Original Paper

High-Amylose Resistant Starch Increases Hormones and Improves Structure and Function of the Gastrointestinal Tract: A Microarray Study

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Key Words

High-amylose maize · Resistant starch · Microarray · Validation · Cecal cells · Gastrointestinal tract

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 downregulated with feeding of HAM-RS2, a microarray was performed. **Methods:** Adult, male Sprague Dawley rats were fed one of the following three diets for a 4-week study period: cornstarch control (CC, 3.74 kcal/g), dietary energy density control (EC, 3.27 kcal/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 versus EC comparison tested effects of fermentation as energy density of the diet was controlled. **Results:** For the RS versus 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. Production of gut hormones may promote healthy functions beyond the GI tract.

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Introduction

Although the mechanism remains unclear, dietary resistant starches (RS) 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 RS improve health. RS are one type of non-digestible, fermentable fiber. Generally, RS are 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 in 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 and then cooled potatoes, and RS4 is considered chemically modified starch with either constituent groups attached or linkages between starch molecules.

RS will potentially 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 3.6–4 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 was a standard AIN-93G diet with added cornstarch (CC = cornstarch control), which is greater in metabolizable energy than the RS diet [15]; the second one had a metabolizable energy density equal to the RS diet due to the addition of cellulose (EC = energy density control). The inclusion of the EC diet allowed for testing of fermentation effects separately from energy density differences.

Methods

Animals and Diets

Forty-five 8-week-old male Sprague Dawley rats (Harlan, Indianapolis, Ind., USA) 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 type 2, HAM-RS2 (table 1). The number of rats in each treatment group was 15, purchased in three separate orders from Harlan Co., i.e. $n = 5$ /shipping for each of the three treatment groups. Note that the staggering of orders equalized for the three treatments was done to control for condition of rats at different shipping times.

The EC and RS diets had 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).

Table 1. Composition of the rat diets

Ingredient	CC	EC	RS
Casein, g	200	200	200
Sucrose, g	100	100	100
Cornstarch, g ^a	530.69	403.69	0
Resistant cornstarch, g ^b	0	0	530.69
Cellulose, g	50	177	50
Soybean oil, g	70	70	70
Mineral Mix (AIN-93M), g	35	35	35
Vitamin Mix (AIN-93), g	10	10	10
Choline chloride, g	1.3	1.3	1.3
L-Cystine, g	3	3	3
BHT, g	0.014	0.014	0.014
Total weight, g	1,000	1,000	1,000
Energy density, kcal/g ^c	3.74	3.27	3.27

BHT = Butylated hydroxytoluene.

^a 100% amylopectin cornstarch (Amioca®; National Starch LLC, Bridgewater, N.J., USA). ^b High-amylose maize cornstarch (Hi-maize®260; National Starch LLC). This product has RS2 and is described in the methods section of the text.

Rats were sacrificed by heart puncture and plasma was collected for analysis of PYY and GLP-1 by radioimmunoassay (Phoenix, Burlingame, Calif., USA) and ELISA (Millipore, Billerica, Mass., USA), 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 cells (colonocytes), 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 RNeasy mini kit (Valencia, Calif., USA) 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, Calif., USA). Only RNA samples with a concentration over 2.3 ng/μl and an RNA integrity number greater than 8.0 were used. The best total RNA samples from each time period (see 'Animals and Diets' 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 Chemiluminescent RT Labeling Kit (Carlsbad, Calif., USA) was used to produce digoxigenin-labeled cDNA for hybridization to the microarray.

Microarray Procedure

We used the Applied Biosystems rat genomic chips (~27,000 genes). From each sample, 10 μg 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 the AB1700 software. Fold change (FC), log₂ FC, p value, Benjamini-Hochberg adjusted p value, and FC bin were provided for each detected gene or transcript.

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 ^a	18	23	17
Unclassified genes p < 0.05 ^b	515	2,711	2,266

S/N = Signal-to-noise ratio.

^a PANTHER was the gene ontology software program used to estimate the number of gene mRNAs in pathways or unclassified. ^b www.pantherdb.org.

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 Bonferroni 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 (TIBCO, Somerville, Mass., USA) 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 versus 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 FCs 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 FC 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 FC calculation using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) [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 FCs.

Results

Microarray

Over 10,000 genes were considered 'present' (the signal-to-noise ratio was ≥ 3) for all three comparisons. The RS versus 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 versus 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 (fig. 1).

Fig. 1. 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.

Validation

The genes chosen from the microarray for validation have their microarray results listed in table 3 with names, abbreviations, FCs, p values, category of function (pathway or unclassified), and NCBI Gene ID number. PANTHER gene expression analysis without the Bonferroni adjustment for the RS versus EC comparison demonstrated that our microarray gene

Table 3. List of genes from microarray with RS versus EC results and used in validation (alphabetical by abbreviation)

Name	Abbreviation	NCBI gene ID	FC	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
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 70 kDa protein 5 (glucose-regulated protein)	Hspa5	25617	1.63	0.000	Parkinson disease

Table 3 (continued)

Name	Abbreviation	NCBI gene ID	FC	p value	Function (pathway or unclassified)
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
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 and cytokine
Mitogen activated protein kinase 3	Mapk3	50689	1.51	0.005	Inflammation-mediated chemokine and 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 and 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
Proteasome (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 and 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
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
Smoothed 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

One gene did not amplify on the 96-well LDA panel platforms and therefore only 93 of 94 test genes are reported in this table. 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 table. All genes in this table are currently (July 2011) recognized as genes at the NCBI website.

Fig. 2. Results of validation for the RS versus EC comparison. Genes are identified by their abbreviation, followed by FC and p value in parentheses, separated by a comma. Names of genes and their abbreviations are listed in table 3.

list contained genes significantly overrepresented in 44 pathways. One gene did not amplify in the TaqMan® custom arrays so we report on 93 genes from the custom arrays (fig. 2–4). For the RS versus EC comparison, there was validation of 86% of the genes as 80 out of 93 genes that had a significant difference in FC 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, 8 were significant using qPCR. Two genes changed from a significant FC <1 to significant with a FC >1. In figure 5, RS versus CC is compared for similarities and differences in numbers of genes that were either upregulated, not different, or downregulated grouped according to four broad categories of function. A hierarchical cluster for the RS versus EC comparison is shown in figure 6. Neurotensin was analyzed separately by individual qPCR. From the microarray and PANTHER results, the FC for RS versus EC for neurotensin was 8, the p value <0.0001, and the pathway assignment was unclassified. The individual qPCR results for neurotensin for RS versus EC, RS versus CC and CC versus EC were: FC = 4.59, p < 0.0001; FC = 4.17, p < 0.0001; FC = 1.1, not significant, respectively. Thus, the microarray results were largely confirmed.

Other Results

At the end of this 4-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

Fig. 3. Results of validation for the RS versus CC comparison. Genes are identified by their abbreviation, followed by FC and p value in parentheses, separated by a comma. Names of genes and their abbreviations are listed in table 3.

[5, 9, 13]. Thus, HAM-RS2 needs to be consumed for more than 4 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, and EC 3.2 $\pm$ 0.9 g pooled error), while the CC and EC groups were not different. pH of the cecal contents was much lower ($p < 0.0001$) for RS than for CC and EC (RS 5.97, CC 8.14, and EC 8.49 $\pm$ 0.05 pooled error), and in the CC group cecal contents' pH was also significantly lower ($p < 0.0001$) than in the EC group. Plasma total PYY (fig. 7a) and GLP-1 (fig. 7b) 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 a 4-week period.

Fig. 4. Results of validation for the CC versus EC comparison. Genes are identified by their abbreviation, followed by FC and p value in parentheses, separated by a comma. Names of genes and their abbreviations are listed in table 3.

Fig. 5. Venn diagrams comparing RS and CC gene expression grouped according to four broad categories of function. The figure compares RS versus CC for similarities and differences in numbers of genes that were either upregulated (A, D, G, J), not different (B, E, H, K), or downregulated (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.

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, replacing other starches, would lower the energy den-

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, similar to that 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 confirmed

Fig. 7. **a** Plasma PYY levels (pg/100 μ l). **b** Plasma GLP-1 levels (pM). Bars with different superscripts are statistically different at $p < 0.0001$.

by measuring short-chain fatty acids in cecal contents after 1 week of feeding of HAM-RS2 [5]. After 4 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 4-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. This may mean that some of the results in our studies may be due to adolescent growth. However, many of our studies use about this age as a starting age.

The results from the microarray indicated that about 38% of the genes of the rat genome (10,000+ out of approx. 27,000) were expressed above an acceptable level of noise (signal-to-noise ratio ≥ 3) from the sampled cells (table 2). It was also validly hypothesized that the RS versus 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 (fig. 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 Bonferroni adjustment. However, our next step was to focus on the RS versus EC comparison for validation of the microarray results. As a result, we expanded the number of PANTHER pathways by removing the Bonferroni 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 versus 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 (fig. 2).

The general picture from the results of the validation of the microarray using the RS versus 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 3,979, EC 4,608; 19% weight of diet as HAM-RS2 $5,555 \pm 172 \mu$ m 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 table 3 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 kinase phosphatases [24–26]. *Areg* mRNA was highly elevated in RS versus in 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- α (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.

Similarly to simultaneous stimulation of growth factors and inhibitors of growth, there was an increase in the mRNA of a pro-inflammatory signal *Il1a* [31]. Along with an increase in gene expression of *Il1a*, there was 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 the 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 versus 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* belongs to the EGF family of polypeptides and is involved in cell proliferation [39], but is also upregulated 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 [41]. In several types of cancer cells, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) increases the protein cellular myc, which represses gene expression of *GADD45 α* and γ . The result is the escape from programmed cell death [42]. *Jun-B* proto-oncogene mRNA was increased and this occurs under the control of TGF- β [43] and is associated with cell proliferation and differentiation [44]. However, knockdown of *Jun-B* in wild-type murine fibroblasts did result in increased proliferation and tumorigenicity [45]. *Rheb* inhibits NF- κ B 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- κ B and increased apoptosis as NF- κ B is known to be an inhibitor of apoptosis and increased activity increases risk of cancer [41]. Psme3 (also known as PA28 γ) 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; the protein is a subunit of the transcription factor Hif-1. This transcription factor plays a role in inducing the transcription of genes whose proteins play a role in survival during hypoxia, which also induces Vegfa [49]. Vegfa mRNA was also increased; the protein product promotes increased blood vessel development [50]. At the same time, Adm mRNA was increased; translation of Adm mRNA results in a proadrenomedullin peptide that upon post-translational modification results in a peptide that promotes vasodilation and hypotension by inhibiting angiotensin II [51]. However, Adm protein is also elevated 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 upregulated. 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 Δ 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.’ Moreover, under various conditions the protein can have tumor-suppressive or oncogenic effects. They reported that downregulation of Atf3 promoted colon cancer and that blocking the production of heat shock protein 90 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 upregulated in the RS versus 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 upregulated in the RS versus EC comparison. Slc16a3 is also a 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 upregulated, but Fabp6, which appears to bind bile acids in the cytoplasm after sodium-dependent uptake by enterocytes by active transport [58] was downregulated by RS. Several other Slc16a transporter proteins were upregulated 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 that were identified by PANTHER as proteins that are components of metabolic pathways were increased in the RS versus EC comparison. 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 Hori et al. [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 aspar-

tate. Cbs is an enzyme catalyzing the reaction of serine plus homocysteine to cystathionine. Enol 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 versus 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 in 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 versus EC and RS versus CC comparisons. However, the CC versus EC comparison results from the validation were quite different from the results of the other two comparisons (fig. 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 versus EC compared to EC versus 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 FOS and wheat bran, demonstrate some general similarity to the results from the currently reported study [18, 76]. Chen et al. [18] reported 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. 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 FCs >1.6, Fukasawa et al. [76] report FCs >1. We also chose to report data as FCs lower than a prescribed number such as 1.5 because many of the relatively lower FCs 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 at $p < 0.05$. However, the risk of a type 1 error is much lower for the latter p value. Small FCs for RS versus 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 versus 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/1,000 g of diet, ~297 g of HAM-RS2/1,000 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/1,000 g of diet and ~50% of the 297 g of HAM-RS2/1,000 g of diet or 148.5 g/1,000 g of diet. The EC diet had 177 g cellulose added/1,000 g 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 versus 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 or 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 versus 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 versus 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 FCs, 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 was 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 appear to improve the structure and function of the GI tract, according to 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.

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