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Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

CD Obesity-Prone Rats, but not Obesity-Resistant Rats, Robustly Ferment Resistant Starch Without Increased Weight or Fat Accretion

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Objective: This study used CD obesity-prone (OP) and obesity-resistant (OR) rats to examine how weight gain and fat accretion relate to fermentation levels and microbiota composition after feeding resistant starch (RS).
Methods: After feeding OP rats and OR rats a high-fat (HF) diet for 4 weeks, rats were stratified into three groups: they were fed either an HF diet (group 1: HF-HF) or were switched to a low-fat (LF) diet (group 2: HF-LF) or an LF diet supplemented with 20% RS by weight for 4 weeks (group 3: HF-LFRS). Energy intake, body weight, fermentation variables, and microbiota composition were determined.
Results: In OP rats, RS elicited robust fermentation (increased cecal contents, short-chain fatty acids, and serum glucagon-like peptide 1). Total bacteria, species of the *Bacteroidales* family *S24-7*, and the archaean *Methanobrevibacter smithii* increased. The robust fermentation did not elicit higher weight or fat accretion when compared with that of control rats fed the same isocaloric diets (HF-LF ± RS). In OR rats, body weight and fat accretion were also not different between HF-LF ± RS diets, but RS elicited minimal changes in fermentation and microbiota composition.
Conclusions: Robust fermentation did not contribute to greater weight. Fermentation levels and changes in microbiota composition in response to dietary RS differed by obesity phenotype.

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Introduction

Gut microbiota composition affects energy balance (1-4). Compared with lean individuals, those predisposed to obesity may have microbial species that promote more fermentation for more efficient extraction from the diet (1,2). Studies have shown that fermentation of resistant starch (RS) reduces abdominal fat accretion in rodents fed isocaloric diets (5-9), as RS fermentation increases fat oxidation without affecting physical activity (5).

We used outbred colonies of rats: CD obesity-resistant (OR) and CD obesity-prone (OP) rats (Charles River Laboratories, Houston, Texas). They differ in respect to their disposition to develop obesity when on a high-fat (HF) diet (10), with both having a fully functioning leptin receptor (10-12). Their polygenic pattern of inheritance is similar to most human obesity phenotypes (11). We investigated the effects of diet on obesity phenotype and in relation to gut microbiota. Belobrajdic et al. (13) reported that Sprague Dawley rats fed HF diets for 4 weeks resulted in OP and OR rats with reduced body fat when fed RS at different doses. Their study did not use isocaloric diets with RS compared with the control group, as diets were matched for carbohydrate content. They observed the effects of diet energy dilution and fermentation of RS on weight gain. In the current study, our main comparisons had isocaloric diets in two phases of the study and focused on the effects of fermentation by gut bacteria without the confounding effects of energy dilution.

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We measured *Methanobrevibacter smithii*, which constitutes up to 10% of anaerobic gut microorganisms (14). A mutual relationship exists between *M. smithii* and bacterial species that ferment carbohydrates to produce hydrogen and formate. Hydrogen accumulation reduces the fermentation rate by inhibiting bacterial nicotinamide adenine dinucleotide (NAD) dehydrogenases and ATP yield (14,15). By consuming hydrogen in methane production, *M. smithii* improves fermentation efficacy (14). Although this function suggests a role for methanogens in greater caloric harvest and weight gain, the role of methanogens in the pathogenesis of obesity is unclear. Because fermentation is beneficial to host health (16), higher levels of *M. smithii* may be beneficial, so we sought to determine how their abundance relates to the obesity phenotype.

Methods

Animals

The rat study was approved by the Louisiana State University (LSU) Institutional Animal Care and Use Committee. Three- and four-week-old male OP ($N = 32$) and OR ($N = 32$) (Charles River Laboratories) rats arrived, and after a 1-week quarantine, they were singly housed in a temperature-controlled vivarium with a 12:12-hour light/dark cycle. Rats began the study at 5 and 6 weeks old.

Study plan

Our basic design (Figure 1, Table 1) was to feed either an HF or LF diet to OP and OR rats for 4 weeks (phase 1). In phase 1, 10 OP and 10 OR rats were fed the LF diet, and 22 OP and 22 OR rats were fed the HF diet. At the end of phase 1, four OP and four OR rats were euthanized from both diet groups. For phase 2, six OP and six OR rats continued on the LF diet for 4 weeks. The remaining 18 OP and 18 OR rats from the HF group in phase 1 were placed into three groups for each rat type. One group continued on HF (HF-HF), a second was switched to LF (HF-LF), and the third was switched to LF with 20% by diet weight as RS (HF-LFRS).

Our hypothesis, based on a previous study in which an HF diet reduced fermentation of RS compared with an LF diet when HF and RS were fed at the same time (7), was that the HF diet would promote dysbiosis in rats and might affect subsequent bacterial types with the feeding of RS with an LF diet. Thus, there was a need for a control group also fed HF in phase 1 and switched to LF without RS in phase 2. Without the effect of dietary energy dilution for this comparison, OP rats might not have responded as well as in the study by Belobrajdic et al. (13). For phase 2, we thought OP rats might have greater accretion of abdominal fat because of a greater energy harvest not offset by dietary energy dilution.

Diets and feeding

The compositions of the modified AIN-93M diets are listed in Table 2. Weight and food intake were determined twice a week. Food and water were provided *ad libitum*. After a 1-week quarantine on chow, there was 1-week acclimation period with the LF diet. Rats were then fasted (~6 hours) to determine serum glucose and insulin levels after retro-orbital bleeding. The homeostatic model of assessment of insulin resistance index was calculated (17). Rats were stratified based on weight and the homeostatic model of assessment of insulin resistance index.

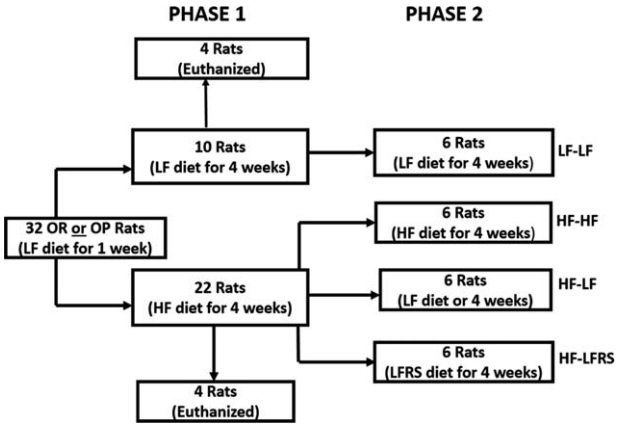

Figure 1 Study design showing two phases with both OP and OR rats.

Serum and gastrointestinal tract collections and analyses

At euthanasia, abdominal fat pads (epididymal, perirenal, and retroperitoneal) were excised. Serum was collected by heart puncture. The weight of the gastrointestinal tract without contents was added to the disemboweled body weight for the emboweled body weight (EBW). Cecal contents were frozen in liquid nitrogen and stored at −80°C. Fermentation (cecum weights, cecal content pH and short-chain fatty acids [SCFAs], and serum glucagon-like peptide 1 [GLP-1] active) was measured as described (7,18,19).

Bacterial DNA extraction

DNA was extracted from 200 mg of cecal contents by using the QIAamp DNA Fast Stool Mini Kit (Qiagen, Valencia, California) according to the manufacturer's protocol, with slight modifications. In the third step, zirconium beads (200 mg; Bio Spec Products Inc.; Cole-Parmer, Vernon Hills, Illinois) and an InhibitEX buffer (Qiagen) were added, and the mixture was subjected to bead beating

TABLE 1 Rat and diet acronyms for phases 1 and 2 of study

	Description
OP	CD obesity-prone rat (Charles River Laboratories)
OR	CD obesity-resistant rat (Charles River Laboratories)
LF	Low-fat diet
HF	High-fat diet
LF-LF	Low-fat diet in phases 1 and 2 of study
HF-HF	High-fat diet in phases 1 and 2 of study
HF-LF	High-fat diet in phase 1 with a switch to a low-fat diet for phase 2
HF-LFRS	High-fat diet in phase 1 with a switch to a low-fat diet with 20% resistant starch by weight in phase 2

TABLE 2 Diets for CD OP and OR rats

Ingredient	Energy value, kcal/g	LF diets		HF diet
		LF (g)	LF-RS (g)	HF (g)
100% amylopectin cornstarch[a]	3.5	521.1	147.1	405.7
HM260[b] (containing RS at 20% of diet[c])	2.8	0	472.4	0
Sucrose	4.0	100	100	100
Casein	3.50	140	140	140
Cellulose	0	150.8	52.4	106.2
Corn oil	8.84	40	40	100
Lard	9.00	0	0	100
Mineral mix (AIN-93M)	0.88	35	35	35
Vitamin mix (AIN-93)	3.87	10	10	10
Choline chloride	0	1.3	1.3	1.3
L-cystine	4	1.8	1.8	1.8
Total weight (kcal)		1,000 g (3,160.2)	1,000 g (3,160.1)	1,000 g (4,182.5)

[a]100% amylopectin cornstarch is the AMIOCA cornstarch product from Ingredion Incorporated (Bridgewater, New Jersey).
[b]HM260 is high-amylose cornstarch (HI-MAIZE 260 RS) from Ingredion Incorporated (Bridgewater, New Jersey).
[c]HM260 lot was 42.3% RS based on wet weight, as used in the diet.
All dietary components other than starches were purchased from Dyets, Inc. (Bethlehem, Pennsylvania).

(FastPrep-24, MP Biomedicals, Santa Ana, California) for 1 minute at 6.5 m/s (two times). After centrifugation (20°C, 20,000*g*), 500 µl of supernatant was collected. Purified DNA was quantified by NanoDrop spectrophotometry and stored at −80°C.

Primer selection

Primers (*16S rRNA*) for *M. smithii* from Dridi et al. (20) and produced by Integrated DNA Technologies (Coralville, Iowa) were F:5′CCGGGTATCTAATCCGGTTC3′ and R:5′CTCCCAGGGTAGAGGTGAAA3′. Primers for total bacteria (*16S rRNA* gene) were F:5′ACGTCRTCCMCNCCTTCCTC3′ and R:5′GTGSTGCAYGGYYGTCGTCA3′, as reported by Belenguer et al. (21). The Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed June 22, 2017) was used to verify *in silico* that primers for *M. smithii ATCC 35061* were specific (100% identity with 123 base pair [bp] double-stranded amplicon) to this species and three others (*M. ruminantium, M. millerae*, and *M. oralis* in genus *Methanobrevibacter* and *Methanosphaera stadtmanae DSM 3091*). Dridi et al. (20) found *M. smithii* in human stool samples but also found *M. stadtmanae* in some samples with a different set of primers. They sequenced the amplicon for their primers for *M. smithii* and found 99% to 100% similarity to *M. smithii ATCC 35061*, so we concluded our results with these primers to reflect *M.smithii*. *In silico* results for the universal *16S rRNA* gene matched a broad range of bacteria.

Polymerase chain reaction amplification and amplicon DNA standards

Using Qiagen's Taq PCR Master Mix Kit, genomic DNA from pooled samples from each treatment in phase 1 was used as a template to prepare amplicon DNA with specific gene sequences and lengths (123 bp for *M. smithii* and 147 bp for universal *16S rRNA* genes). Amplicon DNA size was verified by using 2% agarose gel electrophoresis. Briefly, 100-µL reactions containing 5 µL (0.5 µM) each of the forward and reverse primers, 1,000 ng of template DNA, and 50 µl of the polymerase chain reaction (PCR) mix were used. Cycling conditions were as follows: initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. Annealing temperatures were 52°C for *M. smithii* and 60°C for the *16S rRNA* gene. After the completion of PCR, DNA amounts increased by 314 ng and 1,218 ng for *M. smithii* and the universal primers, respectively. These amounts were used in the formula below to calculate amplicon amounts for standard curves. Post-PCR DNA was cleaned by using the QIAquick PCR Purification Kit (Qiagen) and quantified by using a NanoDrop spectrophotometer (Thermo Fisher, Waltam, Massachusetts). Molar concentrations of amplicon standard DNA were converted into gene copies per microliter by using the formula shown by Oldham and Duncan (22), assuming the average molecular mass of the double-stranded DNA base pairs is 6.6×10^{11} ng/mol and the Avogadro's number of copies per mole is 6.022×10^{23}.

Thus, copies per microliter = DNA concentration (nanograms per microliter) $\times$ 6.02×10^{23}(copies per mole) / amplicon length (bp) $\times$ 6.6×10^{11} ng/mol.

Standard curves ranging from 10^8 to 10^2 copies per microliter were generated by serial 10-fold dilutions of amplicon DNA with nuclease-free water.

Quantitative real-time PCR

The SYBR Green quantitative PCR (qPCR) assay was used to quantify total bacteria and *M. smithii* by using the ABI Prism 7900HT Sequence Detection System (Life Technologies, Foster City, California). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, followed by primer annealing at 52°C for *M. smithii* and 60°C for the *16S rRNA* gene and then 78°C for 30 seconds. No-template controls and amplicon

standards were included in each plate. Amplicon quantities (gene copies per microliter) versus cycles-to-threshold standard curves were used to determine *M. smithii* and total bacteria quantities.

Microbiota analysis by next-generation sequencing using Illumina Mi-Seq

Amplification, sequencing, and bioinformatics of bacterial DNA from phase 2 were performed at the LSU Microbial Genomics Resource Group by using V3 2×300 bp kits as described previously (19,23,24). Analysis using the UPARSE process was with 97% identity for operational taxonomic units (19,24).

Statistical analyses

Phase 1 data were analyzed as a 2×2 factorial (OP and OR) and (LF and HF). Phase 2 data were analyzed as a 2×4 factorial (OP and OR) and (LF-LF, HF-HF, HF-LF, and HF-LFRS). We used the MIXED procedure of SAS 9.4 (SAS Institute, Cary, North Carolina). Main and interactive effects were considered significant at $P < 0.05$ and expressed as means ± SE. Further a priori comparisons used the alpha level divided by the number of comparisons. Next-generation sequencing data were analyzed by linear discriminant analysis as a screening tool for differences in relative abundance of operational taxonomic units among treatments (https://huttenhower.sph.harvard.edu/galaxy/). The Benjamini-Hochberg procedure (25) was applied to *P* values for dependent variables to decrease false discovery.

Results

Fermentation

After phase 1, no differences in weight of full and empty ceca were observed. In phase 2, OP rats fermented RS better than OR rats ($P < 0.0001$, Figure 2A and 2D), as there were increases in weights of full and empty ceca of rats fed RS (diet $P < 0.001$, Figure 2B and 2E). The interaction of diet and phenotype was significant ($P < 0.001$, Figure 2C and 2F). There was a sixfold increase of full cecum weight and a twofold increase in empty cecum weight for the OP HF-LFRS group versus the OP LF-LF and OP HF-LF groups. In OR rats, the cecum weights in rats in the HF-LFRS group were 30% larger than for rats in the HF-LF group ($P < 0.0167$).

The number of SCFAs in cecal contents and the cecal content pH were not different after phase 1, and pH values were above 7 (Supporting Information Table S1). In phase 2, there were significant phenotype, diet, and interaction effects that reflected greater amounts of fermentation in OP rats in the HF-LFRS group (Table 3). A priori comparisons of groups with diets containing RS versus no RS resulted in higher amounts of SCFAs (this may also reflect absorption and/or utilization) and a lower cecal content pH.

GLP-1 active concentrations were not different at the end of phase 1 (Supporting Information Table S1). After phase 2, there were increased values for GLP-1 active for LFRS diet ($P < 0.0083$) (Table 3).

Energy intake, body weight, and fat accretion

Energy intake in OP rats was higher than in OR rats in both phases (Supporting Information Figure S1). Most weight gain occurred in phase 1 (Figure 3). After phase 1, the EBW was significantly higher ($P < 0.0065$) in OP rats compared with OR rats, whereas a priori diet comparisons showed no significant effects within rat type ($P = 0.64$). After phase 2, OP rats had higher EBW ($P < 0.0001$) than OR rats. Diet also had an effect, and OP rats fed the HF diet had the highest increase in EBW, as there was a diet effect

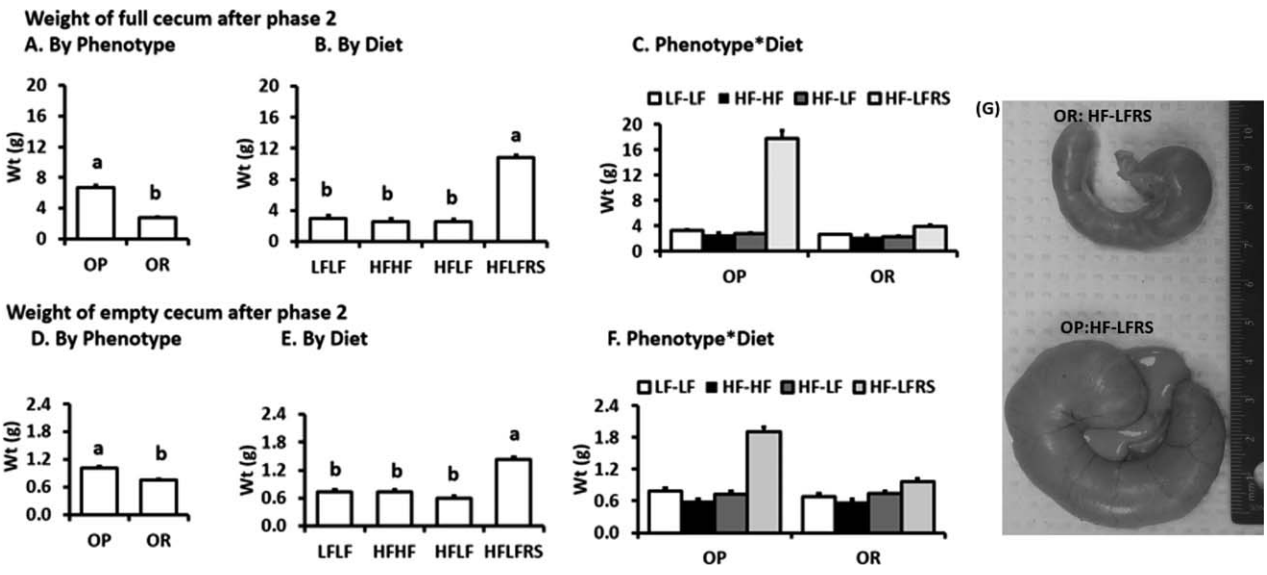

Figure 2 Weight and size of ceca. (**A**-**C**) Full ceca and (**D**-**F**) empty ceca. In panels A and D, letters designate differences between OP and OR rats ($P < 0.0001$). In panels B and E, letters denote a priori differences between HF-LFRS and the three other diets ($P < 0.0167$). In panels C and F, differences ($P < 0.0125$) between a priori comparisons: OP HF-LFRS vs. OP LF-LF, OP HF-LFRS vs. OP HF-LF, OP HF-LFRS vs. OR HF-LF ± RS, and OR HF-LFRS vs. OR HF-LF. (**G**) Representative picture of the ceca of OP and OR rats fed RS.

TABLE 3 Fermentation variables: SCFAs, pH of cecal contents, and GLP-1 active in serum

	Diet	OP	OR	*P* (Phenotype)	*P* (Diet)	*P* (Phenotype by diet)	Pooled SEM
Acetate (mmol/cecum)	LF-LF	0.081	0.045	< 0.0001	< 0.0001	< 0.0001	0.007
	HF-HF	0.056	0.032				
	HF-LF	0.061	0.032				
	HF-LFRS	0.401	0.095				
Propionate (mmol/cecum)	LF-LF	0.015	0.009	< 0.001	< 0.001	< 0.0001	0.001
	HF-HF	0.010	0.006				
	HF-LF	0.030	0.004				
	HF-LFRS	0.049	0.008				
Butyrate (mmol/cecum)	LF-LF	0.020	0.010	< 0.001	< 0.001	< 0.0001	0.0017
	HF-HF	0.014	0.008				
	HF-LF	0.016	0.007				
	HF-LFRS	0.127	0.022				
pH	LF-LF	7.46	7.62	< 0.001	< 0.001	< 0.0001	0.973
	HF-HF	7.56	7.57				
	HF-LF	7.56	7.30				
	HF-LFRS	5.62	6.90				
GLP-1 (pmol/L)	LF-LF	0.97	0.89	0.20	0.0083	0.839	0.073
	HF-HF	1.15	1.13				
	HF-LF	0.88	0.99				
	HF-LFRS	1.66	1.06				

For all Table 2 variables, there were differences ($P < 0.0125$) for four a priori comparisons (OP HF-LFRS vs. OP LF-LF, OP HF-LFRS vs. OP HF-LF, OP HF-LFRS vs. OR HF-LFRS, and OR HF-LFRS vs. OR HF-LF).

($P < 0.008$). The interaction of diet and phenotype was not significant ($P = 0.43$), indicating that the relationship of weight gain among diets with two phenotypes was similar. Among a priori comparisons, the OP HF-LFRS group had greater EBW than the OR HF-LFRS group ($P < 0.0125$) (Figure 3A). The percent increase in EBW during phase 2 was higher in OP rats compared with OR rats ($P < 0.005$), and a diet effect was observed ($P < 0.03$) because OP rats fed the HF diet gained the most weight. Among a priori comparisons, the percent increase in weight in rats switched from an HF to an LFRS diet was not different from those switched to an LF diet without RS (Figure 3B). There was an effect of both phenotype ($P < 0.001$) and diet ($P < 0.001$) on total abdominal fat after phase 2. The interaction was not significant ($P = 0.064$). Total abdominal fat and percent abdominal fat in rats switched from the HF diet to the LFRS diet were not different from those switched to the LF diet (Figure 3C-3D). Although feeding RS to OP rats increased fermentation in comparison to other groups, it did not contribute to greater energy storage.

Total bacteria and *M. smithii* levels

In phase 1, OR rats had more total bacteria compared with OP rats ($P < 0.014$), but diet ($P = 0.86$) and interaction ($P = 0.69$) were not significant. In phase 2 (Figure 4A-4C), RS increased total bacteria more than threefold ($P < 0.005$) only in OP rats fed the LFRS diet. Dietary manipulation did not change total bacteria in OR rats.

Diet and phenotype had no effect on the abundance of *M. smithii* in phase 1, and the HF diet did not change *M. smithii* levels in either OP or OR rats. In phase 2 (Figure 4D-4F), there was a diet effect ($P < 0.02$) because *M. smithii* abundance with greater fermentation increased in the LFRS group by threefold in OP rats ($P < 0.005$, LFRS vs. other three groups). There was an interaction between diet and phenotype ($P < 0.007$) because there was not a similar increase in *M. smithii* in OR rats fed RS (Supporting Information Figure S5).

Proportions of bacteria taxa

Most changes in bacterial composition occurred in OP rats fed RS (Figure 5, Supporting Information Figures S2-S4). Dietary RS increased bacteria in phylum *Bacteroidetes* and reduced bacteria in *Firmicutes* in OP rats compared with OR rats (Supporting Information Figures S2-S4). The abundance of *Bacteroidales* family *S24-7* was highest in OP rats fed RS ($P < 0.001$), whereas the *Firmicutes* families *Lactobacillaceae* and *Lachnospiraceae* were more abundant in OR rats fed RS (linear discriminant analysis effect size, Supporting Information Figure S4, $P < 0.05$, Figure 5, Supporting Information Figure S3).

Discussion

Based on prior findings that RS fermentation reduces the accretion of abdominal fat (5-9), we hypothesized that OR rats would retain a lean phenotype with an HF diet followed by an LFRS diet,

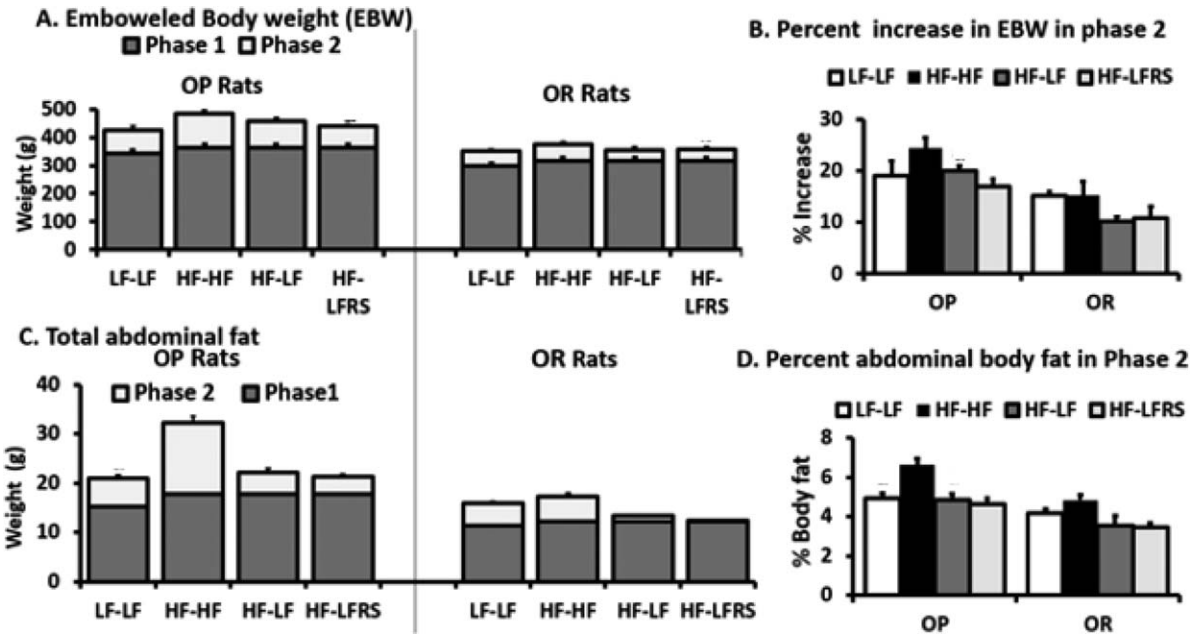

Figure 3 Body weight and body fat. (**A**) Weight of the gastrointestinal tract contents was subtracted from the total body weight to determine EBW for OP and OR rats. (**B**) Percent increases in EBW as phase 2 / phase 1 × 100. (**C**) Total abdominal fat. (**D**) Percent abdominal fat (abdominal fat / EBW × 100) after phase 2. In panels A and C, for the lower darker bars determined from the four animals per LF and HF diet groups euthanized after phase 1. Data for the upper lighter bars determined from the six animals per group after phase 2. Only a priori difference after phase 2 was between OP HF-LFRS vs. OR HF-LFRS ($P < 0.0125$). No significant differences for OP HF-LFRS vs. OP HF-LF, OP HF-LFRS vs. OP LF-LF, and OR HF-LFRS vs. OR HF-LF.

compared with HF-LF diet, by a mechanism that involves fermentation of dietary components despite a presumably higher energy harvest with RS. Because of our results, this hypothesis was rejected; OR rats exhibited modest fermentation of RS, and OP rats robustly fermented RS.

The obesity trait in CD rats mimics characteristics of the human obesity phenotype, in which not all individuals who consume increased calories develop obesity (11), and allows the delineating effects of microbiota composition on obesity phenotype and fermentation of RS. The finding that the OP rats gained greater weight and fat even on an LF diet had not been shown before (Figure 3B and 3D). Previous studies have shown that they gain more weight on an HF diet (10,11). In phase 2, diet components had no effect on body weight and had a minimal effect on fermentation in OR rats. In OP rats, RS elicited very high fermentation, as demonstrated by fermentation variables measured (Figure 2, Table 2). High fermentation presumably ensures more energy harvest and has been proposed to be one of the causes of obesity (1,2). Studies using germ-free and knockout mice have indicated that the extracted energy is stored in adipocytes through a pathway that involves microbial downregulation of intestinal epithelial expression of fasting-induced adipocyte factor, a circulating inhibitor of lipoprotein lipase. Suppression of the fasting-induced adipocyte factor increases lipoprotein lipase activity from adipocytes and enhances storage of liver-derived triglycerides in fat cells (1). In the current study, dramatically increased fermentation in OP rats did not result in higher energy storage because switching rats to a an LF diet or an LF diet with RS did not lead to significant differences in weight and fat accretion in OP rats (Figure 3). The absence of robust fermentation in OR rats fed RS was unexpected, given that this type of rat strain is reported to remain insulin sensitive and maintains a leaner phenotype when fed increased calories (10-11). Neither OP nor OR rats responded to fermentation with decreased body fat accretion. Our past study observed increased fatty acid oxidation (5), and this may be the result of stimulation by increased SCFAs reaching the liver via the portal blood (26), causing reduced accretion of abdominal fat in several rat models fed RS (5-9). The results with OP and OR rats may be explained by their development from breeding of heavier Sprague Dawley rats (Charles River Laboratories). However, it is surprising that both OR and OP models were developed. OR and OP rats may harvest enough energy from fermentation to balance any increase in fat oxidation, and they do not reduce fat accretion or have a reduced metabolic response to fermentation. It is also possible that feeding RS without prior feeding of an HF diet might have produced a different result.

Our study had different results than those in the study by Belobrajdic et al. (13), possibly because we focused on the effects of fermentation with isocaloric diets for our comparisons of the HF-LFRS groups (OP or OR) with the HF-LF groups rather than focusing on the combined effects of fermentation and dilution of energy density. With resistance to digestion, RS has a lower metabolizable energy than control starches (27). Belobrajdic et al. (13) used RS to replace control starches, and RS diets in their study had lower amounts of energy. Secondly, our study used commercially established CD OP and CD OR rat strains, but Belobrajdic et al. (13) used Sprague Dawley rats. We discovered an OR rat model that is a low fermenter of RS. Such a model could serve as a model for humans who are reported to be low fermenters (28-30).

The amounts of total bacteria and the archaean *M. smithii* determined by qPCR were not different between the HF and LF diets

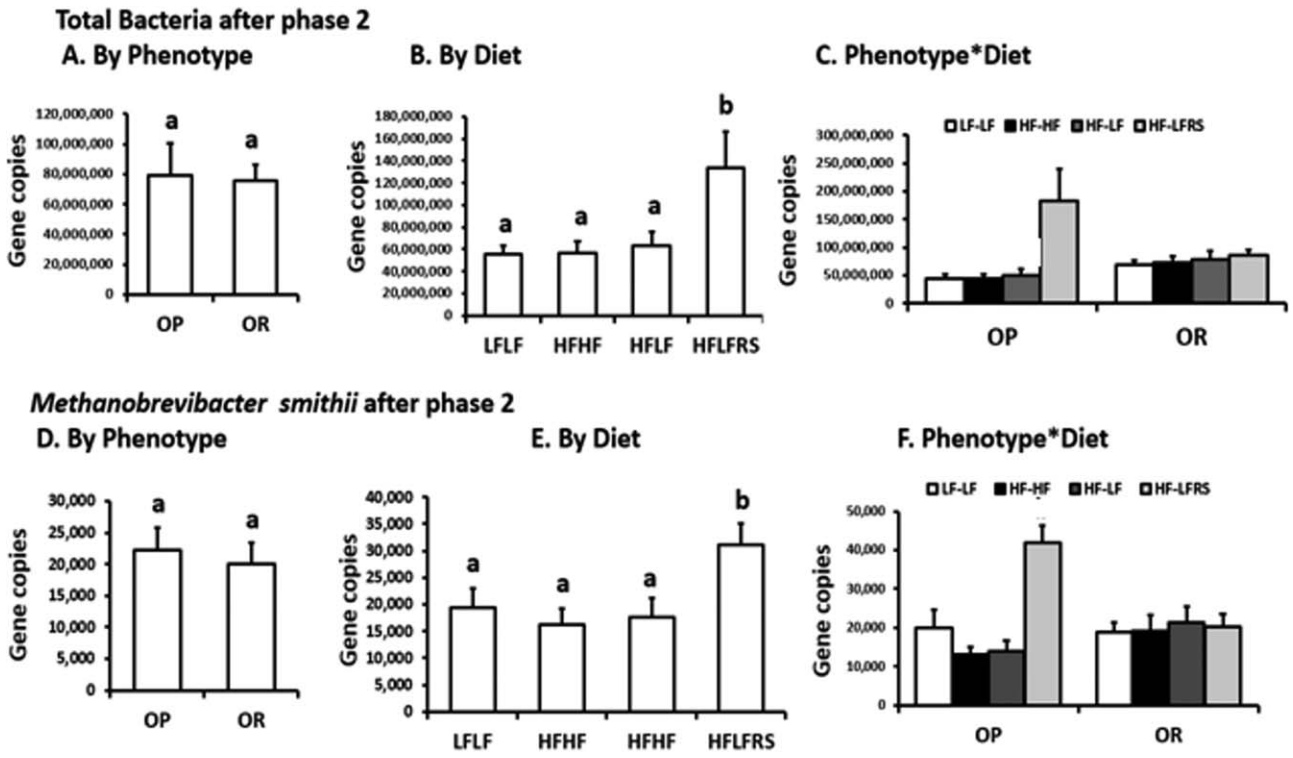

Figure 4 Abundance of total bacteria and *M. smithii* after phase 2 (qPCR). (**A**-**C**) Total bacteria. (**D**-**F**) *M. smithii*. In panels A and D, no significant differences in both total bacteria and *M. smithii* for OP and OR rats. In panels B and E, indicate three a priori differences $P < 0.0167$) between the HF-LF ± RS diet and all other diets. In panels C and F, OP rats, there were a priori differences ($P < 0.0125$) between OP HF-LFRS vs. OR HF-LF, OR HF-LFRS vs. OP LF-LF, and OP HF-LFRS vs. OR HF-LFRS. No significant difference was observed between OR HF-LFRS and OR HF-LF.

(phase 1). OR rats had significantly higher amounts of total bacteria than OP rats, but the amount of *M. smithii* was not significantly different. Including RS in the diet (phase 2) increased both total bacteria and *M. smithii* in OP rats but not in OR rats (Figure 4). In the absence of dietary RS, the role of methanogenic archaeans, and particularly *M. smithii*, in the pathogenesis of obesity is not clear. Mathur et al. (15,31) has shown that methanogenic archaeans contribute to altered metabolism and weight gain in the host. They observed higher proportions of *M. smithii* in humans with obesity, compared with their lean counterparts, and in mice that have genetic

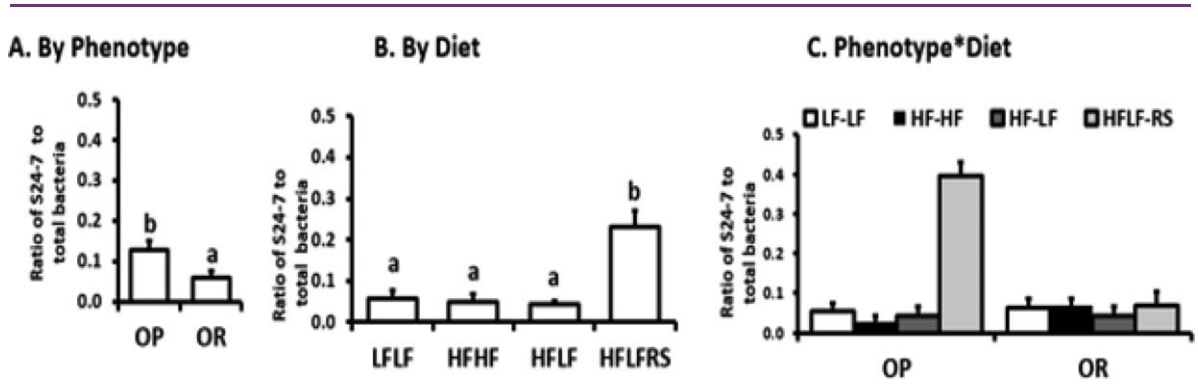

Figure 5 Relative abundance of the family *S24-7* gene copies (qPCR) data after phase 2 (S24-7 / total bacteria). (**A**) OP rats had a higher abundance of *S24-7* compared with OR rats ($P < 0.003$), indicated by different letters. (**B**) HF-LFRS diet had increased *S24-7* abundance compared with all other diets ($P < 0.0005$). Letters indicate a priori differences between OP HF-LFRS and OP LF-LF or OP HF-LF and OR HF-LFRS ($P < 0.0125$). (**C**) Interaction between diet and phenotype influenced *S24-7* abundance ($P < 0.0001$). There were a priori differences ($P < 0.0125$) between OP HF-LFRS vs. OP LF-LF, OP HF-LFRS vs. OP LF-LF, and OP HF-LFRS vs. OR HF-LFRS. No difference was observed between OR HF-LFRS and OR HF-LF.

obesity when compared with their lean littermates. Gut colonization with *M. smithii* correlated with and predicted the degree of weight gain (15). In contrast, our results showed that *M. smithii* levels were not affected by the obesity phenotype but were affected by the fermentation of RS. Furthermore, the increase in *M. smithii* levels was beneficial to the host, as it appeared to contribute to enhanced fermentation with no extra weight gain or fat accretion. Mathur et al. (15) and Mathur and Barlow (31) did not elaborate on the components of diet, particularly on the amounts of fermentable fiber. The observed higher proportions of *M. smithii* in humans with obesity and mice that have genetic obesity may have been errantly attributed to the obesity phenotype rather than to greater amounts of fermentable fiber.

Another bacterial composition increase in OP rats in response to RS was in the abundance of *S24-7*, an uncultured gram-negative family of order *Bacteroidales* (Figure 5). *S24-7* is involved in host-microbe interactions that impact gut function and health, and abundance is altered with different conditions (32); in the current study, it was altered in response to RS feeding. Analysis of *16S rRNA* gene databases from metagenomics by Ormerod et al. (32) showed that members of the family *S24-7* are fermentative but have alternative modes of energy production as they encode elements of an electron transport chain. Like other families in the *Bacteroidales* order, carbohydrate-active enzymes constitute about 6% of the *S24-7* coding sequences. Based on enzyme abundance, *S24-7* genes encode glycoside hydrolases, largely α-amylases, suggesting starch as a key substrate with the ability to ferment several other carbohydrate moieties (32).

We conclude that increases in *M. smithii* and *S24-7* are markers of bacterial fermentation of RS because both rat phenotypes had similar amounts of these potential markers in phase 1 when no RS was fed. We previously observed increased *S24-7* in Zucker diabetic fatty (ZDF) rats with obesity that robustly fermented RS (19), and we hypothesize that we would have observed an increase in *M. smithii* if these were measured in other studies. Increases in *S24-7* and *M. smithii* may be under a homeostatic mechanism of host dynamics that regulates the gut community responses to promote host fermentation. The robust fermentation in OP rats did not elicit reduced food intake and did not result in extra weight or fat accretion, which demonstrated that fermentation does not appear to be the cause of obesity in OP rats. OR rats may be a good model for humans reported to be low fermenters of RS, and the cause of their low response to RS is unknown. It may be related to their microbiota and inability to increase *M. smithii* and *S24-7* levels. In OP rats, the host environment favored RS fermentation, and *M. smithii* and *S24-7* were then beneficial to the host for increased fermentation. O

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