condition of rats at one order time vs. another time. The EC and RS diets have equal energy density. Energy content of the starches was calculated based on an approximate metabolizable energy content of 2.8 kcal/g for the high-amylose cornstarch [10, 16] and using the Atwater factor of 4.0 kcal/g for the rapidly-digestible cornstarch (4.0 kcal/g). A modified version of the Englyst method [17] was used to derive an estimate of 56% resistant starch for the HAM-RS2. Thus, the HAM-RS2 diet had 530.7 g of Hi-maize® starch and 297 g of resistant starch, or 29.7% of the weight of the diet (Table 1).

Rats were killed by heart puncture and plasma was collected for analysis of peptide YY and glucagon-like peptide-1 by RIA (Phoenix, Burlingame, CA) and ELISA (Millipore, Billerica, MA), respectively. The full ceca were excised and weighed, and an aliquot of cecal contents was removed for measurement of pH. In addition, three abdominal fat pads, retroperitoneal, perirenal and epididymal, were also removed and weighed. After cecal contents were removed, the cecum was carefully opened with scissors and the inner surface of the cecum was rinsed with sterilized saline three times. Cecal cell samples used for RNA extraction were collected by gently scraping the inner cecal surface. This layer of cells includes epithelial (colonocytes) cells, nerve cells, endocrine cells and immune cells, which represent all cell types that would be exposed to short chain fatty acids, the fermentation products of RS in the cecum. Previously Chen et al. [18] used a similar approach for collecting cells of the proximal colon for a gene array with dietary fibers.

RNA and cDNA

RNA was isolated from cecum cell scrapings using the Qiagen (Valencia, CA) RNeasy mini kit with an additional on column DNase digestion. RNA samples were eluted with RNase free water. RNA concentrations and integrities were determined using an Agilent Bioanalyzer (Foster City, CA). Only RNA samples with a concentration over 2.3 ng/ul and an RNA integrity number greater than 8.0 were used. The best total RNA samples from each time period **(see animal section above)** were used (maximum of three except for one time period with n=4 for RS). Thus, the total n for the three groups was: RS, n=10; and CC and EC, n=9. The Applied Biosystems (Carlsbad, CA) Chemiluminescent RT Labeling Kit was used to produce digoxygenin (DIG)-labeled cDNA for hybridization to the microarray.

Microarray Procedure

We used the Applied Biosystems rat genomic chips (~27,000 genes). From each sample, 10 ug of labeled cDNA was hybridized to a microarray and processed for scanning using the Applied Biosystems Chemiluminescent Detection Kit. Microarrays were scanned by an Applied Biosystems 1700. ABarray software (Bioconductor.org) was used to quantile normalize the signal across all arrays. It filtered the data to retain genes having a signal to noise ratio 3. The data were also filtered to remove data derived from array spots which were flagged as defective by AB1700 software. Fold-change, $\log_2$ fold-change, p-value, Benjamini Hochberg adjusted p-value, and fold-change bin were provided for each detected gene or transcript. Three gene lists from ABarray pairwise comparisons CC-EC, CC-RS, and EC-RS were generated. The Protein Analysis through Evolutionary Relationships (PANTHER, www.pantherdb.org) software was used to determine if significantly affected

genes were known to be assigned to a biochemical pathway or were unclassified at the present time. Analysis was performed with and without Bonferoni adjustment to determine statistical significance of pathway gene hits. In addition, the software was used to determine if molecular functions of the unclassified genes were known. Additionally, Spotfire® software was used for hierarchical clustering.

Validation Procedure

We used Applied Biosystems 384 well TaqMan® Custom Arrays for qPCR to focus on and confirm 94 genes from the microarray results. The 96a format was chosen which includes one mandatory control, 18SrRNA, and allows four samples per 384 well array. Another control, cyclophilin F, was also included. The total n for the three groups was: RS and EC, n=8; and CC, n=7 and each of these biological samples were run in triplicate. The RS vs. EC comparison was considered the most important of the three comparisons as it represents the effect of resistant starch beyond simple energy dilution of the diet. Narrowing our list to 94 genes was accomplished by choosing genes with the greatest fold changes and with the lowest p values. Only genes that were inventoried by Applied Biosystems could be included in the custom arrays. Additionally, neurotensin, a peptide hormone with a high fold change and very low p value, was run separately from the custom arrays. The 18S rRNA gene was used as the reference gene in analyses as it was more consistently expressed than cyclophilin F from sample to sample and between experimental groups. The custom array data were normalized using 18S followed by fold-change calculation using the CT method (2^{-Ct}) [19]. These values were submitted to ABqPCR software to determine p values using t-tests for comparisons of CC-EC, CC-RS, and EC-RS. The average values for each gene in each group were used to determine fold changes (FC).

Results

Microarray

Over 10,000 genes were considered "present" (the signal to noise ratio was 3) for all three comparisons. The RS vs. CC comparison had the greatest number of differences in mRNA expression for genes out of the possible ~27,000 genes measured on the microarrays. The greater number of differentially expressed genes for RS vs. CC appears logical as this comparison represents the greatest dietary differences that include dilution of dietary energy density and fermentation with the addition of HAM-RS2 to the diet. A summary of the microarray results is shown in table 2. Strong global differences among the three groups are depicted in a hierarchical cluster using Z scores from signal intensities of genes that had significantly different FC at p<0.01 (Figure 1).

Validation

The genes chosen from the microarray for validation have their microarray results listed in tables 3, 4 and 5 with names, abbreviations, fold changes, p values, category of function (pathway or unclassified), and NCBI Gene ID number. Panther gene expression analysis without the Bonferoni adjustment for the RS vs. EC comparison, demonstrated that our microarray gene list contained genes significantly over-represented in 44 pathways. One gene did not amplify in the TaqMan® custom arrays so we report on 93 genes from the

custom arrays (Figures 2-4). For the RS vs. EC comparison, there was validation of 86% of the genes as 80 out of 93 genes that had a significant difference in fold change with the microarray showed a significant difference with the custom arrays. Chosen from the microarray were 74 genes with a FC>1 and low p values. Seventy-two of these genes were significant with the custom arrays. Of the 19 genes with a FC<1 and low p values with the microarray, eight were significant using qPCR. Two genes changed from a significant FC<1 to significant with a FC>1. In Figure 5, RS vs. EC is compared to RS vs. EC for similarities and differences in numbers of genes that were either up-regulated, not different, or down regulated grouped according to four broad categories of function. A hierarchical cluster for the RS vs. EC comparison is shown in figure 6. Neurotensin was analyzed separately by individual qPCR. From the microarray and PANTHER results, the FC for RS vs. EC for neurotensin was 8, the p value<0.0001, and the pathway assignment was unclassified. The individual qPCR results for neurotensin for RS vs. EC, RS vs. CC and CC vs. EC were: FC = 4.59, p<0.0001; FC = 4.17, p<0.0001; FC = 1.1, NS; respectively. Thus, the microarray results were largely confirmed.

Other Results

At the end of this four-week study period, the RS and EC groups had reduced abdominal fat pads and body weights compared to the CC group, but RS and EC were not statistically different (data not shown). This is in contrast to our previous observations where rats fed HAMS-RS2 for 12 weeks had a significantly lower abdominal fat than the rats fed the EC diet [5, 9, 13]. Thus, HAM-RS2 needs to be consumed for more than four weeks to elicit effects on adiposity **when rodents are consuming an equal energy density diet and the difference between diets is the presence of higher levels of fermentable carbohydrate**. The greater body weights and abdominal fat for CC rats were likely the result of their greater energy intake for CC compared to RS and EC (data not shown). Food intake (grams) was similar among groups, but the higher energy density of the CC diet resulted in greater energy intake. The RS groups had significantly greater (p<0.0001) full cecal weights than the CC and EC groups (RS, 16.7; CC, 2.6; EC 3.2±0.9 g pooled error), while the CC and EC groups were not different. Cecal contents pH was much lower (p<0.0001) for RS than CC and EC (RS, 5.97; CC 8.14; EC, 8.49±0.05 pooled error), and CC cecal contents pH was also significantly lower (p<0.0001) than EC. Plasma total PYY (Figure 6a) and GLP-1 (Figure 6b) were increased for RS compared to CC and EC. The main goal of this microarray study was to examine the changes of gene expression profile in cecal cells with enhanced gut fermentation caused by dietary HAM-RS2. These results demonstrate that the fermentation of HAM-RS2 is established at the end of four week period.

Discussion

The aim of the current study was to use microarray technology to analyze expression of many genes in a single experiment quickly and efficiently to understand the fundamental aspects of mechanisms related to colonic fermentation of HAM-RS2 diets that may improve health. Adding HAM-RS2 to a diet by replacing other starches would lower the energy density of the diet, which is an important contributor for causing change in the gene expression profile in the gut. We used an additional control group (EC group) to control for

this confounding factor. Adding HAM-RS2 to a diet also increases the amount of fermentation in the large intestine, particularly the cecum. Fermentation of HAM-RS2 in the cecum was previously shown to be associated with increased expression of cecal genes for PYY and proglucagon [5, 11], increased plasma levels of PYY and GLP-1 [5, 9, 12], reduced abdominal fat [5, 8, 9, 13], and increased proopiomelanocortin (POMC) in the arcuate nucleus of the hypothalamus [9]. Thus, in the current study, we used three treatment groups to assess the effects of simple energy dilution (CC vs. EC), fermentation with similar energy density of diets (RS vs. EC) or both energy density dilution and fermentation (RS vs. CC) on global gene expression of cells from the cecum of rats.

Fermentation of HAM-RS2 leads to an increase in short chain fatty acids in the lumen of the cecum, which affects all cells on the cecal inner surface. Our cecal cell collection method, also similar in a recent study [18], allowed us to examine a broad view of gene expression affected by HAM-RS2 fermentation. In a previous study, fermentation was evident through measuring short chain fatty acids in cecal contents after 1 week of feeding of HAM-RS2 [5]. After four weeks in the current study, HAM-RS2 fed rats had increased full cecal weights, decreased cecal contents pH, and increased plasma levels of PYY and GLP-1. All of these are well known indicators of fermentation. Thus, the four week study period allowed us to focus on changes in gene expression profile in cecal cells that resulted from fermentation of HAM-RS2. Beginning the study with 8 week old rats meant that the rats may be considered not quite adults. However, many of our studies use about this age as a starting age. This may mean that some of the results in our studies may be due to adolescent growth.

The results from the microarray indicated that about 38% of the genes of the rat genome (10,000+/~27,000) were expressed above an acceptable level of noise (S/N 3) from the sampled cells (Table 2). It was also validly hypothesized that the RS vs. CC comparison should have the greatest number of gene differences because this comparison represents both a difference in energy density of the diet and greater fermentation with RS compared to CC. Hierarchical clusters of the microarray results (Figure 1) also demonstrated that there were global differences in the differential expression results of genes for RS compared to both the CC and EC groups.

Originally the search for PANTHER pathways was for those that had a significant number of genes overrepresented and differentially expressed in an amount greater than by chance. Initially the searches were restricted with Bonferoni adjustment. .However, our next step was to focus on the RS vs. EC comparison for validation of the microarray results. As a result we expanded the number of PANTHER pathways by removing the Bonferoni adjustment. This was all dictated by our major goal, which was to examine the beneficial effects of fermentation beyond simple energy density dilution of the diet; and to broaden the scope for validation. Thirty three of the 44 pathways were represented with the TaqMan®Custom Array panels for the validation focusing in on the RS vs. EC comparison. Other genes included in the panels were for peptide hormones, growth and immune factors. The results from the validation demonstrate that our broadening was an effective strategy as 86% of the genes from the microarray chosen for the panels remained significantly differentially expressed (Figure 2).

The general picture from the results of the validation of the microarray using the RS vs. EC comparison, is one of growth of the gut with appropriate signaling pathways for cell division and apoptosis. We previously showed increased thickness of the cecal mucosal layer with feeding of HAM-RS2 to rats (unpublished data: CC[a], 3979; EC[b], 4608; 19% weight of diet as HAM-RS2[c], 5555; ±172 μm pooled error). Other researchers have reported increased colon proliferation in neonatal pigs fed fructooligosaccharide (FOS) [20], and this is considered healthy as there is increased surface area for absorption of nutrients [18]. Several genes were listed in PANTHER as part of the integrin pathway. These included Actn1 and Actn4, Fn1, Frk, Mapk3, Arf2 and Arf3 (see tables 3-5 for gene abbreviations and names). Functions listed in PANTHER for this list of genes are in line with Rust et al. [21] who describe integrins as cell surface receptors that regulate many intracellular processes. These processes include "growth, death (apoptosis), adhesion, migration, and invasion by activating several signaling pathways." Integrins are also considered good targets for chemotherapeutic agents to treat cancer. Other genes also fit these functions. Mras appears to function in reorganization of the actin cytoskeleton [22]. Bmps are a group of growth factors also called cytokines that were originally discovered for induction of the formation of bone and cartilage, but they are now known to be morphogenetic factors important for tissue architecture throughout the body [23]. Dusp proteins respond to oxidative stress and also negatively regulate cell proliferation by inactivation of mitogen-activated protein (MAP) kinase phosphatases [24-26]. Areg mRNA was highly elevated in RS vs. EC rats (FC, 11.3, p value = 4×10^{-16}), and the protein product of this gene is part of the epidermal growth factor (EGF) family and interacts with EGF and transforming growth factor-alpha (TGF-α) to promote growth and regeneration of normal epithelial cells [27] and inhibit growth of certain aggressive cancer cell lines [28]. However, increased Areg is also associated with tumors [29]. Le Leu et al. [30] have reported that dietary RS promotes apoptosis of pre-cancerous cells, while not reducing normal cell proliferation. Thus, the data indicate that dietary RS supports normal healthy growth of the gut while likely preventing growth of tumors.

Similar to simultaneous stimulation of growth factors and inhibitors of growth was an increase in the mRNA of a pro-inflammatory signal Il1a [31]. Along with an increase in gene expression of Il1a there was an increased expression for a regulator and promoter of the cell cycle, Cdk2 [32]. Apoptosis is required to turn off inflammation. Thus, there was also increased gene expression of Cdkn1a, the cyclin-dependent kinase inhibitor which promotes apoptosis of potentially inflammatory cells [33]. Several caspase proteins are involved in activation of pro-inflammatory cytokines that function in innate immunity. However, for Casp4 no substrate has been identified and so its function remains unknown [34]. Tnfrsf1a is a receptor for the TNF proteins [35]. Upon binding of TNF proteins to TNF receptors one of three pathways can be stimulated based on cell type and conditions [36, 37]. TNF can be involved in either promoting or preventing apoptosis and stimulating the immune response. Thus, there was increased expression of genes for **inflammation**, cell proliferation and apoptosis.

Several other factors that had significantly increased mRNA for the RS vs. EC comparison also support increased healthy growth of the gut. Eif2s1 is the alpha subunit of Eif that

catalyzes the first regulated step of protein synthesis initiation, promoting the binding of the initiator tRNA to ribosomal subunits [38]. Ereg also belongs to the EGF family of polypeptides and is involved in cell proliferation [39], but Ereg is also up-regulated in cancer cell growth [40]. Several of the Gadd45 proteins promote apoptosis by activating mitogen-activated protein kinase kinase (MEKK/MTK1), which then increases mitogen-activated protein kinase kinase 4 (MKK4) and then phosphorylation of c-Jun N-terminal kinase (JNK) [41]. In several types of cancer cells, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) increases the protein cellular myc (c-myc) which represses gene expression of GADD45α and γ and the result is the escape from programmed cell death [42]. Jun-B proto-oncogene mRNA was increased and this occurs under control of TGFβ [43], and is associated with cell proliferation and differentiation [44]. However, knock down of Jun-B in wild type murine fibroblasts did result in increased proliferation and tumorigenicity [45]. Rhob inhibits NF-κB activity, which would then promote apoptosis of damaged cells [46]. Gdf15 is produced in response to injury and regulates [47] inflammation, cell survival, proliferation, and apoptosis in several types of injured tissues with varying disease processes. Additionally, Nfkbib mRNA was increased indicating the binding of NF-κB and increased apoptosis as NF-κB is known to be an inhibitor of apoptosis and increased activity increases risk of cancer [41]. Psme3 (also known as PA28γ) promotes an interaction between tumor suppressor p53 and murine double minute oncogene (MDM2) and this may be the mechanism by which Psme3 inhibits apoptosis and promotes cell cycle progression [48].

Additionally, mRNAs of several genes whose proteins regulate blood flow were increased. Hif1a mRNA was increased and the protein is a subunit of the transcription factor Hif-1. This transcription factor plays a role in inducing transcription of genes whose proteins play a role in survival during hypoxia [49]. Hypoxia also induces Vegfa [49], and Vegfa mRNA was increased and the protein product promotes increased blood vessel development [50]. At the same time, Adm mRNA was increased and translation of Adm mRNA results in a proadrenomedullin peptide that upon post-translational modification results in a peptide that promotes vasodialtion and hypotension by inhibiting angiotensin II [51]. However, Adm protein is also elevated in disorders in patients with disorders of the cardiovascular system [51]. All of these conditions, excluding the cardiovascular disorders, are necessary for growth of tissue.

Atf3 gene expression was highly up-regulated. When the protein product of this gene was discovered to be a transcription factor, it was assumed that it was an activating factor because the majority of transcription factor proteins are activating factors and very few repressors have been discovered [52]. However, it was later discovered that there are several different alternatively spliced isoforms. Atf3 functions as a repression element by its binding to promoters of genes and stabilizing inhibitory co-factors. Another version of the protein, Atf3 Zip, lacks the ability to bind to DNA as it lacks the leucine zipper domain. This version of the protein actually activates gene transcription as it appears to bind to inhibitory co-factors and prevent their interaction with gene promoter regions [52]. Hackl et al [53] discuss that Atf3 is involved in "the complex process of cellular stress response." Also under various conditions the protein can have tumor suppressive or oncogenic effects. They reported that down-regulation of Atf3 promoted colon cancer and that blocking the

production of heat shock protein 90 Hsp90, increased Atf3 production [53]. Another recent publication reported that Atf3 can have positive or negative effects on proliferation and survival of adult T-cell leukemia cells depending on the conditions [54].

Genes for several detoxifying enzymes were up-regulated with the RS vs. EC comparison. Gsta5 represented this group in the validation. It appears that increased levels of expression of these genes represent sensitivity to noxious chemicals [55]. Compounds known to protect against chemical carcinogenesis, such as coumarins and phenolic antioxidants, are known to increase amounts of glutathione S-transferase enzymes.

Additionally, several fatty acid binding proteins were up-regulated by the RS vs. EC comparison. Slc16a3 is also monocarboxylate transporter 4 that transports lactate, pyruvate and the ketone bodies, acetoacetate and β-hydroxybutyrate [56]. Fabp2 which binds long chain fatty acids [57] was up-regulated, but Fabp6 appears to bind bile acids in the cytoplasm after sodium-dependent uptake by enterocytes by active transport [58] was down-regulated by RS. Several other Slc16a transporter proteins were up-regulated in the gene array, but were not included in the validation. For example, Slc16a1, also called monocarboxylate transporter 1, transports short chain fatty acids into cells [59, 60].

Several other gene mRNAs were increased with the RS vs. EC comparison that were identified by PANTHER as proteins that are components of metabolic pathways. Acat3 combines cholesterol and fatty acids to form cholesterol esters in several tissues. Acat1 is ubiquitous, but Acat3 is limited to intestine and liver [61]. Currently at the NCBI website the Entrez Gene ID number from PANTHER for Acat2 discussed in [61] refers to Acat3. Idh2 is a mitochondrial, NADP$^+$-enzyme that catalyzes the third step in the citric acid cycle converting isocitrate to α-ketoglutarate [62]. Asns produces asparagine from aspartate. Cbs is an enzyme catalyzing the reaction of serine plus homocysteine to cystathionine. Eno1 is an enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis. Pdxk converts pyridoxal to pyridoxal phosphate. All of the proteins from these genes would be important for normal cellular metabolism and their function is commonly known.

Three peptide hormone mRNAs, Gcg, Pyy, and neurotensin (Nts), that were increased for RS vs. EC in the microarray were also validated with the panels or separately (Nts) by individual qPCR. Plasma levels of GLP-1 and PYY were also measured in this study and were elevated for RS compared to either CC or EC groups. Our lab group has previously demonstrated in several studies that Gcg and Pyy gene expression and plasma peptide levels are increased with feeding of HAM-RS2 to rodents. [5, 8, 9, 11, 12] Thus, Gcg and Pyy were used as positive controls for the microarray and validation. Gcg is the glucagon gene that is also referred to as preproglucagon or proglucagon. The protein is produced in pancreatic α cells, brain and intestine and undergoes different post-translational modification depending on the tissue [63]. In the L-endocrine cells of the intestine the products are GLP-1, glucagon-like peptide 2 and oxyntomodulin. PYY is also produced by L-endocrine cells in the intestine. In the gut these peptides are associated with improved health. Nts is produced by NT cells in the intestine [64] and is also produced in neural cells

throughout the body. In the gut, Nts is reported to control gut motility [65]. GLP-1 and PYY are involved with glucose control [66, 67] and energy metabolism [68-75].

For the most part, but with some exceptions, gene expression results for the validation were similar for the RS vs. EC and RS vs. CC comparisons. However, the CC vs. EC comparison results from the validation were quite different from the results of the other two comparisons (Figures 2-4). This was expected as the differences between the CC and EC groups were lower energy density in the EC diet and a greater amount of cellulose. Clearly then, the greatest amount of gene expression was altered in RS vs.EC compared to EC vs. CC. Thus, increased fermentation in RS rats had a fairly similar response for many genes to rats fed the CC diet with a greater energy density diet. The numerical results are summarized with Venn-diagrams in figure 5; and specific similarities and differences between these two groups of rats can be determined using figures 2 and 3. The differences in results between RS and CC groups further demonstrate that fermentation of RS impacted the cecal gene expression profile more than energy dilution in rats.

Previous reports of gene array and validation results with other sources of fermentable fibers, such as fructooligosaccharide (FOS) and wheat bran, demonstrate some general similarity to the results from the currently reported study [18, 76]. Chen et al [18] report greater amounts of changes in gene expression with FOS than with wheat bran, presumably because of higher rates of fermentation and short chain fatty acid production. Wheat bran is considered to ferment more slowly than FOS and its fermentation is less complete than FOS. Chen et al. [18] also speculate that different transit times can affect fermentation and gene expression results. RS is generally used in rodent diets in greater amounts than for similar studies with FOS. In the current study, the amount of HAM-RS2 used was an amount that promotes abdominal body fat loss in rodents (>25% of weight of diet) [5, 8, 9, 13]. Unlike Chen et al. [18] who report fold changes greater than 1.6, Fukasawa et al. [76] report fold changes greater than 1. We also chose to report data as fold changes lower than a prescribed number such as 1.5 because many of the relatively lower fold changes for gene expression had extremely small p values. Obviously there is no difference in significance at $p<0.05$ and $p<0.01$ as significance is set by convention of $p<0.05$. However, the risk of a type 1 error is much lower for the latter p value. Small fold changes for RS vs. EC with a very small p value may represent important treatment differences because of very low variation of the mean.

One major advantage of our current microarray and validation study is that we employed an energy dilution control. Energy dilution occurs when dietary fiber components replace starches or other carbohydrates in diet formulations. Fukasawa et al [76] increased FOS at the expense of sucrose resulting in two groups similar to our RS vs. CC comparison as their FOS group would have a lower energy density than their control group. On the other hand, Chen et al. [18] controlled for dietary fiber, but not energy density as their control diet with 10% by weight of diet as cellulose had a lower energy density than their experimental diets with either FOS or wheat bran. However, this difference is relatively small with a maximum of a 7% difference in energy density as the total fiber in all diets is only 10% of the diet. In the current study, the CC diet is essentially an AIN-93 diet [15] and has greater energy density than the RS and EC diets. Based on an approximate calculation using the

metabolizable energy density of 2.8 kcal/g for the Hi-maize® high amylose cornstarch [10] ~50% of the HAM-RS2 in our HAM-RS2 diet is fermented. What this means is the following: 1) Hi-maize® high amylose cornstarch is ~56% HAM-RS2, 2) based on the amount of Hi-maize® added to the diet, 530.7 g/1000 g of diet, ~297 g of HAM-RS2/1000 g of diet were added, 3) ~50% of the 297 g of HAM-RS2 is not fermented. Using this approximation and information, the HAM-RS2 diet has ~11% more non-fermentable fiber than the EC diet, but the same energy density. This includes 50 g of cellulose/1000 g of diet and ~50% of the 297 g of HAM-RS2/1000 g of diet or 148.5 g/1000 g of diet. The EC diet had 177 g cellulose added/1000g of diet. Thus, in the current study, we controlled for dietary energy density, were close in non-fermentable fiber, and tested for fermentable fiber with the RS vs. EC comparison.

In summary, a microarray and validation of the microarray were conducted with a total RNA extract from cecal cells in rats fed one of three treatment diets, RS, CC and EC. About 10,000 of ~27,000 genes were expressed for all three groups. The greatest number of significantly differentially expressed genes was observed with the RS vs. CC comparison as this comparison measured both differences in energy density of the diet and fermentation effects. However, the focus of the validation was primarily on the RS vs. EC comparison because the two treatment diets were equal in energy density and specific fermentation effects were tested. Genes chosen for the validation had higher fold changes, lower p values and had to be inventoried by Applied Biosystems. The validation was "successful" as 86% of the genes chosen from the microarray were confirmed as significantly differentially expressed.

Further, the results demonstrated that fermentation of HAM-RS2 altered expression of genes that would result in cell growth, proliferation and differentiation of the gut, as well as expression of genes involved in apoptosis and control of cell proliferation. Additionally, expression of several genes related to metabolic pathways and gut peptide hormones were altered, such as GLP-1 and PYY. Plasma levels for these hormones were also increased. GLP-1 and PYY can have effects throughout the body. Overall, the increased gene expressions observed appears to **improve the structure and function of the** GI tract, based on their functions and supporting information in the literature. Because fermentation of dietary resistant starch changes the gene expression profile in cecal cells, the physiological relevance of these gene expression changes requires further investigation to explore the beneficial effects of HAM-RS2 beyond simple dietary energy dilution as a fiber.

Acknowledgments

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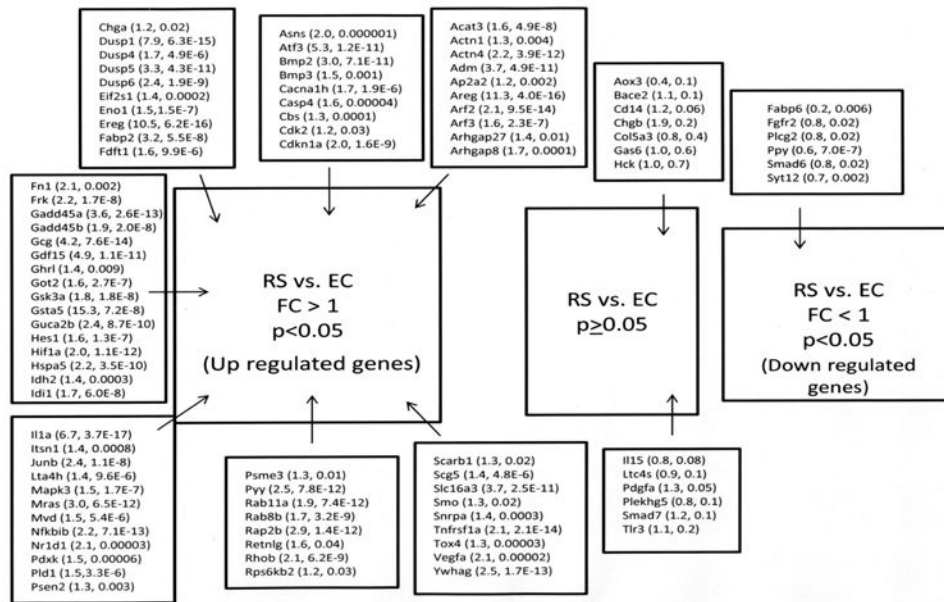
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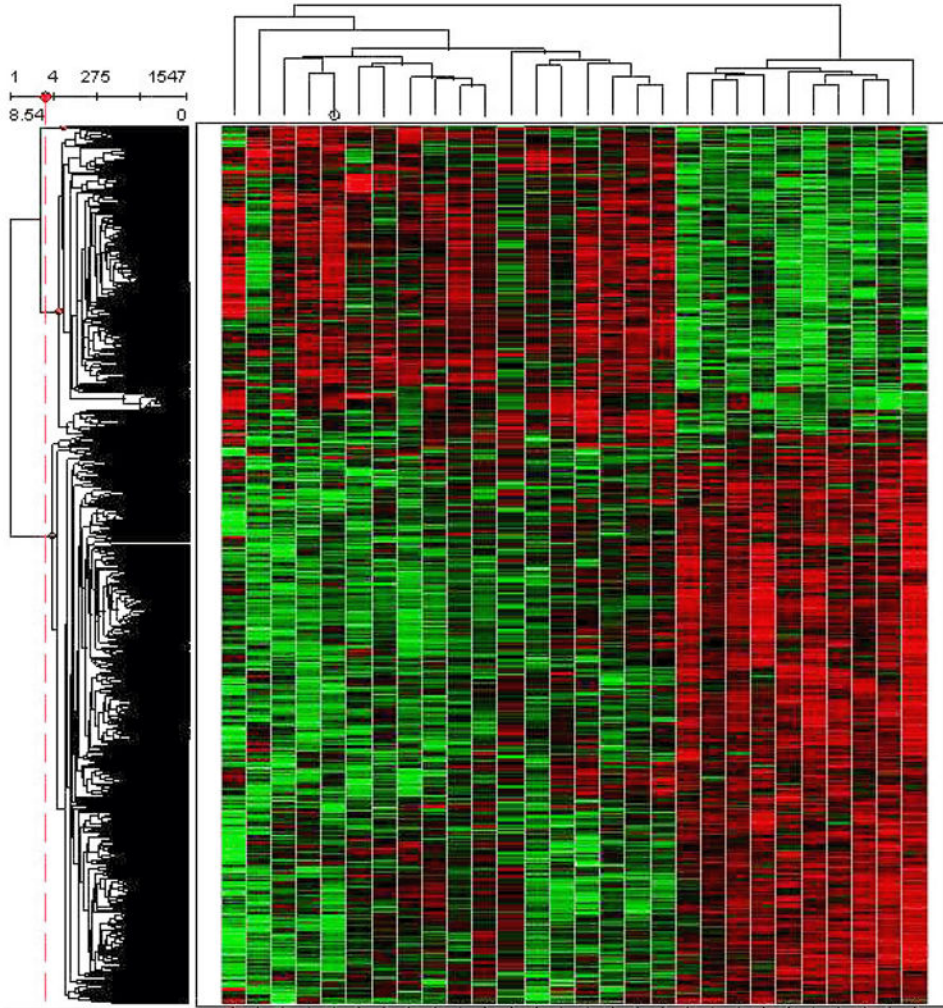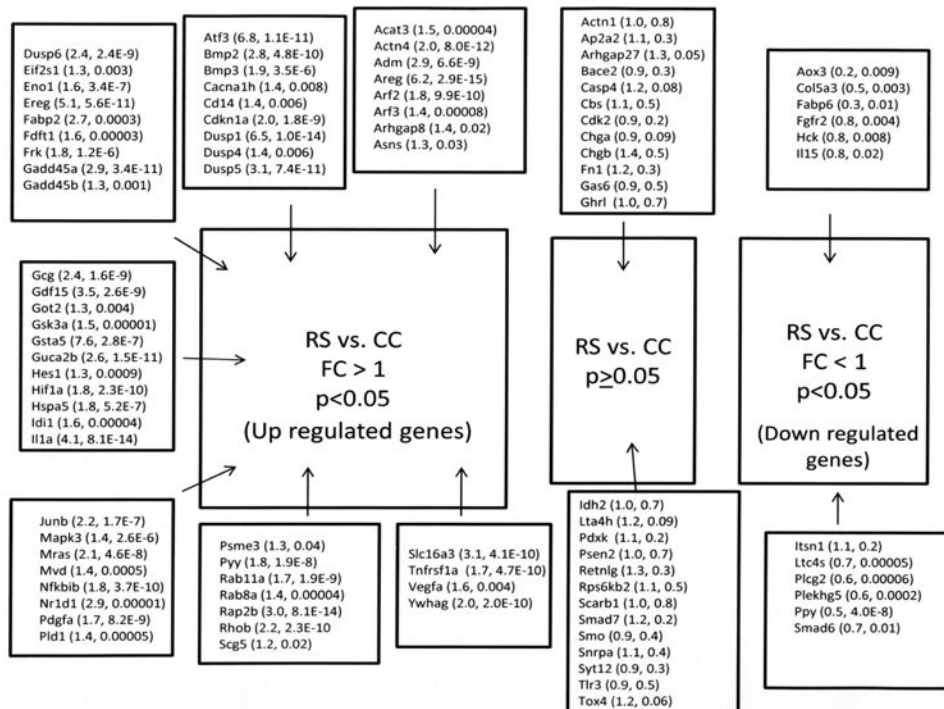
Figure 1.
A hierarchical cluster of the Z scores of the microarray signals for RS, CC and EC groups for genes that were significantly different at p<0.01. This visualization shows data in a dendrogram (a tree graph) based on the similarity between treatment replicates. The Z-scores were determined by calculating the three groups combined mean for each gene, and then individual differences of treatment replicates from the mean were divided by the standard deviation. This is one type of normalization for making gene-gene comparisons. Different genes are shown in each separate row. The columns are individual replicates of treatments and from left to right they are: EC3, CC3, CC14, CC2, CC8, CC12, CC4, CC15, EC15, EC13, EC12, EC6, CC7, EC5, EC2, CC6, EC9, EC7, RS3, RS9, RS15, RS8, RS13, RS4, RS12, RS14, RS7, RS5.

| Chga (1.2, 0.02) |
| Dusp1 (7.9, 6.3E-15) |
| Dusp4 (1.7, 4.9E-6) |
| Dusp5 (3.3, 4.3E-11) |
| Dusp6 (2.4, 1.9E-9) |
| Eif2s1 (1.4, 0.0002) |
| Eno1 (1.5,1.5E-7) |
| Ereg (10.5, 6.2E-16) |
| Fabp2 (3.2, 5.5E-8) |
| Fdft1 (1.6, 9.9E-6) |

| Asns (2.0, 0.000001) |
| Atf3 (5.3, 1.2E-11) |
| Bmp2 (3.0, 7.1E-11) |
| Bmp3 (1.5, 0.001) |
| Cacna1h (1.7, 1.9E-6) |
| Casp4 (1.6, 0.00004) |
| Cbs (1.3, 0.0001) |
| Cdk2 (1.2, 0.03) |
| Cdkn1a (2.0, 1.6E-9) |

| Acat3 (1.6, 4.9E-8) |
| Actn1 (1.3, 0.004) |
| Actn4 (2.2, 3.9E-12) |
| Adm (3.7, 4.9E-11) |
| Ap2a2 (1.2, 0.002) |
| Areg (11.3, 4.0E-16) |
| Arf2 (2.1, 9.5E-14) |
| Arf3 (1.6, 2.3E-7) |
| Arhgap27 (1.4, 0.01) |
| Arhgap8 (1.7, 0.0001) |

| Aox3 (0.4, 0.1) |
| Bace2 (1.1, 0.1) |
| Cd14 (1.2, 0.06) |
| Chgb (1.9, 0.2) |
| Col5a3 (0.8, 0.4) |
| Gas6 (1.0, 0.6) |
| Hck (1.0, 0.7) |

| Fabp6 (0.2, 0.006) |
| Fgfr2 (0.8, 0.02) |
| Plcg2 (0.8, 0.02) |
| Ppy (0.6, 7.0E-7) |
| Smad6 (0.8, 0.02) |
| Syt12 (0.7, 0.002) |

| Fn1 (2.1, 0.002) |
| Frk (2.2, 1.7E-8) |
| Gadd45a (3.6, 2.6E-13) |
| Gadd45b (1.9, 2.0E-8) |
| Gcg (4.2, 7.6E-14) |
| Gdf15 (4.9, 1.1E-11) |
| Ghrl (1.4, 0.009) |
| Got2 (1.6, 2.7E-7) |
| Gsk3a (1.8, 1.8E-8) |
| Gsta5 (15.3, 7.2E-8) |
| Guca2b (2.4, 8.7E-10) |
| Hes1 (1.6, 1.3E-7) |
| Hif1a (2.0, 1.1E-12) |
| Hspa5 (2.2, 3.5E-10) |
| Idh2 (1.4, 0.0003) |
| Idi1 (1.7, 6.0E-8) |

RS vs. EC
FC > 1
p<0.05

(Up regulated genes)

RS vs. EC
p≥0.05

RS vs. EC
FC < 1
p<0.05
(Down regulated genes)

| Il1a (6.7, 3.7E-17) |
| Itsn1 (1.4, 0.0008) |
| Junb (2.4, 1.1E-8) |
| Lta4h (1.4, 9.6E-6) |
| Mapk3 (1.5, 1.7E-7) |
| Mras (3.0, 6.5E-12) |
| Mvd (1.5, 5.4E-6) |
| Nfkbib (2.2, 7.1E-13) |
| Nr1d1 (2.1, 0.00003) |
| Pdxk (1.5, 0.00006) |
| Pld1 (1.5,3.3E-6) |
| Psen2 (1.3, 0.003) |

| Psme3 (1.3, 0.01) |
| Pyy (2.5, 7.8E-12) |
| Rab11a (1.9, 7.4E-12) |
| Rab8b (1.7, 3.2E-9) |
| Rap2b (2.9, 1.4E-12) |
| Retnlg (1.6, 0.04) |
| Rhob (2.1, 6.2E-9) |
| Rps6kb2 (1.2, 0.03) |

| Scarb1 (1.3, 0.02) |
| Scg5 (1.4, 4.8E-6) |
| Slc16a3 (3.7, 2.5E-11) |
| Smo (1.3, 0.02) |
| Snrpa (1.4, 0.0003) |
| Tnfrsf1a (2.1, 2.1E-14) |
| Tox4 (1.3, 0.00003) |
| Vegfa (2.1, 0.00002) |
| Ywhag (2.5, 1.7E-13) |

| Il15 (0.8, 0.08) |
| Ltc4s (0.9, 0.1) |
| Pdgfa (1.3, 0.05) |
| Plekhg5 (0.8, 0.1) |
| Smad7 (1.2, 0.1) |
| Tlr3 (1.1, 0.2) |

Figure 2.
Results of validation for the RS vs. EC comparison. Genes are identified by their abbreviation. In parentheses are the (fold change, p value). Names of genes and their abbreviations are listed in tables 3 through 5.

Figure 3.
Results of validation for the RS vs. CC comparison. Genes are identified by their abbreviation. In parentheses are the (fold change, p value). Names of genes and their abbreviations are listed in tables 3 through 5.
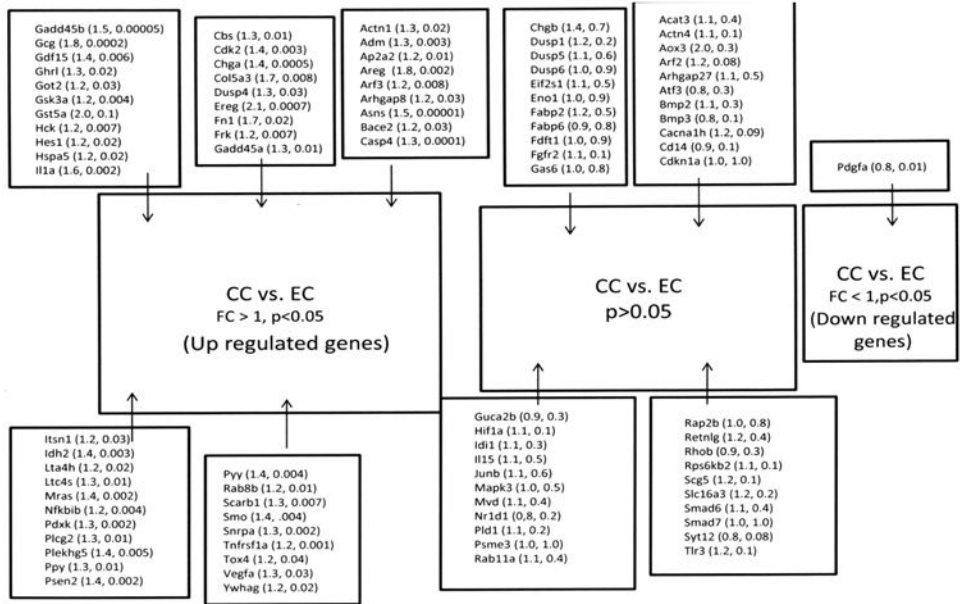
Figure 4.
Results of validation for the CC vs. EC comparison. Genes are identified by their abbreviation. In parentheses are the (fold change, p value). Names of genes and their abbreviations are listed in tables 3 through 5.
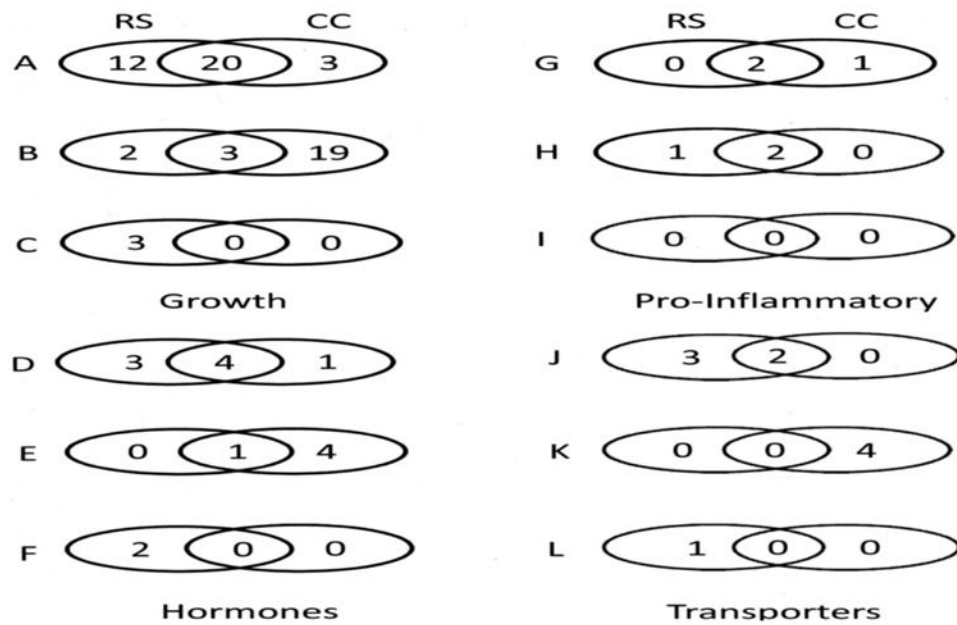
Figure 5.
Venn diagrams comparing RS and CC gene expression grouped according to four broad categories of function. The figure compares RS vs. EC with RS vs. EC for similarities and differences in numbers of genes that were either up-regulated (A, D, G, J), not different (B, E, H, K), or down- regulated (C, F, I, L). The four broad categories are 1. growth and differentiation, regulators of cell cycle, and angiogenesis; 2. Hormones and secretory proteins; 3. pro-inflammatory; and 4. Transporters.
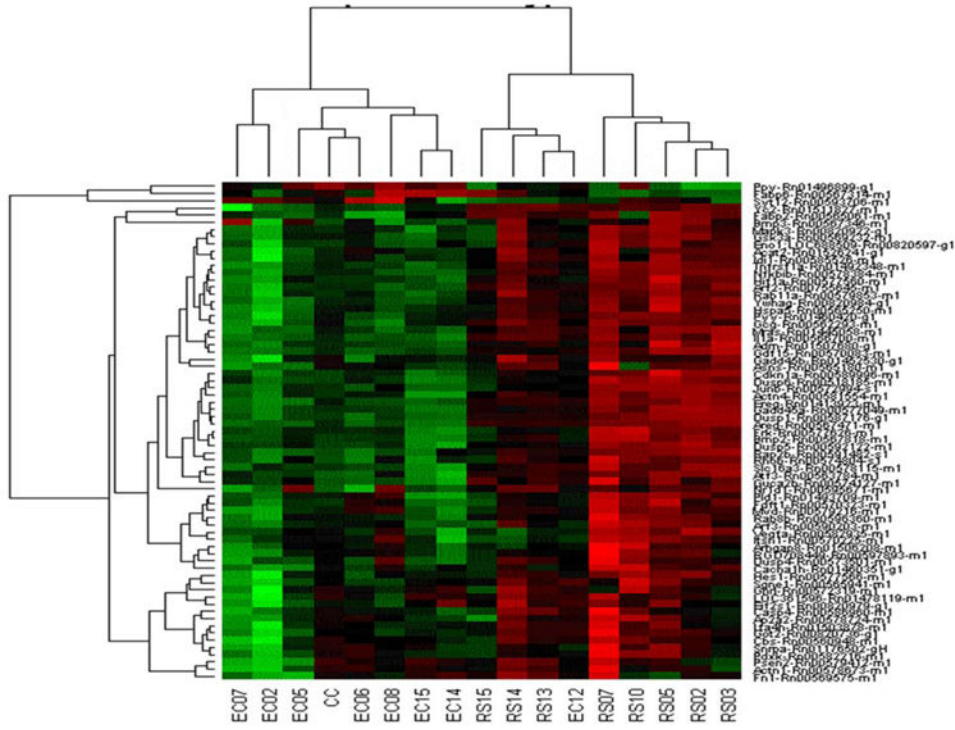
Figure 6.
A hierarchical cluster of the Z scores of the antilogs of the $2^{-\Delta CT}$ of RS vs. EC from the TaqMan Custom Array panel results from the validation. The Z-scores were determined by calculating the three groups combined mean for each gene, and then individual differences of treatment replicates from the mean were divided by the standard deviation. This is one type of normalization for making gene-gene comparisons. Different genes are shown in each separate row. The columns are individual replicates of treatments and from left to right they are: EC7, EC12, EC5, mean of CC group, EC6, EC8, EC15, EC14, RS15, RS14, RS13, EC12, RS7, RS10, RS5, RS2, RS3.
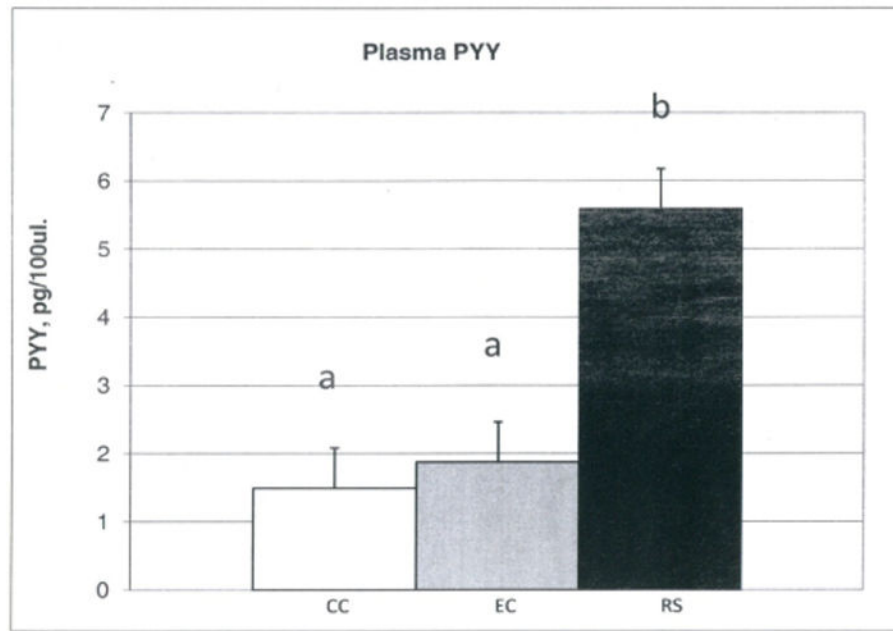
7A
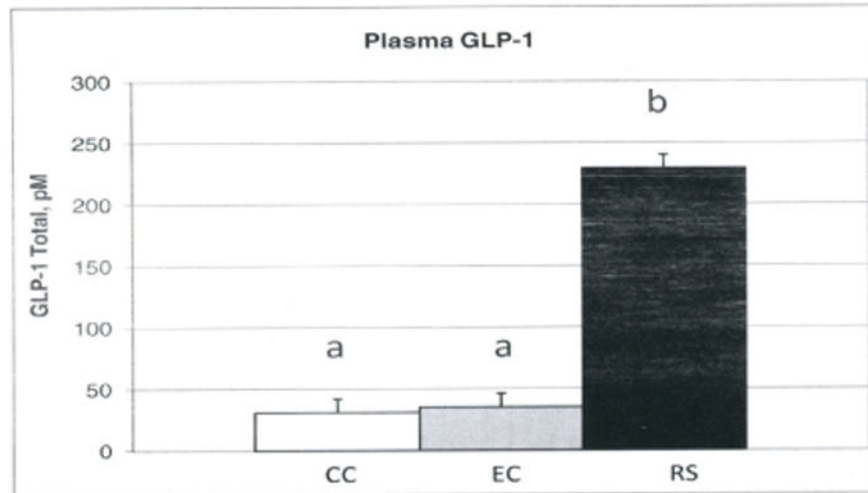
7B

Figure 7.
A. Plasma PYY levels (pg/100μl). B. Plasma GLP-1 levels (pM). Bars with different superscripts are statistically different at p<0.0001.

Table 1

Composition of the rat diets.

Ingredient (g)	CC	EC	RS
Casein	200	200	200
Sucrose	100	100	100
Cornstarch[1]	530.69	403.69	0
Resistant Cornstarch[2]	0	0	530.69
Cellulose	50	177	50
Soybean oil	70	70	70
Mineral Mix (AIN-93M)	35	35	35
Vitamin Mix (AIN-93)	10	10	10
Choline Chloride	1.3	1.3	1.3
L-Cystine	3	3	3
BHT	0.014	0.014	0.014
Total grams	1000	1000	1000
Energy density[3]	3.74 kcal/g	3.27 kcal/g	3.27 kcal/g

[1]100% amylopectin cornstarch (Amioca®, National Starch LLC, Bridgewater, NJ).

[2]High-amylose maize cornstarch (Hi-maize®260, National Starch LLC, Bridgewater, NJ). This product has RS2 and is described in the methods section of the text.

Table 2

Microarray results.

Results	CC vs. EC	CC vs. RS	RS vs. EC
"Present" Gene expression (S/N 3)	10,023	10,573	10,388
Gene differences p<0.05	629	3,192	2,640
Gene differences p<0.01	158	1,769	1,301
PANTHER Gene Pathways p<0.05[1]	18	23	17
Unclassified Genes p<0.05[2]	515	2,711	2,266

[1] PANTHER was the gene ontology software program used to estimate the number of gene mRNAs in pathways or unclassified.

Table 3

List of genes from microarray with RS vs. EC results and used in validation[1,2] (alphabetical by abbreviation).

Name	Abbrev	NCBI Gene ID	Fold Change	p Value	Function (Pathway or Unclassified)
Acetyl-coenzyme A acyltransferase 3	Acat 3	308100	1.61	0.000	Unclassified
Actinin alpha 1	Actn1	81634	1.98	0.000	Integrin
Actinin alpha 4	Actn4	63836	1.97	0.000	Integrin
Adrenomedullin	Adm	25026	5.32	0.000	Unclassified
Aldehyde oxidase 3	Aox3	493909	0.56	0.004	Adenine and hypoxanthine salvage
Adaptor protein complex AP-2	Ap2a2	81637	1.51	0.004	Huntington Disease
Amphiregulin	Areg	29183	25.64	0.000	EGF receptor signaling
ADP-ribosylation factor 2	Arf2	79119	1.51	0.006	Huntington Disease + Integrin
ADP-ribosylation factor 3	Arf3	140940	1.78	0.002	Huntington Disease + Integrin
Rho GTPase activating protein 27	Arhgap27	303583	1.45	0.002	PDGF signaling
Rho GTPase activating protein 8	Arhgap8	300115	2.15	0.002	Angiogenesis + PDGF signaling + VEGF signaling + Cytoskeleton regulation by RhoGTPase
Asparagine synthetase	Asns	25612	2.73	0.000	Asparagine and aspartate biosynthesis
Activating transcription factor 3	Atf3	25389	3.26	0.000	Apoptosis
Beta-site APP-cleaving enzyme 2	Bace2	288227	0.5	0.004	Alzheimer disease-amyloid secretase
Bone morphogenetic protein 2	Bmp2	29373	1.31	0.009	TGF beta signaling
Bone morphogenetic protein 3	Bmp3	25667	2.48	0.030	Unclassified
Calcium channel, voltage-dependent, T type, alpha 1H subunit	Cacna1h	114862	3.12	0.005	Unclassified
Caspase 4	Casp4	114555	1.59	0.000	Unclassified
Cystathionine beta synthase	Cbs	24250	1.52	0.008	Cysteine biosynthesis
CD14 antigen	Cd14	60350	0.56	0.005	Toll-like receptor
Cyclin-dependent kinase	Cdk2	362817	1.59	0.006	p53 + p53 feedback loops 2
Cyclin-dependent kinase inhibitor 1A	Cdkn1a	114851	2.18	0.000	Interleukin + p53 feedback loops 2 + p53
Chromogranin A	Chga	24258	0.52	0.000	Unclassified
Chromogranin B	Chgb	24259	0.54	0.000	Unclassified
Collagen, type 5, alpha 3	Col5a3	60379	2.72	0.003	Integrin
Dual specificity phosphatase 1	Dusp1	114856	5.99	0.000	Unclassified

Name	Abbrev	NCBI Gene ID	Fold Change	p Value	Function (Pathway or Unclassified)
Dual specificity phosphatase 4	Dusp4	60587	2.96	0.000	Oxidative stress response
Dual specificity phosphatase 5	Dusp5	171109	1.65	0.001	Oxidative stress response
Dual specificity phosphatase 6	Dusp6	116663	1.68	0.001	Oxidative stress response
Eukaryotic translation initiation factor 2, subunit 1	Eif2s1	54318	1.42	0.004	Apoptosis
Enolase 1, alpha	Eno1	24333	1.59	0.000	Glycolysis
Epiregulin	Ereg	59325	7.81	0.000	EGF receptor signaling
Fatty acid binding protein 2	Fabp2	25598	4.55	0.000	Unclassified
fatty acid binding protein 6, ileal (gastrotropin)	Fabp6	25440	0.12	0.012	Unclassified
farnesyl diphosphate farnesyl transferase 1	Fdft1	29580	1.55	0.008	Cholesterol biosynthesis
fibroblast growth factor receptor 2	Fgfr2	25022	0.56	0.000	Angiogenesis
fibronectin 1	Fn1	25661	2.28	0.027	Integrin
fyn-related kinase	Frk	79209	1.57	0.000	B-cell activation + Integrin + Axon guidance
growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	25112	2.70	0.000	p53 +PI3kinase
growth arrest and DNA-damage-inducible 45 beta	Gadd45b	299626	2.12	0.014	p53
growth arrest specific 6	Gas6	58935	0.58	0.005	Unclassified
glucagon	Gcg	24952	6.9	0.001	Unclassified
growth differentiation factor 15	Gdf15	29455	2.97	0.007	Unclassified
ghrelin precursor	Ghrl	59301	0.66	0.014	Unclassified
glutamate oxaloacetate transaminase 2	Got2	25721	1.56	0.001	Asparagine and aspartate biosynthesis
glycogen synthase kinase 3 alpha	Gsk3a	50686	1.89	0.001	Insulin IGF PKB +Angiogenesis + Interleukin + PI3Kinase + Hedgehog signaling
glutathione S-transferase Yc2 subunit	Gsta5	494500	10.6	0.000	Unclassified
guanylate cyclase activator 2b	Guca2b	64055	1.51	0.008	Unclassified
hemopoietic cell kinase	Hck	25734	0.58	0.001	Parkinson disease + Angiogenesis = Integrin + Interleukin
hairy and enhancer of split 1 (Drosophila)	Hes1	29577	1.44	0.003	Notch signaling
hypoxia inducible factor 1, alpha subunit	Hif1a	29560	1.59	0.000	Angiogenesis
heat shock 70kDa protein 5 (glucose-regulated protein)	Hspa5	25617	1.63	0.000	Parkinson disease
Isocitrate dehydrogenase 2 (NADP+)	Idh2	361596	1.52	0.003	Tricarboxylic acid
isopentenyl-diphosphate delta isomerase	Idi1	89784	2.23	0.000	Cholesterol biosynthesis
interleukin 15	Il15	25670	0.69	0.006	Interleukin

Name	Abbrev	NCBI Gene ID	Fold Change	p Value	Function (Pathway or Unclassified)
interleukin 1 alpha	Il1a	24493	5.03	0.000	Interleukin
intersectin 1	Itsn1	29491	2.06	0.008	Unclassified
Jun-B oncogene	Junb	24517	2.38	0.000	TGF Beta
leukotriene A4 hydrolase	Lta4h	299732	1.5	0.000	Unclassified
leukotriene C4 synthase	Ltc4s	114097	0.57	0.000	Inflammation-mediated chemokine & cytokine
mitogen activated protein kinase 3	Mapk3	50689	1.51	0.005	Inflammation-mediated chemokine & cytokine + B-cell activation +Parkinson disease +Apoptosis + Integrin + Leukotriene
muscle and microspikes RAS	Mras	25482	3.72	0.000	Integrin
mevalonate (diphospho) decarboxylase	Mvd	81726	2.21	0.004	Cholesterol biosynthesis
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	Nfkbib	81525	1.41	0.001	Apoptosis + Toll-like receptor
nuclear receptor subfamily 1, group D, member 1	Nr1d1	252917	2.58	0.04	Circadian clock system
platelet derived growth factor, alpha	Pdgfa	25266	2.10	0.000	Unclassified
pyridoxal (pyridoxine, vitamin B6) kinase	Pdxk	83578	2.97	0.004	Pyridoxal phosphate salvage
phospholipase C, gamma 2	Plcg2	29337	0.59	0.000	Inflammation-mediated chemokine & cytokine + B-cell activation + Angiogenesis + PDGF signaling + VEGF signaling
phospholipase D1	Pld1	25096	1.50	0.003	Angiogenesis + Ras
pleckstrin homology domain containing, family G (with RhoGef domain) member 5	Plekhg5	310999	0.52	0.004	Cytoskeleton + Axon guidance
pancreatic polypeptide	Ppy	24677	0.43	0.01	Unclassified
presenilin 2	Psen2	81751	2.07	0.03	Notch signaling + Alzheimer disease
proteaseome (prosome, macropain) 28 subunit, 3	Psme3	287716	1.54	0.000	Cell cycle
peptide YY (mapped)	Pyy	287730	1.62	0.001	Unclassified
RAB11a, member RAS oncogene family	Rab11a	81830	1.55	0.000	Huntington disease
RAB8B, member RAS oncogene family	Rab8b	266688	1.42	0.000	TGF beta signaling
RAP2B, member of RAS oncogene family	Rap2b	170923	2.16	0.000	Integrin
resistin-like gamma	Retnlg	288135	3.6	0.006	Unclassified
ras homolog gene family, member B	Rhob	64373	1.56	0.002	Inflammation-mediated chemokine & cytokine + Angiogenesis + Integrin + Axon guidance + Ras + Cytoskeleton regulation by RhoGTPase
ribosomal protein S6 kinase, polypeptide 2	Rps6kb2	361696	1.44	0.001	PDGF signaling + PI3Kinase
scavenger receptor class B, member 1;	Scarb1	25073	2.52	0.004	Unclassified

Name	Abbrev	NCBI Gene ID	Fold Change	p Value	Function (Pathway or Unclassified)
Secretogranin V	Scg5	25719	1.55	0.001	Unclassified
monocarboxylate transporter	Slc16a3	80878	2.36	0.000	Unclassified
SMAD family member 6	Smad6	367100	0.67	0.000	TGF beta signaling
MAD homolog 7 (Drosophila)	Smad7	81516	0.66	0.001	TGF beta signaling
smoothened homolog (Drosophila)	Smo	25273	2.69	0.005	Hedgehog signaling
small nuclear ribonucleoprotein polypeptide A	Snrpa	292729	1.83	0.006	mRNA splicing
synaptotagmin 12	Syt12	191595	0.31	0.003	Unclassified
toll-like receptor 3	Tlr3	364594	0.74	0.007	Toll-like receptor
tumor necrosis factor receptor superfamily, member 1a	Tnfrsf1a	25625	1.64	0.000	Apoptosis
TOX high mobility group box family member 4	Tox4	286990	1.93	0.006	Nucleoside, nucleotide and nucleic acid metabolism
vascular endothelial growth factor A	Vegfa	83785	2.82	0.000	Angiogenesis + VEGF signaling
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	Ywhag	56010	1.85	0.000	FGF signaling + PI3Kinase

[1] One gene did not amplify on the 96 well LDA panel, platforms and, therefore, only 93 of 94 test genes are reported in tables 3 through 5. The gene was Cga (Gene ID 116700), glycoprotein hormones, alpha subunit, with FC 0.390 and p value 0.02. Two genes were used as controls and not listed in the tables.

[2] All genes in table are currently (July 2011) recognized as genes at the NCBI website.