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Satiety Hormone and Metabolomic Response to an Intermittent High Energy Diet Differs in Rats Consuming Long-Term Diets High in Protein or Prebiotic Fiber

Raylene A. Reimer,*[,†,‡] Alannah D. Maurer,[‡] Lindsay K. Eller,[†] Megan C. Hallam,[‡] Rustem Shaykhutdinov,[§] Hans J. Vogel,[§] and Aalim M. Weljie[§,#]

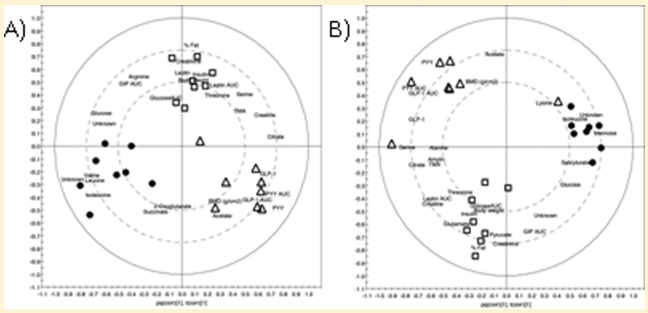
[†]Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, T2N 4N1, Canada

[‡]Faculty of Kinesiology, University of Calgary, 2500 University Drive NW, Calgary, Alberta, T2N 1N4, Canada

[§]Department of Biological Sciences, Bio-NMR Center, University of Calgary, Calgary, Alberta, T2N 1N4, Canada

S *Supporting Information*

ABSTRACT: Large differences in the composition of diet between early development and adulthood can have detrimental effects on obesity risk. We examined the effects of an intermittent high fat/sucrose diet (HFS) on satiety hormone and serum metabolite response in disparate diets. Wistar rat pups were fed control (C), high prebiotic fiber (HF) or high protein (HP) diets (weaning to 16 weeks), HFS diet challenged (6 weeks), and finally reverted to their respective C, HF, or HP diet (4 weeks). At conclusion, measurement of body composition and satiety hormones was accompanied by ^{1}H NMR metabolic profiles in fasted and postprandial states. Metabolomic profiling predicted dietary source with >90% accuracy. The HF group was characterized by lowest body weight and body fat ($P < 0.05$) and increased satiety hormone levels (glucagon-like peptide 1 and peptide-YY). Regularized modeling confirmed that the HF diet is associated with higher gut hormone secretion that could reflect the known effects of prebiotics on gut microbiota and their fementative end products, the short chain fatty acids. Rats reared on a HF diet appear to experience fewer adverse effects from an intermittent high fat diet in adulthood when rematched to their postnatal diet. Metabolite profiles associated with the diets provide a distinct biochemical signature of their effects.



KEYWORDS: *nuclear magnetic resonance, obesity, dietary fiber, prebiotic, adiposity, metabonomics*

INTRODUCTION

Long-term health is profoundly influenced by environmental conditions experienced early in life.[1] The ability to respond to environmental cues, or plasticity, is critical to survival but can result in adaptations that influence disease risk later in life.[1] We previously demonstrated that a high protein diet consumed from weaning to four months of age predisposed rats to greater obesity risk in adulthood following a metabolic challenge with a high energy diet.[2] Body weight, fat mass and glycemia in adult males was higher following a high fat/sucrose challenge in rats that consumed a high protein versus a high prebiotic fiber diet from weaning.[2] Plasma concentrations of the anorexigenic hormone, glucagon-like peptide-1 (GLP-1), were higher in the high prebiotic compared to high protein-fed rats, and leptin was elevated in high protein versus high prebiotic rats.[3] Whether recovery from the high fat/sucrose diet in terms of body fat and satiety hormones is better in rats consuming a long-term high protein versus high fiber diet is not known.

Consumption of a high fat diet is known to produce numerous detrimental metabolic effects including excess adiposity, insulin resistance and leptin resistance.[4] What is less well understood is whether a transient high fat diet is less detrimental when introduced intermittently into a long-term dietary pattern high in protein or dietary fiber. A systematic evaluation of the metabolic response to a transient high fat diet is warranted.

While it is common to measure a limited number of metabolites in serum, such as glucose, in response to dietary intervention or in disease states such as diabetes, it is now possible to measure global metabolic response using proton nuclear magnetic resonance-based metabolomic (^{1}H NMR) analysis.[5,6] NMR has the advantage of providing the simultaneous quantitative measurement of many metabolites that could provide a more complete picture of metabolic

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response to diet and provide novel information that can be used to probe unrecognized mechanisms.[7−9]

Therefore, our objective was to examine the metabolomic profile of adult Wistar rats exposed to a temporary high fat/sucrose diet in the context of a long-term high protein or high fiber diet. Body composition and satiety hormone response during an oral glucose tolerance test were also measured.

MATERIALS AND METHODS

Animals and Diets

Ethical approval was obtained from The University of Calgary Health Sciences Animal Care Committee and was consistent with the National Research Council's guide for the care and use of laboratory animals. Female Wistar rats were obtained from Charles River (Montreal, PQ, Canada) and housed in a temperature and humidity controlled room with a 12-h light, 12-h dark cycle. After a period of acclimatization, females were mated with Wistar males in wire-bottom cages. On the day a copulation plug was found, the females were isolated and given free access to control diet (AIN-93G[10]). One day following birth, the litters were culled to 10 pups (5 males and 5 females where possible) to minimize differences in feeding between litters. At weaning (21 d), the male rats were randomized to one of 3 experimental diets: control (C), high fiber (HF, 21% wt/wt) and high protein (HP, 40% wt/wt). The females were not examined further for the purposes of this study. Details of the composition of the diets have been previously published.[2] A combination of the prebiotic fibers, inulin and oligofructose, at a ratio of 1:1 (by weight) were used in the HF diet. Rats consumed these diets until 15 weeks of age when all rats were given a high fat/high sucrose (HFS) diet for 6 weeks. The HFS diet provided 40% of energy from fat and 45% from sucrose and was composed of (g/100 g) cornstarch (5), casein (14), sucrose (51), soybean oil (10), lard (10), Alphacel (5), AIN-93 M mineral mix (3.5), AIN-93 vitamin mix (1), L-cystine (0.3), and choline bitartrate (0.25). After 6 weeks of HFS consumption, the rats were placed back on their respective weaning diet for an additional 4 weeks. Diets met all nutritional requirements of growing rats based on AIN-93G recommendations, and those of adult rats for maintenance once the rats reached 10 weeks (AIN-93M). Rats were individually housed, and food and water provided ad libitum throughout the experiment.

Body Weight, Food Intake, and Plasma Collection

Body weight was measured weekly throughout the experiment. Food intake was measured throughout the study by subtracting the weight of the feed cup from the previous days' weight. Energy intake was calculated by multiplying food intake by the energy density of each diet (i.e., 3.7 kcal/g for C and HP, and 3.3 kcal/g for HF). At the end of the study, body composition was measured (fat mass, lean mass, bone mineral content (BMC), and bone mineral density (BMD)) using dual energy X-ray absorptiometry (DXA) with software for small animal analysis (Hologic QDR 4500, Hologic, Inc., Bedford, MA). An oral glucose tolerance test (OGTT) was performed at the end of the study. After an overnight fast, rats were anesthetized with isoflurane, and a fasting cardiac blood sample was taken for metabolomics and glucose and satiety hormone analysis. Rats were then given 50% glucose (wt/vol) by gavage at a dose of 2 g of glucose/kg. Additional samples were taken at 15, 30, 60, and 90 min postgavage according to our previous work.[11] Separate serum samples for metabolomics analysis were collected at fasting (time = 0) and one postprandial time point (t = 30 min). Blood glucose concentrations were measured immediately using a blood glucose meter (Accu-Chek Blood Glucose meter, Laval, QC). Blood for satiety hormone analysis was collected in tubes containing diprotinin-A (0.034 mg/mL of blood; MP Biomedicals, Irvine, CA); Sigma protease inhibitor (1 mg/mL of blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1 mg/mL of blood; Roche, Mississauga, ON, Canada) and then centrifuged at 1600g for 15 min at 4 C. Plasma was stored at −80 °C until analysis. After the final blood sample, the rats were over-anesthetized followed by an aortic cut. The liver, stomach, small intestine, cecum, and colon were weighed, and a sample was snap frozen in liquid nitrogen and then stored at −80 °C.

Plasma Analysis for Satiety Hormones

Ghrelin (active), amylin (active), insulin, leptin, GIP (total), GLP-1 (active) and PYY (total) concentrations were quantified using a Rat Gut Hormone Panel Milliplex kit (Millipore, St. Charles, MO) and Luminex instrument according to the manufacturer's specifications. The sensitivity for the Milliplex kit (in pg/mL) is 2 (ghrelin), 20 (amylin), 1 (GIP), 28 (insulin), 27 (leptin), 16 (PYY) and 28 (GLP-1).

RNA Extraction and Real-Time PCR

Total RNA was extracted from the ileum and colon using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed with an input of 1 μg of total RNA using the first strand cDNA synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA USA) with oligo d(T)15 as a primer. Real time PCR using primers for proglucagon and PYY was performed according to our previous work (Maurer et al., 2009).

Sample Preparation and Metabolomics Analysis

Serum samples were stored at −80 °C prior to analysis. Samples were thawed and filtered using 3-kDa Nanosep microcentrifuge filters, prewashed to reduce contamination. The filtrate was transferred to clean microfuge tubes; the final sample volume ranged from 200 to 300 μL. Samples were brought to 650 μL by addition of D_2O, 130 μL of phosphate buffer containing dimethyl-silapentane-sulfonate (DSS, final concentration 0.5 mM) and 10 μL of sodium azide. Final sample pH was adjusted to 7.00 ± 0.05.

All NMR experiments were performed on a Bruker Advance 600 spectrometer (Bruker Biospin, Milton, Canada) operating at 600.22 MHz and equipped with a 5 mm TXI probe at 298 K. All one-dimensional ^{1}H NMR spectra of aqueous samples were acquired using a standard Bruker noesygppr1d pulse sequence in which the residual water peak was irradiated during the relaxation delay of 1.0 s and during the mixing time of 100 ms. A total of 1024 scans were collected into 63 536 data points over a spectral width of 12 195 Hz with a 90° pulse width of 10.4 μs and a 5 s repetition time. A line broadening of 0.1 Hz was applied to the spectra prior to Fourier transformation, phasing, and baseline correction. Additional two-dimensional NMR experiments were performed for the purpose of confirming chemical shift assignments, including total correlation spectroscopy (2D ^{1}H−^{1}H TOCSY) and heteronuclear single quantum coherence spectroscopy (2D ^{1}H−^{13}C HSQC), using standard Bruker pulse programs.

Processed spectra were imported into Chenomx NMR Suite software (version 4.6, Edmonton, AB, Canada) for quantification. Each compound concentration was then normalized to

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account for differences in sample filtration during preparation by dividing the measured concentration into the total concentration of all metabolites in that sample (excluding glucose and lactate because of excessively large concentrations that otherwise dominate the normalization).

Statistical Analysis

All data are presented as mean ± SEM. Differences between the diets were determined using a one-way ANOVA with Tukey's multiple comparison posthoc test. Parameters with serial measurements were analyzed with a repeated measures ANOVA [with time as a within subject variable and diet as the between subject variable] with Bonferroni adjustment when applicable. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using SPSS v 17.0 software (SPSS Inc., Chicago, IL).

For metabolomics analysis, in addition to univariate tests, multivariate analysis was conducted using SIMCA-P+ 12.0.1 software (Umetrics, Sweden) to better assess the concentration changes. Data was preprocessed by mean-centering and unit variance scaling. A supervised partial least-squares discriminant analysis (PLS-DA) approach was chosen to compare the variance of metabolite concentrations between three sample classes (three diet types: control diet, HF diet and HP diet). A supervised orthogonal partial least-squares analysis was used (HF diet versus HP diet model) for a direct comparison of the variance between diet type (Y variable) and metabolite concentrations (X variable). The results from the metabolomics analysis were also combined together with the plasma analysis of satiety hormones and other biological measurements and regressed to the diet type using O2-PLS-DA (orthogonal PLS-DA). Data filtration was accomplished using an alternate modeling procedure for variable selection, "lasso regression",[12] which is designed to handle the multivariate collinearity in high-dimensional "omics" studies. This was accomplished using the "gmlnet" package in gnu R (http://www.R-project.org).[13]

RESULTS

Body Weight, Energy Intake, and Body Composition

Body weight over the course of the study was influenced by week ($P < 0.001$), diet ($P < 0.001$) and their interaction ($P < 0.008$). In the first phase, rats consumed the C, HF, or HP diet from weaning until 15 weeks of age. At the end of this period the rats fed HF diet had significantly lower body weight than rats fed C and HP ($P < 0.001$, Figure 1A). Rats then consumed a HFS diet for 6 weeks as a metabolic challenge. Throughout the entire 6 week period, rats fed the HF diet maintained a lower body weight than rats fed C and HP ($P < 0.01$). After the final 4 week period consuming their respective long-term diet, rats fed HF had lower body weight than HP but not C ($P < 0.01$). Similar to body weight, there was a significant effect of week ($P < 0.001$), diet ($P < 0.03$) and week × diet ($P < 0.001$) on energy intake. Rats fed HP diet had consistently higher energy intake across all three dietary periods that was higher than C and HF at 12 weeks ($P < 0.04$, Figure 1B) and higher than HF at 21 weeks ($P < 0.02$). In the last 3 weeks of the study HP energy intake was higher than C ($P < 0.01$), which was in turn higher than HF ($P < 0.05$). Body composition was measured at study termination, at which time rats fed HF diet had significantly lower percent body fat than HP rats ($P < 0.03$, Table 1). Bone mineral density was lower in rats fed C and HP diet versus HF ($P < 0.05$). Liver weight was lower ($P < 0.05$) and cecum weight higher ($P < 0.001$) in rats fed HF versus C and HP.

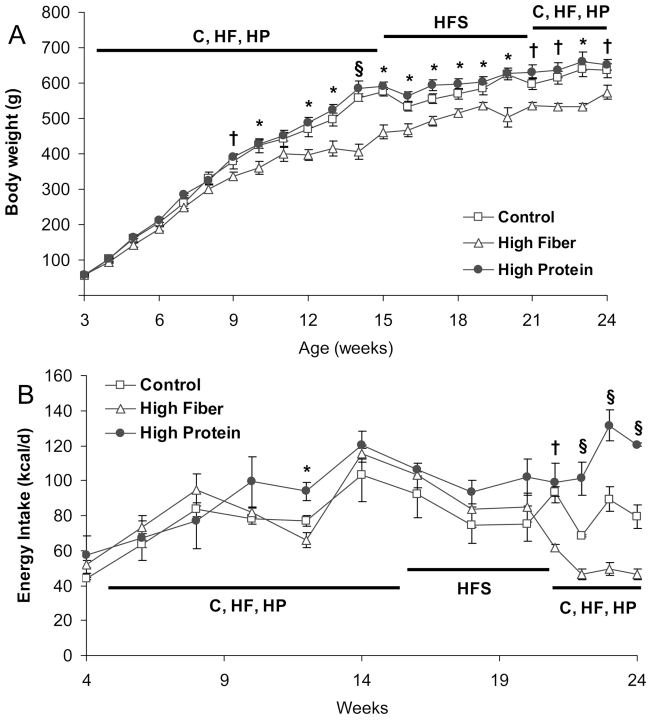

Figure 1. Body weight and energy consumption of rats rematched to control, high fiber, or high protein weaning diets following a high fat, sucrose diet challenge in adulthood. Results are presented as mean ± SEM, $n = 10$ per group. Panel (A) represents longitudinal body weight. Panel (B) represents energy intake measured throughout the 16 weeks of C, HF, or HP diet, followed by 6 weeks of high fat/sucrose diet, and 4 weeks of rematching to C, HF, or HP diet. In Panel (A), the * represents a difference ($P < 0.05$) between HF versus C and HP. The † represents a difference ($P < 0.05$) between HF and HP. The § represents a difference ($P < 0.05$) among all 3 groups. In Panel (B), the * represents a difference ($P < 0.05$) between HP versus C and HF. The † represents a difference ($P < 0.05$) between HF versus C and HP. The § represents a difference ($P < 0.05$) among all 3 groups.

Table 1. Final Body Composition of Rats Rematched to Control, High Fiber, or High Protein Weaning Diets Following a High Fat, Sucrose Diet Challenge in Adulthood

	control	high fiber	high protein
final body weight (g)	634.9 ± 21.1^{a,b}	573.8 ± 20.1^{a}	651.3 ± 15.8^{b}
body fat (%)	29.2 ± 2.6^{a,b}	23.6 ± 1.1^{b}	29.9 ± 2.0^{a}
lean mass (g)	449.6 ± 19.7	443.3 ± 16.5	441.0 ± 11.6
bone mineral density (g/cm^3)	0.184 ± 0.002^{a}	0.192 ± 0.002^{b}	0.185 ± 0.001^{a}
cecum weight (g)	1.0 ± 0.1^{a}	7.7 ± 2.5^{b}	1.2 ± 0.1^{a}
liver weight (g)	18.0 ± 1.1^{a}	13.5 ± 1.2^{b}	20.7 ± 1.5^{a}

^{a,b}Values are mean ± SE with $n = 6–8$ per group. Treatments with different superscript letters are significantly different ($p < 0.05$).

Blood Glucose and Satiety Hormone Response

At the end of the study the HF group showed significantly lower glucose AUC than C ($P < 0.01$, Figure 2B). Insulin levels during the OGTT were higher in C versus HF rats at 15 and 30 min ($P < 0.02$, Figure 2C) and higher in HP rats at 90 min ($P < 0.03$). Reflective of glucose, insulin AUC was lower in rats fed HF versus C and HP ($P < 0.05$, Figure 2D). Fasting leptin was higher in rats fed C versus HF and higher in C versus HP at 30

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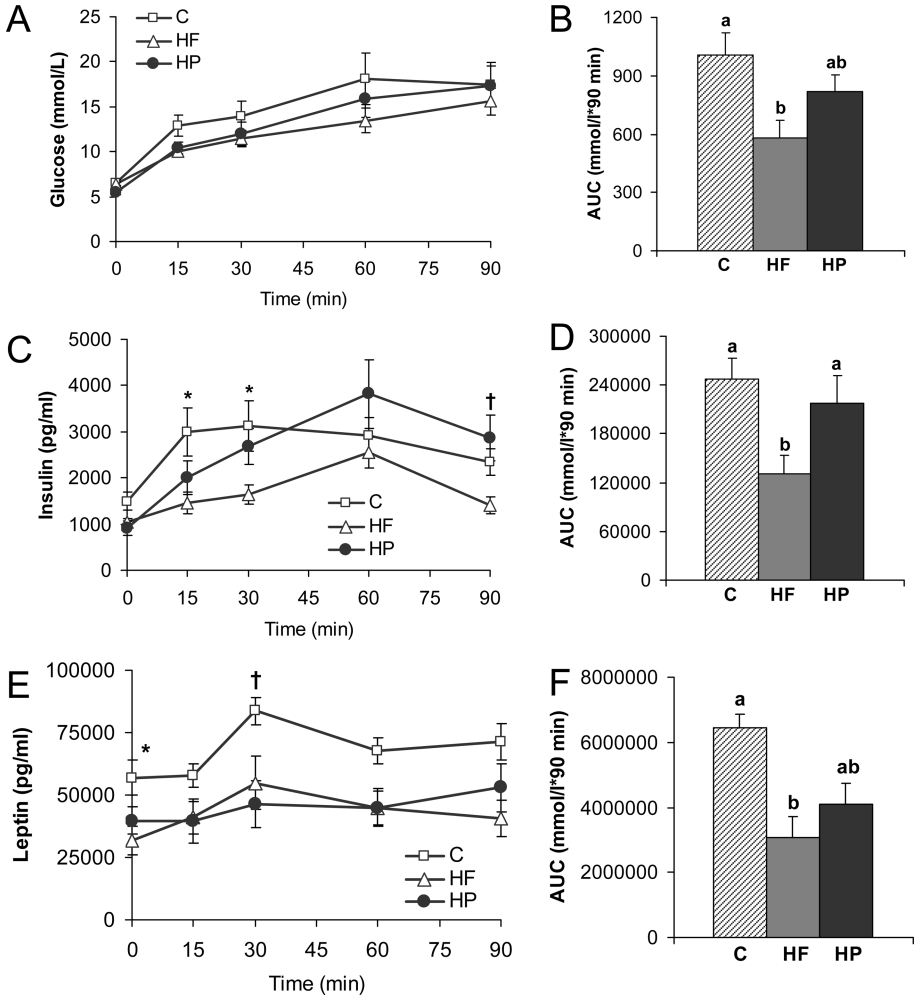

Figure 2. Concentrations of blood glucose and plasma insulin and leptin in rats during an oral glucose tolerance test following the rematching period. Results are presented as mean ± SEM, n = 8−9 per group. Panel (A) represents the serial values and panel (B) the tAUC for blood glucose during the OGTT. Means with different superscripts are different (P < 0.05). Panel (C) represents the serial values and panel (D) the tAUC for plasma insulin during the OGTT. In Panel (C), the * represents a difference (P < 0.05) between HF versus C and the † a difference between HF and HP. In panel (D), means with different superscripts are different (P < 0.05). Panel (E) represents the serial values and panel (D) the tAUC for plasma leptin during the OGTT. In panel (E), the * represents a difference (P < 0.05) between C versus HF and the † a difference between C and HP. In Panel (F), means with different superscripts are different (P < 0.05).

min (P < 0.05, Figure 2E). Leptin AUC was lower in HF versus C (P < 0.01, Figure 2F).

Rats fed the C and HP diets had very similar profiles of GLP-1, PYY and GIP secretion during the OGTT (Figure 3A−F). Rats fed the HF diet, however, had markedly higher GLP-1 and PYY levels and lower GIP levels compared to C and HP rats (P < 0.01, Figure 3B,D,F). With the exception of the 30 min time point for GLP-1, levels of GLP-1 and PYY were higher in rats fed HF versus C and HP for every time point during the OGTT (Figures 3A,C, P < 0.05). Rats fed the HF diet had lower levels of GIP at 60 min compared to C and HP and lower than HP at 90 min (P < 0.03). There were no significant differences in ghrelin between the groups.

Intestinal Expression of PYY and Proglucagon

There was an approximate 5-fold increase in PYY mRNA levels in the colon of rats fed the HF diet (Supporting Information, Figure S1), which was significantly higher than C and HP (P < 0.05). Similarly, proglucagon mRNA levels in the colon were 11-fold higher in rats fed the HF versus C and HP diet. In the ileum, PYY mRNA levels were significantly higher in HF versus C and HP (P = 0.002), whereas the approximately 3-fold increase in proglucagon in the ileum with HF did not differ from the other groups (P = 0.14).

Fasting Metabolic Profile in Serum at the End of the Rematching Period

A total of 50 metabolites were screened in serum at the end of the study. The PLS-DA scores plot of serum showed a significant separation of the C, HF and HP groups in the fasted state (P < 0.05, Figure 4A; coefficient plots shown in Supporting Information, Figure S2). This result suggests that the overall metabolic changes in metabolite levels are reflective of individual diets. The most important metabolites involved in the discrimination of the HP group in the fasted state were increases in leucine, isoleucine, isobutyrate, mannose and reductions in creatine, citrate, and serine (Figure 5A and Table 2). The fasted HF group was characterized by a decrease in arginine. Both groups were distinguished from the control group by a decrease in creatinine. In order to probe more precisely the metabolic shifts, an additional shared-and-unique structure (SUS) analysis was conducted[14] (Figure 6). In this

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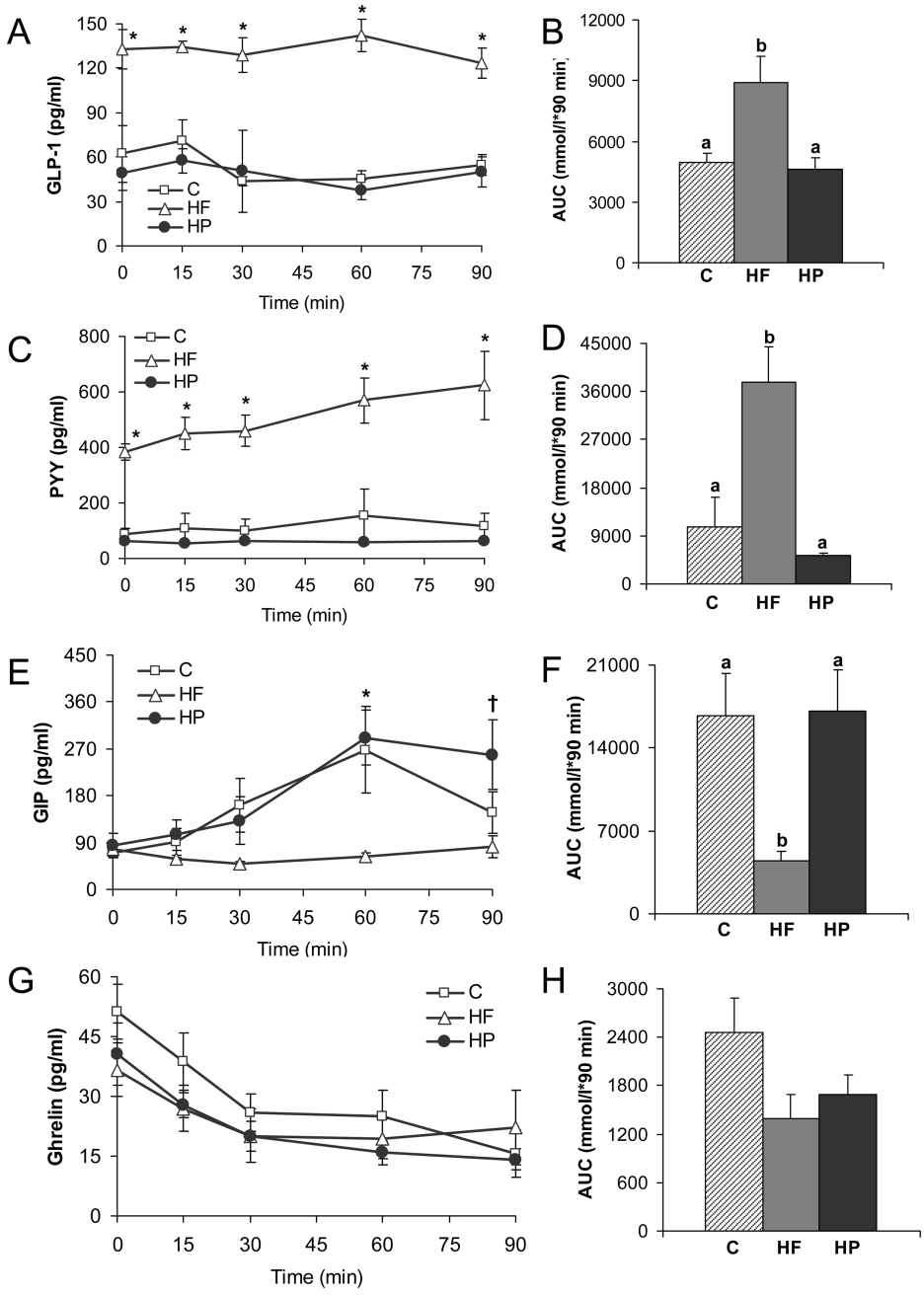

Figure 3. Concentrations of plasma GLP-1, PYY, GIP, and ghrelin in rats during an oral glucose tolerance test following the rematching period. Results are presented as mean ± SEM, n = 8–9 per group. Panel (A) represents the serial values and panel (B) the tAUC for GLP-1 during the OGTT. In panel (A), the * represents a difference (P < 0.05) between HF versus C and HP. In panel (B), means with different superscripts are different (P < 0.05). Panel (C) represents the serial values and panel (D) the tAUC for PYY during the OGTT. In panel (C), the * represents a difference (P < 0.05) between HF versus C and HP. In panel (D), means with different superscripts are different (P < 0.05). Panel (E) represents the serial values and panel (D) the tAUC for GIP during the OGTT. In panel (E), the * represents a difference (P < 0.05) between C versus HF and HP and the † a difference between HF and HP. In panel (F), means with different superscripts are different (P < 0.05). Panel (G) represents the serial values and panel (H) the tAUC for ghrelin during the OGTT. No significant differences were detected for ghrelin.

analysis metabolites that are altered in the same way in HP and HF (compared to control diets) will fall along the positive diagonal. Any difference in metabolite levels will fall in the off-diagonal areas. Interestingly, changes in levels of the short chain fatty acids (SCFA) acetate and an unidentified SCFA were elevated in the HF group (positive coefficients on x-axis), while having coefficients close to zero in the HP group (y-axis). Conversely, levels of glucose were decreased in HF while elevated in HP.

Postprandial Metabolic Profile in Serum at the End of the Rematching Period

Blood was also collected at 30 min following an oral glucose load and 50 metabolites screened in the postprandial state. Similar to the fasted state, the PLS-DA scores plot of postprandial serum showed a significant separation of the C, HF and HP groups (P < 0.05, Figure 4B). Similar to the fasted state, the HP group was characterized by an increase in mannose and decrease in serine, although interestingly no other

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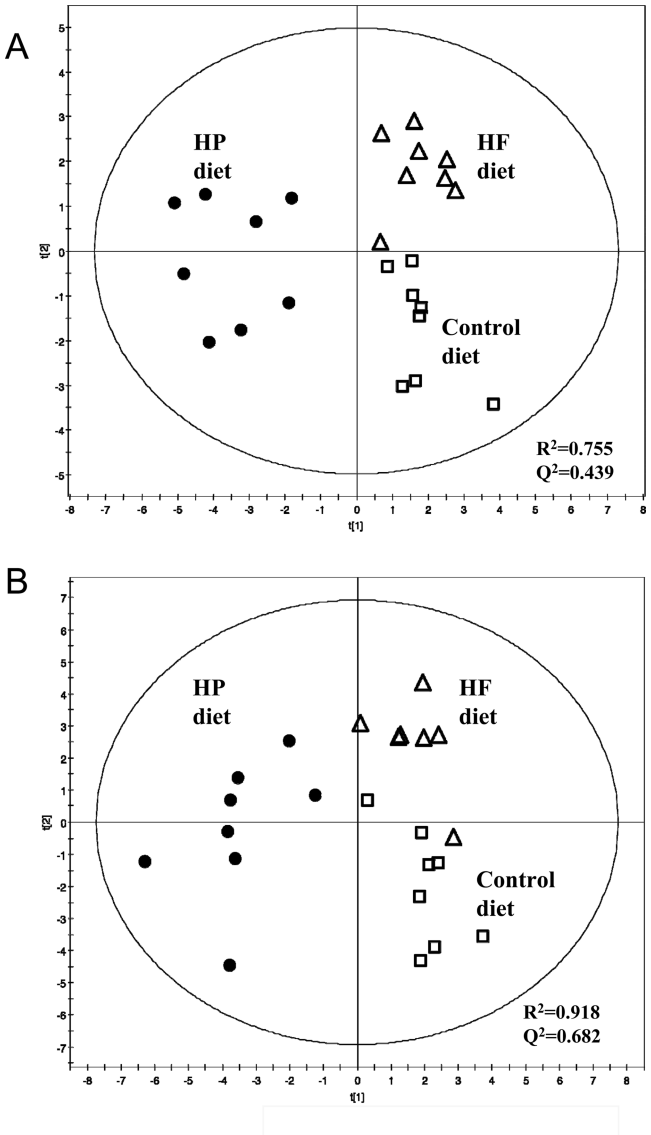

Figure 4. Scores plot and loadings plot for the (A) fasted and (B) postprandial state in rats at the end of the rematching period. PLS-DA score plots of serum samples. One data point represents the combined metabolite measurement from one rat: ■ control, ● high fiber, ♦ high protein. The $t[1]$ and $t[2]$ values represent the scores of each sample in principle components 1 and 2, respectively. R^2 is the explained variance; Q^2 is the predictive ability of the model. The plots represent $n = 8$ per treatment. The postprandial state represents blood collected at 30 min following an oral glucose load (2 g/kg BW).

metabolites were shown to be significantly important (Figure 5B and Table 2). Both HP and HF groups were differentiated from the control group by decreases in creatine, creatinine, glutamate, and threonine.

Relationship between Satiety Hormones, Biological Measurements, and Metabolomics

In order to better elucidate the metabolic relationships between the satiety hormone measurements, biological measurements, and metabolomics data, integrated O2PLS-DA models were generated in which all of the data was regressed to the diet type (Figure 5A, fasted; Figure 5B, postprandial). Together, these analyses provide a detailed view of which metabolites are altered in response to dietary shifts. When examined together with an independent regularized modeling procedure (lasso regression) (Table 2), the HF diet is associated with increases in plasma PYY and total AUC for PYY in both states. Similarly, the HF diet is associated with decreases in fat mass (%) and tAUC for glucose in both states compared to the control group, and interestingly, the fasted measurements for HF are associated with increased BMD.

■ DISCUSSION

Organisms continually respond to cues in their environment, particularly nutritional cues.[15] In humans, growth and development can be segregated into five overlapping periods including prenatal, infantile, childhood, juvenile, and pubertal growth.[1] The transitions between these periods are times of enhanced plasticity or ability to adapt.[1] We previously showed that introduction of a HP diet at weaning (similar to infantile in humans) predisposed rats to higher percent body fat and worse glucose control following a high fat and sucrose diet challenge in adulthood, in contrast to rats weaned onto a diet high in prebiotic fiber that were protected.[2,16] From that study, however, we did not know if returning to the diet consumed throughout growth would mitigate any of the damage caused by the transient high energy diet intake phase. Our objective in this study, therefore, was to determine if long-term consumption of these same high protein and fiber diets protects rats from the deleterious effects of a transient high fat, sucrose diet. Our major findings include (1) a lower final body fat and glucose AUC in HF rats and no difference between C and HP; (2) markedly elevated plasma and intestinal mRNA levels of the anorexigenic hormones, PYY and GLP-1 (proglucagon) in HF compared to C and HP rats; (3) significantly reduced secretion of GIP in HF versus C and HP rats; (4) metabolomic profiles that predicted class separation between the diets with >90% accuracy; and (5) a clearly distinguishable spectrum of metabolites for each diet that shows a meaningful relationship with biological measures such as satiety hormone secretion and body fat. Overall, the HF rats had lower body weight, % body fat and glucose, leptin and GIP AUC and higher GLP-1 and PYY AUC and BMD compared to the HP rats. In addition, they had lower energy intake and insulin AUC compared to HP and C rats.

As predicted from our previous work,[2,16] rats fed the HF diet from weaning to 15 weeks of age gained weight at a slower pace than rats consuming C or HP diets. Rats fed the HP diet maintained the highest body weight throughout the study, and this was consistently higher than the rats fed the HF diet. From the longitudinal growth curves, it is clear that reduced weight gain by the rats fed HF during the first phase of the study provided them an advantage throughout the entire study. Whereas the rats fed the HF diet gained an average of 405 g of body weight in the first 15 weeks, the rats fed the C and HP diets gained 520 and 533 g, respectively. Weight gain during the HFS and final rematched periods were similar across groups, thereby resulting in a final body weight that was significantly lower in HF compared to HP rats. Consistently higher body weight in the rats fed the HP diet appeared to be maintained in part because of higher energy intake, particularly in the final rematching period when their energy intake was markedly higher than HF rats throughout and significantly higher than C rats in the final 3 weeks. Although this study is limited in having body fat measures only at termination due to restrictions on animal movement in and out of the facility, it is interesting that the rats fed HP diet had nearly identical body fat (%) to C rats. This is in contrast to our previous work in which body fat was

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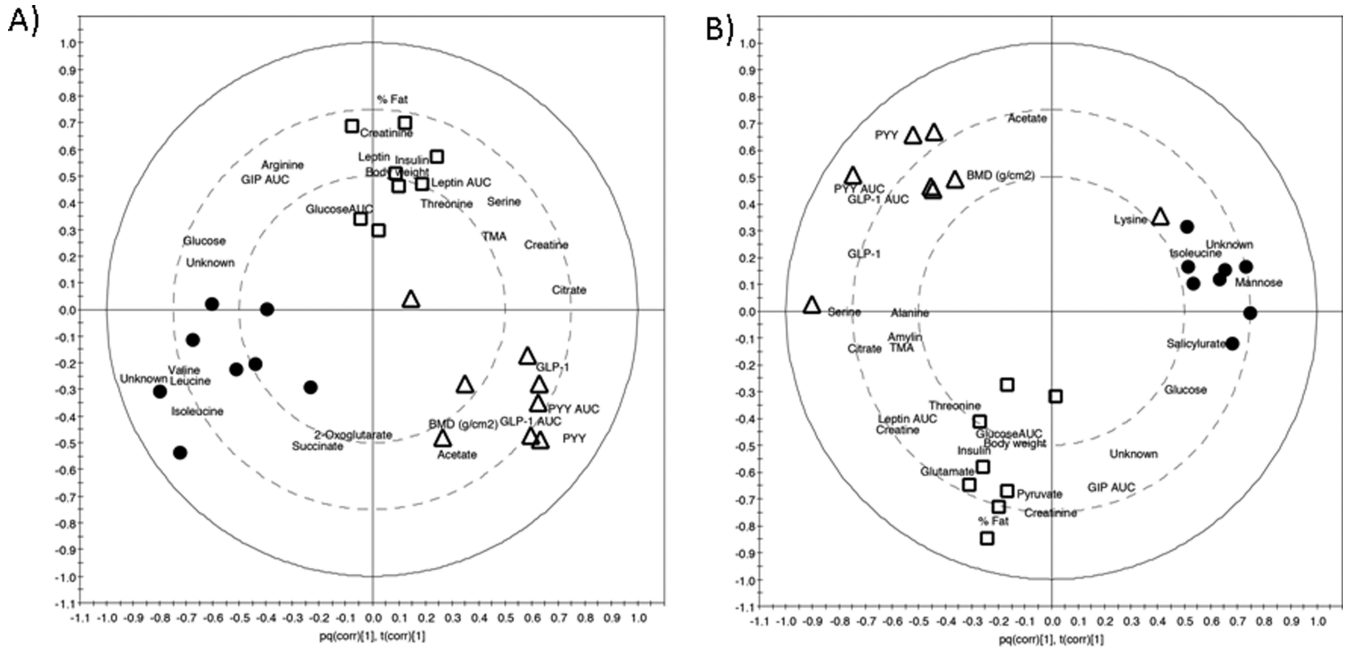

Figure 5. Integration of metabolomics data with biological parameters through O2PLS-DA modeling. (A) Fasted metabolomics measurements ($p = 4.2 \times 10^{-10}$, $R^2 = 0.857$, $Q^2 = 0.795$), and (B) postprandial metabolomics measurements ($p = 4.0 \times 10^{-9}$, $R^2 = 0.833$, $Q^2 = 0.74$). Scores and loading biplot from the O2PLS-DA modeling are shown with scores as large points, and the loadings as small points labeled. Only the variables with the largest influence on projection (VIP) are shown (VIP $\geq$ 1). (Note that the reversal of axis between the two plots is a function of the mathematics and does not impact the relationships between the measured parameters and diets).

Table 2. Comparison of Measurements Deemed Most Significant in the Fasted and the Fed States As a Function of Diet[a]

		metabolomics measurement	satiety hormone measurement	biological measurements
fasted	high fiber	↓arginine	↑PYY	↑BMD (g/cm^2)
			↑PYYAUC	
	high protein	↓citrate		
		↓creatine		
		↑isoleucine		
		↑leucine		
		↑mannose		
		↑isobutyrate		
		↓serine		
	control	↑creatinine	↑insulin	↑body weight
				↑% fat
				↑glucoseAUC
fed	high fiber		↑PYY	
			↑PYYAUC	
	high protein	↑mannose		
		↓serine		
	control	↑creatine		↑% fat
		↑creatinine		↑glucoseAUC
		↑glutamate		
		↑threonine		

[a]The most significant features (either metabolomic or biological) associated with each diet type using regularized general modeling techniques.

measured following the HFS phase that showed significantly higher body fat in HP versus C and HF.[2,16] Two possibilities exist to explain this discrepancy. Either the HP rats in the current study also had increased body fat (%) following the HFS phase and as a result of being rematched to their long-term diet had a beneficial reduction in fat mass by the end of the study, or the control rats were somehow different in this study. We have previously seen that some rats fed the AIN-93 control diet gain more fat mass than expected (unpublished results), and therefore it is possible the C group differs between studies even though they were all Wistar rats from the same supplier. What remains consistent between studies, however, is that the HF group had significantly lower body fat (%) compared to the HP group.

We previously showed that following a HFS diet challenge in adulthood, rats that consumed a HP diet during growth had higher blood glucose levels at 30, 60, and 90 min during an OGTT compared to rats fed HF during growth.[16] This elevated glycemic response appears to have been partially corrected in the HP rats when placed back on their long-term diet given that there were no significant differences in glucose AUC between HF and HP at the end of the study. Nevertheless, taking into account the reduced insulin needed to manage the glucose load in the HF rats (Figure 2D), it would appear that HF as opposed to C or HP was associated with greater insulin sensitivity.

Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are released from intestinal L cells and reduce food intake.[17] The ability of prebiotic fibers to increase proglucagon mRNA levels and GLP-1 secretion is well supported.[16,18−20] Increased proglucagon expression typically takes place in the context of increased cecal weight due to the markedly increased bacterial fermentation that occurs with consumption of prebiotics.[16,18] Both GLP-1 and PYY were markedly elevated in rats fed the HF diet, and the advanced regularized modeling confirmed a positive relationship between the metabolite profile of the HF diet and plasma PYY concentrations in the fasted and

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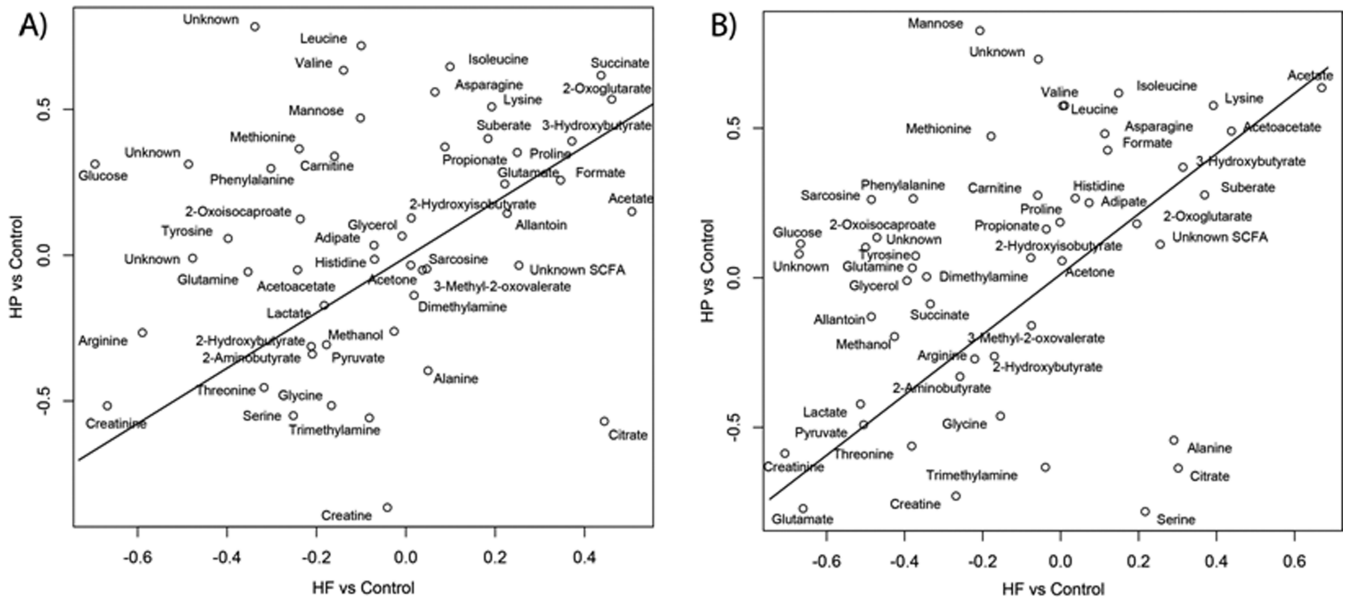

Figure 6. Shared and unique structures (SUS) plots for the (A) fasted and (B) postprandial states. Metabolites that have similar loadings in both the HF vs control (*x*-axis) and HP vs control (*y*-axis) OPLS-DA models will fall along the diagonal with a positive slope. Conversely, metabolites elevated in only one or the other will fall in the off-axis area. For example, citrate is elevated in the HF/control comparison under fasted and fed conditions (positive loadings value) and reduced in the HP/control comparison under both conditions (negative loadings value).

postprandial state. It is interesting to note that the prebiotic fiber diet appears to have acute and lasting effects on the ability of L cells to produce and secrete GLP-1 and PYY. In the current study, measurements for GLP-1 and PYY were made at the end of a 4 week period when fiber was physically in the diet, but we have also shown higher GLP-1 secretion in HF rats at the end of the HFS diet period, a time when the diet is devoid of prebiotics.[3]

Metabolomic profiling allows for the global assessment of endogenous metabolites within an organism.[5,6,21] The use of ^{1}H NMR in this study was able to clearly distinguish between the three diets examined. Partial least-squares-discriminant analysis of serum samples showed clear separation of the C, HF and HP rats at the end of the study. Essentially, the metabolite profile provided a distinct biochemical signature or snapshot of the combined gene function, enzyme activity and physiological response to the experimental diets. Others have similarly shown clearly distinguished phylometabonomic patterns across five inbred strains of mice fed a high fat or normal carbohydrate diet.[22]

External validation of the metabolomic analysis is provided by our glucose measurements. Glucose was measured in two ways in the study, using ^{1}H NMR analysis and using a blood glucose meter during the OGTT. Glucose AUC from the OGTT was significantly lower in rats fed HF versus C, and this was supported by O2PLS-DA modeling in which glucose was shown to be lower with HF diet. Furthermore, we were able to show that the most important metabolites involved in the discrimination of the HP group in the fasted state were increases in isobutyrate, mannose and the branched chain amino acids (BCAA) leucine and isoleucine and reductions in creatine, citrate, and serine. The HF group was characterized by a decrease in arginine.

While BCAA are recognized as key metabolites involved in protein synthesis and cell growth, there is controversial evidence surrounding their role in cellular metabolism linked to the development of obesity-associated insulin resistance.[23] On the one hand, using principal component analysis, Newgard et al[23] identified a distinctive profile related to elevated BCAA in obese versus lean subjects.[23] Evidence suggests that altered metabolism,[23,24] particularly a reduction in the catabolism of BCAA in liver and adipose tissue may contribute to higher plasma BCAA and potentially insulin resistance. On the other hand, oral supplementation with BCAA has been shown to improve insulin sensitivity and reduce diet-induced obesity.[25,26] We detected elevated levels of the BCAA in our rats fed a long-term high protein diet; however, given that the sole protein source in our diets was casein, a milk-derived protein that is rich in BCAA, it is not clear whether these levels simply reflect the diet or metabolic alterations per se.

Several metabolites are known to be of gut microbial origin including dimethylamine and trimethylamine.[24] Connor et al.[24] identified a decrease in trimethylamine in db/db versus db/+ mice using urinary NMR-based metabolomic analysis. In our data set, trimethylamine was consistently shown to positively contribute to the separation between groups and may reflect the higher bacterial fermentation occurring in the rats fed HF.[18] Also consistent with increased bacterial fermentative action in the colon of the HF rats were the higher levels of the SCFA, acetate and an unidentified SCFA shown in the rats consuming the prebiotic fiber. SCFA have been proposed as a chief mechanism responsible for increased expression and secretion of the anorexigenic hormones PYY and GLP-1.[19,27,28] Recently, we have also demonstrated that prebiotic fibers are associated with marked increases in *Bifidobacterium* spp. in diet-induced[29] and genetically lean and obese JCR:LA cp rats.[18] *Clostridium leptum*, a member of the Firmicutes phylum was also markedly reduced with prebiotic fiber intake in diet-induced obese rats.[29] Interestingly, Martin et al.[30] used ^{1}H NMR-based metabolic profiling of fecal matter to probe the complex interaction between host and the gut microbiota. In that study the combination of a prebiotic (galactosyl-oligosaccharide) with a probiotic (*Lactobacillus paracasei*) was associated with increased fecal acetate over time,[30] which supports our observation of

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increased serum acetate in the HF group, assuming appropriate transport into the bloodstream.

A point of interest is the ability of the independent O2PLS-DA models (Figure 5A,B) and regularized modeling results (Table 2) to identify the significant relationship between the HF diet and increased bone mineral density. Indeed, prebiotic fibers have been shown to significantly improve mineral absorption and metabolism and bone composition and architecture in animal models and increasingly in human studies.[31]

On a technical note, regularized modeling is an under-utilized tool in the study of metabolomics data. This approach is designed for analysis of data when multicollinearity is present among variables. This is clearly the situation when multiple metabolites belonging to similar pathways are being measured simultaneously. Briefly, the principle for variable selection lies in the application of penalty terms to variable coefficients (the lasso or ridge regression,[13] or a combination, elastic net) to shrink those coefficients not required for modeling accuracy. In contrast to the use of regularized modeling, the use of projection-based methods such as PCA or PLS is relatively prevalent in metabolomics analysis. Rigorous statistical comparisons of variable selection using PLS methods and regularized modeling have shown that each approach has individual strengths.[32] In our own study, certain differences are noted between the two approaches, such as the association of mannose to the HP diet in the fasted state using regularized modeling, but not using O-PLS-DA. Such differences are to be expected on the basis of the unique treatment of statistical variance in each approach and might provide interesting information about so-called "edge-cases", which are are not clear-cut associations and differences. As a result, we suggest expanding the metabolomics statistical toolbox to include regularized modeling as a worthwhile pursuit.

CONCLUSIONS

In conclusion, we demonstrate that despite higher energy intake, rats rematched to a high protein diet following a transient high fat, sucrose diet have similar percent body fat to those rematched to the control diet. This is in contrast to the increased fat mass that rats raised on a high protein diet display following a terminal high fat, sucrose diet.[2,16] In contrast, a diet high in prebiotic fiber was protective against excess body fat and hyperglycemia throughout the study. In fact, rats reared on a high fiber diet were least affected by an intermittent high fat diet in adulthood when it was followed by a return to their long-term diet. The high fiber diet could potentially provide a degree of metabolic rescue. Finally, a clearly distinguishable spectrum of metabolites was associated with each diet. Using ^{1}H NMR it is possible to identify a unique metabolic phenotype for the experimental diets examined and their relationship to physiological and biochemical outcomes associated with consumption of those diets.

ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: reimer@ucalgary.ca. Phone: (403) 220-8218. Fax: (403) 284-3553.

Present Address

[#]Department of Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania, United States

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BCAA, branched chain amino acids; BMD, bone mineral density; DXA, dual energy X-ray absorptiometry; HFS, high fat−high sucrose; HF, high prebiotic fiber; HOMA-IR, homeostatic model of insulin resistance; HP, high protein; LBM, lean body mass; OGTT, oral glucose tolerance test; tAUC, total area under the curve

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