RESEARCH ARTICLE

The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses

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Abstract

Dietary interventions might prevent or reverse age-related declines in health through modification of the activity and composition of the intestinal microbiota. As a first step toward more comprehensive evaluations of single dietary components on healthy aging, 16S rRNA gene amplicon sequencing was applied to determine the structure of the bacterial communities in the ceca of 20-month-old healthy mice fed energy-controlled diets containing 0, 18, or 36% type 2 resistant starch (RS) from high-amylose maize (HAM-RS2). The cecal microbiota of mice fed a diet depleted in RS and containing the readily digestible carbohydrate amylopectin were dominated by bacteria in the Firmicutes phylum and contained low levels of Bacteroidetes and Actinobacteria. In contrast, mice fed diets containing HAM-RS2 were colonized by higher levels of Bacteroidetes and *Bifidobacterium*, *Akkermansia*, and *Allobaculum* species in proportions that were dependent on the concentration of the dietary fiber. The proportions of *Bifidobacterium* and *Akkermansia* were positively correlated with mouse feeding responses, gut weight, and expression levels of proglucagon, the precursor of the gut anti-obesity/diabetic hormone GLP-1. This study showed that aging mice harbor a distinct microbiota, which can be modulated by RS and enriched for bacteria that are associated with improved health.

Introduction

Aging is characterized by a progressive decline in organismal fitness and deterioration of physiological function. A loss of appetite and food digestion and absorption are associated with aging and result in reduced energy intake and weight loss (Chapman, 2007; Moss *et al.*, 2012). This so-called anorexia of aging predisposes elderly individuals to higher morbidity and mortality (Chapman, 2007). In 2010, 13% of the US population was aged 65 years or older (http://quickfacts.census.gov/qfd/states/00000.html), and it is estimated that this will increase to 20% by 2030 (Vincent & Velkoff, 2010). Therefore, it is important to understand the major nutrition-related physiological changes that occur during aging to provide noninvasive mechanisms to promote an extended health-span and longevity.

Perturbations in the intestinal microbiota were recently identified in elderly individuals (Mariat *et al.*, 2009; O'Toole & Claesson, 2010; Claesson *et al.*, 2011). Intestinal microorganisms include approximately 10^{14} bacterial cells representing over 1000 different species that perform numerous beneficial functions in healthy individuals (Sekirov *et al.*, 2010). Perturbations of the intestinal microbiota have been associated with immune, metabolic, and neurological diseases (Ley *et al.*, 2006; Finegold *et al.*, 2010; Hong *et al.*, 2010; Larsen *et al.*, 2010). The distal gut of young healthy adults is typically dominated by bacteria in the phyla Firmicutes (60–80% of total bacteria) and Bacteroidetes (20–40% of total bacteria) (Dethlefsen *et al.*, 2007). Among the elderly, proportions of these phyla differ compared with younger individuals (Mariat *et al.*, 2009; O'Toole & Claesson, 2010; Tiihonen *et al.*, 2010). At the genus level, depletion of specific beneficial gut bacteria such as *Bifidobacterium* and *Akkermansia* spp. has been reported for elderly individuals (Bartosch *et al.*, 2004; van Tongeren *et al.*, 2005; Collado *et al.*, 2007;

Biagi *et al.*, 2010; Hippe *et al.*, 2011). Aged persons can also exhibit extreme inter-individual variability in the composition of the intestinal microorganisms (Claesson *et al.*, 2011).

Because intestinal microorganisms can directly influence the amount of energy harvested from the diet (Turnbaugh *et al.*, 2006), modulation of the gut microbiota composition offers an opportunity to promote or restore digestive health among the elderly. Modifying components of the diet are a means to target specific bacterial species or functional groups of microorganisms for enrichment (or depletion). In younger individuals, the potential of this so-called prebiotic approach was shown for a variety of carbohydrates, which were found to alter the composition of the intestinal microbiota and consequently epithelial cell metabolism and immune and endocrine cell signaling, resulting in changes in the host physiology (Jacobasch *et al.*, 1999; Cummings *et al.*, 2001; Louis *et al.*, 2007; Abell *et al.*, 2008; Shen *et al.*, 2011). Similarly, resistant starch (RS) affects postprandial glycemia and promotes the production of short-chain fatty acids (SCFA) in the colon as a result of bacterial fermentation. This dietary fiber thus has potential in the treatment of metabolic conditions such as diabetes, hyperlipidemia, cardiovascular disease, and colon cancer (Jenkins & Kendall, 2000). Diets supplemented with RS resulted in changes in the activity and composition of the gut microbiota of healthy adults (Abell *et al.*, 2008; Martínez *et al.*, 2010; Walker *et al.*, 2011). RS, specifically type 2 RS from high-amylose maize (HAM-RS2), improved host energy homeostasis mainly in the context of metabolic diseases such as diabetes and obesity in adult rodents (Zhou *et al.*, 2006, 2008, 2009; Shen *et al.*, 2011). Effects of HAM-RS2 are likely mediated through mechanisms involving bacterial fermentation of the dietary fiber in the colon and stimulation of the expression of anti-obesity/diabetic hormones GLP-1 and PYY (Zhou *et al.*, 2006, 2008, 2009; Shen *et al.*, 2011).

In a recent study, the effects of HAM-RS2 on aged, healthy C57BL/6J male mice were investigated (Zhou *et al.*, 2012). The mice were 18–20 months old (analogous to 56–65 years in humans) (Flurkey *et al.*, 2007) and were fed energy-controlled diets containing 0%, 18%, or 36% HAM-RS2 for 10 weeks (Zhou *et al.*, 2012). The RS was well-tolerated by the mice, resulted in higher cecal weights, and elevated expression of proglucagon (the precursor of GLP-1) and PYY in the cecum (Zhou *et al.*, 2012). Here, we describe and compare the structure of the bacterial communities in the ceca of a subset of animals randomly selected from these mice in an effort to correlate changes in microbiota to altered hormonal and feeding responses in the host.

Materials and methods

Mouse study design and physiological measurements

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 18–20 months old, were described by Zhou *et al.* (2012). Six mice were randomly selected from each of three groups receiving diets enriched in either Amioca corn starch (100% amylopectin, 0% RS) (Control1 to Control6) or 18% (18%RS1 to 18%RS6) or 36% (36%RS1 to 36% RS6) type 2 RS from high-amylose maize (HAM-RS2, Hi-maize 260®, National Starch LCC, Bridgewater, NJ) (Supporting Information, Table S1). These amounts of HAM-RS2 follow standards for studying the mechanisms by which RS influences host physiology in rodents, including reductions in body fat (Scribner *et al.*, 2008; Zhou *et al.*, 2012). The diets contained the same metabolizable energy density and micro-/macro-nutrient contents (Table S1). Caloric amounts available in the diets were estimated based on knowledge that HAM-RS2 contains approximately 60% indigestible starch (a type of insoluble dietary fiber) and 40% digestible (glycemic) starch. Mice were selected from separate shoe box cages, except mice designated as 36%RS4 and 36%RS5, which shared a cage. Animals were given free access to food and water on a 12 h light/dark cycle. After 8 weeks on the different diets, the feed was removed from the cages; 12–13 h later, the regular-assigned diets were again provided, and food intake was measured at 1, 2, and 4 h. At 10 weeks, the mice were sacrificed and the gastrointestinal tracts and abdominal fat pads were collected and weighed. Measurement of proglucagon and PYY transcript amounts in the cecal cells was performed by real-time PCR as described previously (Zhou *et al.*, 2003). Briefly, the $2^{-\Delta\Delta Ct}$ method was used to express the change in gene expression compared to Controls (Livak & Schmittgen, 2001).

DNA extraction from cecal contents

Mouse cecal contents were washed three times in 10 volumes of PBS buffer (pH = 7), suspended in lysis buffer containing 200 mM NaCl, 100 mM Tris pH 8.0, 20 mM EDTA, 20 mg mL^{-1} lysozyme, and 300 mg of zirconium beads (0.1 mm, BioSpec Products), and incubated for 30 min at 37 °C. The bacterial cells were mechanically lysed by bead-beating for 2 min at maximal speed (Fast Prep 24, MP, Solon, OH), and the genomic DNA was purified using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA). The 260 nm/280 nm ratios of the purified cecal DNA ranged from 1.8 to 2.0 according to spectrophotometric measurements on the Nanodrop (Thermo Scientific, Wilmington, DE).

16S rRNA gene amplification and pyrosequencing

Barcoded 16S rRNA gene amplification and pyrosequencing was performed at the Core for Applied Genomics and Ecology (CAGE) facilities (University of Nebraska-Lincoln, NE). The V1 to V3 regions of the extracted 16S rRNA genes were amplified by PCR from cecal DNA using primers and PCR reaction conditions that were previously described by Martinez *et al.* (Martínez *et al.*, 2010), except that the ratio B-8FM to B-8FMBifido was 5:1. PCR product concentrations were quantified using the Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Carlsbad, CA) and quality controlled on an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). Amplicons were then mixed in equal amounts and subjected to emulsion PCR according to protocols developed by Roche/454 Life Sciences. Sequencing was performed from the A end using the 454/Roche A sequencing primer kit and Roche 454 GS-FLX Titanium chemistry on a Roche-454 GS-FLX Pyrosequencer (454 Life Sciences, Branford, CT).

DNA sequence analyses

Sequences were analyzed with the software package Quantitative Insights into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010b). The analysis removed low-quality sequences that did not perfectly match the PCR primer, were shorter than 200 bp and longer than 500 bp, contained undetermined nucleotides (N), or did not match a barcode. Chimeras were identified using the ChimeraSlayer algorithm (Haas *et al.*, 2011) and removed from the data set. Sequences were binned by barcode, and taxonomic categories were assigned using the CLASSIFIER program of the Ribosomal Database Project (RDP) (Wang *et al.*, 2007) within QIIME. Diversity among samples was assessed using the weighted UniFrac metric (Lozupone *et al.*, 2006), visualized on a principal coordinates analysis (PCoA) plot and confirmed using jackknifed hierarchical clustering [unweighted pair group method with arithmetic mean (UPGMA)] according to default settings in QIIME. Phylogenetic trees were constructed after sequence alignment with PyNast (Caporaso *et al.*, 2010a) using FastTree (Price *et al.*, 2009) in QIIME and visualized using Dendroscope (Huson *et al.*, 2007).

16S rRNA gene copy number quantification by real-time PCR

Total bacterial 16S rRNA gene copy number was quantified using universal primers (Belenguer *et al.*, 2006). *Clostridium* cluster IV- and *Clostridium* cluster XIV-specific primers used were previously described by Shen *et al.* (Shen *et al.*, 2011). Real-time PCR amplification was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA). Each well contained SsoFast EvaGreen Supermix with low ROX (BioRad, Hercules, CA), 400 nM of each primer (Invitrogen, Carlsbad, CA), and 2.5 ng cecal genomic DNA as the template. PCR amplification was initiated at 95 °C for 20 sec, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 30 s.

For validation of the real-time PCR primers, genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) from *Clostridium leptum* VPI T7-24-2 (ATCC29065) (for cluster IV primers) and *Clostridium coccoides* CLC-1 (ATCC29236) (for cluster XIV primers) after anaerobic growth in chopped meat medium (ATCC593) for 48 h at 37 °C, and from *Lactobacillus plantarum* WCFS1 (for universal primers) grown in MRS medium for 18 h at 37 °C. PCR primers efficiencies assessed on 10-fold serial dilutions of the corresponding genomic DNA from 10 ng to 1 pg were 83% for both *Clostridium* cluster IV- and *Clostridium* cluster XIV-specific primers and 97.7% for the universal primers. Samples were assessed in triplicate.

Statistical analyses

Before statistical analysis, phylotype proportions of 0 were replaced with 0.5/total reads, and all proportion values were log10-transformed (Benson *et al.*, 2010). Nonparametric methods were used because of the low sample size ($n = 6$), and the comparisons were performed using Anastats tools (http://www.anastats.fr/). The significance of the difference among mice fed the different diets for a given measurement was assessed by the Kruskal and Wallis test. Linear relationships and statistical significance between variables were assessed using the Spearman correlation test.

Results

Pyrosequencing characterization of gut microbiota in aged mice fed different quantities of RS

The cecal microbial populations of 20-month-old mice were characterized after feeding energy-controlled diets containing 0 (Control), 18, or 36% type 2 RS from high-amylose maize (HAM-RS2) for 10 weeks. The mice were colonized by similar amounts of total bacteria, as indicated by the comparable quantities of total genomic DNA extracted from the cecum contents of each mouse (not shown) and by real-time PCR (Fig. S2). Pyrosequencing of the V1 to V3 regions of the 16S rRNA genes extracted from the cecum contents of 18 mice (six per group) resulted in the collection of 59 145 high-quality, filtered classifiable

sequences with a mean of 3286 ± 976 sequences per mouse. Using QIIME (Caporaso *et al.*, 2010b), bacterial phylotypes were distinguished at the level of 97% sequence identity and were further analyzed to compare the composition of the gut microbiota among mice.

Mice fed the same amount of HAM-RS2 tended to share a similar gut microbiota according to the weighted UniFrac distance metric (Fig. 1). This β diversity measure takes phylotype proportions and variation into account (Lozupone *et al.*, 2006). Principal coordinate analysis (PCoA) showed that 43% of the variation in the gut microbiota could be explained along PC1 and clearly separated the gut microbiota according to presence or absence of HAM-RS2 in the diet (Fig. 1). This result was confirmed by a high jackknife support (75–100%). An additional 22% of the variation was distinguished along PC2 and separated the gut microbiota according to the concentration of HAM-RS2 fed to the animals (Fig. 1). Overall, inter-individual differences in the microbiota were greater among mice fed HAM-RS2 compared with the Control animals (Fig. 1).

The cecal microbiota of several mice did not cluster together with the other animals fed the same diet (Fig. 1). Mouse 18% RS6 shared a gut microbiota most similar to the Control mice. Conversely, mice designated as Control5 and Control6 were colonized by microbiota that were more related to mice fed 18% HAM-RS2 than the other Controls. Lastly, the cecal bacterial composition in 18%RS1 clustered with the 36% HAM-RS2-fed mice (Fig. 1). These differences among the microbiota could not be explained by cage-specific variations and are consistent with the taxonomic variation in the individual mice as described below.

Firmicutes and Bacteroidetes are the dominant phyla in the ceca of aged mice

Taxonomic assignments of the sequences showed that bacteria encountered in the aged mice were divided into eight phyla, 25 families, and 31 genera. For all of our mice, Firmicutes was the dominant phylum constituting between 50% and 98% of the cecal bacteria (Fig. 2). Bacteroidetes was the second most abundant phylum comprising 1–24% (Fig. 2), and the proportions of Firmicutes and Bacteroidetes were inversely correlated ($r = -0.70$, $P < 0.01$). Members of the Actinobacteria and Verrucomicrobia phyla were also found in the ceca in proportions ranging from 0.1 to 30% and 0 to 17% of total bacteria, respectively (Fig. 2). Representatives in the Proteobacteria, Deferribacteres, Tenericutes, and TM7 phyla were present at lower levels (< 2.3%).

Taxonomic assignment was efficient such that 97% of all sequences were classified to a phylum, an average of 82% to a family, and 27% to a genus. The majority of the sequences that were not assigned a genus constituted members of the Firmicutes phylum, and specifically the Lachnospiraceae family (53%) (Fig. S1a). This result was not unexpected considering the large amount of sequences identified as Lachnospiraceae that are not assigned to a genus in the RDP database (62%, http://rdp.cme.msu.edu/). However, differences among the identified taxa were sufficient to discriminate the mice and to identify bacterial families and genera that were affected by HAM-RS2 consumption and associated with physiological responses to HAM-RS2.

Dietary RS promoted Bacteroidetes colonization in the aged mouse gut

Members of the Bacteroidetes phylum constituted 4.6 ± 3.5% of the total bacteria identified in the cecal microbiota of mice fed amylopectin (0% RS) (Fig. 2). In comparison, Bacteroidetes constituted 8.2 ± 5.3% and 12.6 ± 7.2% of the microbiota in mice fed either 18% or 36% HAM-RS2, respectively. Remarkably, the relative amounts of the Bacteroidetes genera *Bacteroides*, *Alistipes*, and *Parabacteroides* did not correlate with the increase in overall abundance of this phylum. The proportions of

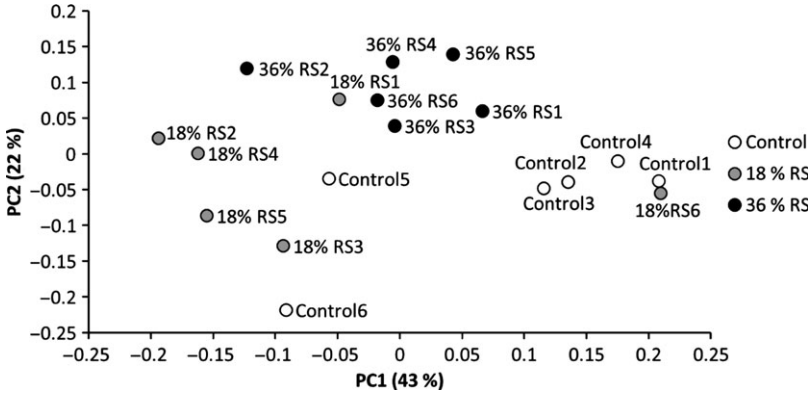

Fig. 1. Principal coordinates analysis (PCoA) plot of the weighted UniFrac distances of cecal microbiota from aged mice fed 0% (Control), 18% and 36% HAM-RS2. PC1 and PC2 are plotted on *x*- and *y*-axes. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. Each point corresponds to the composition of the microbiota of one mouse from Control (open circle), 18% HAM-RS2 (gray circle), and 36%RS2(closed circle)-fed mice.

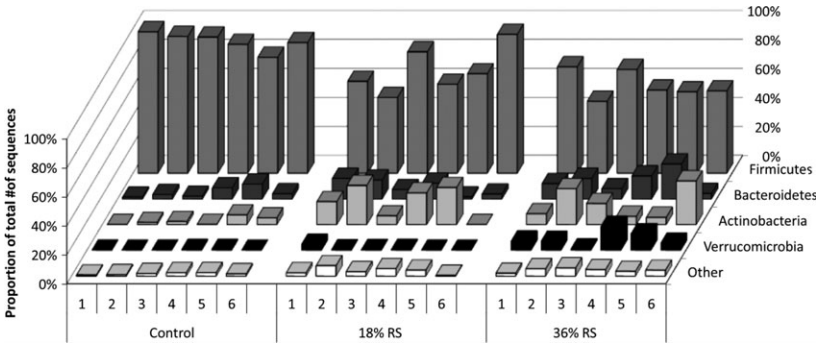

Fig. 2. Phylum-level distributions of the cecal bacteria of mice fed diets containing different amounts of HAM-RS2. Only the dominant phyla are represented, the other phyla and the unclassified sequences are grouped under the 'Other' category. The *x*-axis displays the mouse identification number, assigned depending on the percent RS contained in the diet: 0 (Control), 18 (18%RS), and 36 (36% RS).

Bacteroides species were not significantly affected by diet, and the levels of *Alistipes* and *Parabacteroides* were lower in mice fed HAM-RS2 compared to Controls (Fig. S1b). Instead, the higher relative representation of the Bacteroidetes was due to higher levels of unclassified members of this phylum (Fig. S1b).

RS selects for specific bacteria in the Firmicutes phylum although total proportional amounts of this phylum decline

The proportions of Firmicutes were moderately lower (1.5-fold, $P < 0.05$) in 36% HAM-RS2-fed mice compared with the Controls. Firmicutes were also 1.3 ± 0.2-fold less abundant in mice fed a diet consisting of 18% HAM-RS2; however, the difference was not significant. This reduction was most obvious for members of the families Lachnospiraceae and Ruminococceae. Lachnospiraceae represented 51 ± 14% of total bacteria in Control mice and 36 ± 14% in mice fed HAM-RS2. Similarly, Ruminococceae were reduced from 7.7 ± 4.3% of total bacteria among the Controls to 4.5 ± 2.6% in mice fed HAM-RS2. Proportions of specific Firmicutes genera were also reduced by the presence of dietary RS including *Roseburia* and *Butyrivibrio* (Fig. 3). These results were consistent with quantitative real-time PCR that showed that mice fed 36% HAM-RS2 carried lower levels of *Clostridium* from the clusters IV and XIV (Kruskal and Wallis, $P < 0.05$) (Fig. S2).

In contrast to the dominant Firmicutes genera, *Lactobacillus* tended to be enriched in mice fed 18% HAM-RS2 ($P < 0.1$) (Fig. 3). There was a high inter-individual variation in *Lactobacillus* proportions, ranging from 6.6-fold lower to 38.0-fold higher compared with mice that did not consume RS. *Allobaculum* was also enriched by HAM-RS2 consumption (10.0 ± 10.7-fold) (Fig. 3), and its abundance was positively correlated with the proportions of *Bifidobacterium* ($R = 0.78$, $P < 0.0005$) and negatively with *Turicibacter* ($R = -0.71$, $P < 0.0005$) and *Oscillibacter* ($R = -0.56$, $P < 0.01$) (Table 2).

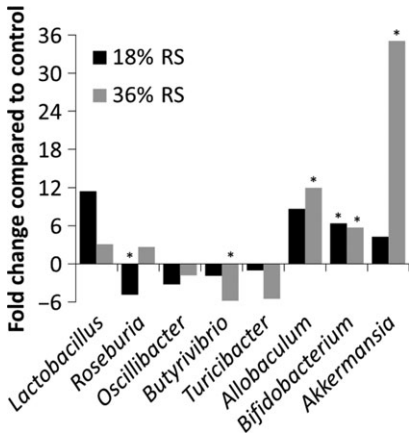

Fig. 3. Bacterial genera that were significantly affected by dietary RS in aged mice. The genera shown represented on average more than 0.5% of the total bacteria and exhibited at least a two-fold change in abundance in mice fed HAM-RS2 compared with the Control animals. Statistically significant differences compared to Control mice according to the Kruskal and Wallis test are indicated by * ($P < 0.05$).

RS consumption resulted in increased proportions of *Bifidobacterium* and *Akkermansia* in the mouse intestine

The ceca of aged mice that consumed HAM-RS2 were enriched for bacteria from the phylum Actinobacteria (Fig. 1); the majority (> 93%) of which was represented by members of the genus *Bifidobacterium*. Mice fed 18% or 36% HAM-RS2 were colonized by *Bifidobacterium* at the same level (15.2 ± 10.4 and 13.6 ± 9.9% of total bacteria, respectively), representing an average six-fold increase compared with the Controls (Fig. 3). When the outlier mice (Control5 and Control6, 18%RS1, and 18% RS6) were excluded from the analysis, an even greater 17-fold increase in bifidobacteria abundance was observed. Noticeably, differences in the proportions of bifidobacteria found among those mice at least partly explain their separation from others fed the same diet in Fig. 1.

Closer inspection of the *Bifidobacterium* 16S rRNA gene sequences showed that a diet containing 36% HAM-RS2 selected for specific members of the *Bifidobacterium* genus (Fig. S3). Aged mice fed the Control diet or 18% HAM-RS2 were predominantly colonized by close relatives of *Bifidobacterium pseudolongum* subsp. *pseudolongum* and *globosum*. In contrast, mice fed 36% RS were enriched with *Bifidobacterium* most closely related to *B. animalis*, including the subspecies *animalis* and *lactis*.

Akkermansia muciniphila in the phylum Verrucomicrobia constituted nearly all (> 99%) of the bacteria identified for that phylum in the aged mice. The relative abundance of *A. muciniphila* was highest in animals fed HAM-RS2 in amounts that constituted 36% of the weight of the diet (Fig. 3). Those mice harbored a 35.1-fold ($P < 0.05$) increase in *A. muciniphila* compared with the other animals (Fig. 2).

RS-responsive genera correlated with health markers in aged mice

HAM-RS2 consumption was associated with changes in intestinal weight and gut signaling pathways (Zhou *et al.*, 2012) (Table 1). HAM-RS2 did not result in abdominal fat and weight gain in animals, but instead, cecal (empty and full) and total gastrointestinal tract weights were significantly greater with increasing concentrations of HAM-RS2 in the diet. These conclusions confirm the results obtained for a larger set of mice including those examined here (Zhou *et al.*, 2012). Moreover, we also report that the feeding response after a mild (overnight) fast tended to be improved in mice fed 36% HAM-RS2 (Table 1). Food intake after an overnight fast was directly associated with the proportions of *Akkermansia*, *Bifidobacterium,* and *Allobaculum* in the ceca (Table 2). The proportions of those genera were also related to markers of gastrointestinal fermentation, that is, full cecum, empty cecum, and full gastrointestinal tract weights (Table 2). Conversely, abundances of the HAM-RS2-depleted genera *Butyrivibrio* and *Turicibacter* were negatively correlated with food intake after fasting and gut weight (Table 2).

Proglucagon gene expression was significantly increased with the presence of HAM-RS2 in the diet and was positively correlated with the intestine weight and eating behavior 1 h after refeeding following a mild fast (Table 1). Remarkably, proportions of *Akkermansia* and *Bifidobacterium* were positively correlated with proglucagon transcript levels in the cecum (Table 2). Conversely, PYY expression level was not significantly affected by HAM-RS2 consumption but was negatively correlated with the abundance of the genus *Turicibacter* (Table 2).

Discussion

To the best of our knowledge, this is the first study to apply high-throughput sequencing to identify the microbiota of aged mice. Because intestinal weight and eating behavior are generally associated with improved gut health and gut signaling of energy deficits, we hypothesized that feeding RS would attenuate the anorexia commonly associated with aging (Moss *et al.*, 2012). We showed that HAM-RS2 consumption enriched for specific bacterial genera and that the changes in the intestinal microbiota were correlated with increased food intake, gastrointestinal tract weight, and production of proglucagon by the animals. Our findings are in agreement with studies showing that dietary RS is associated with health benefits in young human and rodent populations (Abell *et al.*, 2008; Shen *et al.*, 2011). In young rodent adults, RS consumption stimulated food intake and induced body fat loss that was associated to intestinal fermentation and production of SCFA (Zhou *et al.*, 2009), suggesting a role of intestinal bacteria in the physiological effects of RS.

Remarkably, mice fed HAM-RS2-enriched diets were colonized by proportions of bacterial phyla that were more similar to the proportions found in younger animals and human adults than to the aged-matched Controls (Ley *et al.*, 2006; Hoffmann *et al.*, 2009; Martínez

Table 1. Physiological parameters measured in mice

	Food intake (g)*		Weight (g)*					Gene expression†	
	1–2 h	2–4 h	Full gi tract	Full cecum	Empty cecum	Abdominal fat	PBWC‡	Proglucagon	PYY
Control	0.15 ± 0.13	0.28 ± 0.20	2.19 ± 0.25	0.28 ± 0.07	0.07 ± 0.01	0.59 ± 0.25	9.27 ± 10.91		
18%RS	0.21 ± 0.13	0.50 ± 0.17	2.92 ± 0.47	0.54 ± 0.14	0.11 ± 0.02	0.58 ± 0.24	15.00 ± 9.02	1.07 ± 0.44¶	−1.06 ± 1.86
36%RS	0.43 ± 0.20§	0.82 ± 0.36¶	4.04 ± 0.68¶	1.03 ± 0.27¶	0.16 ± 0.06¶	0.48 ± 0.31	10.19 ± 9.41	1.72 ± 1.28¶	1.00 ± 1.75

*Values are average ± standard deviation for six mice.

†Values are fold change in expression compared to Controls [$\log2(2^{-\text{averaged}\Delta\Delta Ct})$ with averaged ΔCt for Control mice as the reference condition].

‡PBWC = percent body weight change after 65 days of feeding.

§Indicates a trend toward increased amounts compared to Controls (Kruskal and Wallis test, $P < 0.1$).

¶Indicates that the difference between mice fed RS and Controls is significant (Kruskal and Wallis test, $P < 0.05$).

Table 2. Correlations between selected genera abundance and physiological measurements

	Bifido bacterium	*Allo baculum*	*Turicib acter*	*Butyri vibrio*	*Oscillib acter*	*Lacto bacillus*	*Rose buria*	1–2 h	2–4 h	Full gi tract	Full cecum	Empty cecum	Proglu cagon	PYY	Abdo minal fat	% weight change after 65 days		
Akkermansia	ns	ns	ns	−0.4	ns	ns	ns	0.55	ns	0.59	0.69	0.65	0.53	ns	ns	ns		
Bifidobacterium		0.78	−0.8	ns	−0.6	ns	ns	ns	0.47	0.65	0.64	0.54	0.44	ns	ns	ns		
Allobaculum			−0.7	ns	ns	0.4	ns	ns	0.49	0.56	0.56	0.48	ns	ns	ns	0.78		
Turicibacter				ns	0.55	ns	ns	ns	−0.4	−0.5	−0.5	−0.5	ns	−0.4	ns	−0.8		
Butyrivibrio					ns	0.44	ns	−0.5	−0.5	−0.5	−0.6	ns	−0.6	ns	0.43	ns	Positive	Negative
Oscillibacter						ns	0.68	ns	ns	ns	ns	ns	ns	ns	ns	−0.6	correlation	correlation
Lactobacillus							ns	ns	ns	ns	ns	0.4	ns	ns	0.4	ns	$P < 0.0005$	$P < 0.0005$
Roseburia								ns	ns	ns	ns	ns	ns	ns	ns	ns	$P < 0.01$	$P < 0.01$
1–2 h									0.55	0.51	0.53	ns	0.41	ns	ns	ns	$P < 0.05$	$P < 0.05$
2–4 h										0.62	0.61	0.5	ns	ns	ns	0.47		
Full gi tract											0.97	0.76	0.78	ns	ns	0.65		
Full cecum												0.85	0.77	ns	ns	0.64		
Empty cecum													0.65	ns	ns	0.54		
Proglucagon														ns	ns	ns		
PYY															ns	ns		
Abdominal fat																ns		

Values are Spearman correlation coefficients color coded according to their level of significance.

et al., 2010; Murphy *et al.*, 2010; Arumugam *et al.*, 2011; Bailey *et al.*, 2011). The most significant change in the microbiota of mice fed HAM-RS2 was the higher proportion of bacteria in the phyla Bacteroidetes, Actinobacteria, and Verrucomicrobia. These findings differ compared with younger human adults for which regular consumption of type 2 or type 3 RS did not result in phylum-level shifts in the composition of fecal microorganisms (Martínez *et al.*, 2010; Walker *et al.*, 2011). Therefore, the dramatic effects of HAM-RS2 on the aged animals might have been the consequence of a skewed microbiota in the Control animals fed the low-fermentable fiber or a restoration to a 'normal' composition by the addition of the RS substrate into the mouse diet.

Members of the *Bifidobacterium, Bacteroides, Ruminococcus,* and *Eubacterium* genera are able to metabolize amylose for energy and growth (Leitch *et al.*, 2007). Here, only *Bifidobacterium* was significantly enriched in mice fed 18% and 36% HAM-RS2 and therefore were the likely primary degraders of the RS substrate. This enrichment is comparable to the increase in *Bifidobacterium* found upon the addition of prebiotic carbohydrates into the diets of mice (Everard *et al.*, 2011) and humans (Davis *et al.*, 2011). Although the specific mechanisms involved in *Bifidobacterium* effects on the host are not well understood, *Bifidobacterium* species are typically the target organisms of prebiotic applications (Macfarlane *et al.*, 2008) because of their associations with good digestive tract function and health (Ventura *et al.*, 2009).

Other intestinal microorganisms might have also benefited from the increased availability of HAM-RS2-derived sugars and fermentative end products (e.g. lactate) from the metabolism of primary HAM-RS2-degraders. Possible secondary HAM-RS2-degraders include species of *Allobaculum*, which were enriched in the ceca of mice fed HAM-RS2 and are able to consume either lactate or mono- and disaccharides for growth (Greetham *et al.*, 2004). Interestingly, the major end product of *Allobaculum* fermentation is butyrate, which production is of particular relevance in the gut because this SCFA is rapidly taken up by colonocytes where it serves as a main energy source (Donohoe *et al.*, 2011) and has been shown to stimulate the expression of gut peptides (PYY and proglucagon) in cecal tissues (Zhou *et al.*, 2006). The proportional amounts of other butyrate producing bacteria such as *Butyrivibrio*, *Roseburia*, and species from the *Clostridium* clusters IV and XIV were either not affected or reduced in mice fed HAM-RS2. Although these bacteria are typically regarded to be beneficial for gut function and health (Louis & Flint, 2009), their reduction might indicate a shift toward a more balanced microbiota in the aged mice. This possibility is supported by the findings from Claesson *et al.* (Claesson *et al.*, 2011) that elderly individuals were

colonized by higher amounts of bacteria in *Clostridium* cluster IV than younger persons (Claesson *et al.*, 2011).

Comparisons of the aged mice microbiota also showed that HAM-RS2 enriched for specific bacterial genera in a concentration-dependent manner (Fig. 3). *Lactobacillus* spp. were specifically enriched in mice on diets containing 18% HAM-RS2; whereas the phylum Verrucomicrobia, and specifically *A. muciniphila*, was abundant in mice fed diets composed of 36% HAM-RS2. *Akkermansia muciniphila* is regarded as a beneficial member of the gut microbiota (Derrien *et al.*, 2011) and is an efficient mucin degrader (Derrien *et al.*, 2008; van Passel *et al.*, 2011). Enrichment in *A. mucinphila* was also recently reported for genetically obese (*ob/ob*) mice fed prebiotic carbohydrates (Everard *et al.*, 2011). These bacteria might have benefited from increases in cecal mucin production induced by the dietary carbohydrates (Morita *et al.*, 2004). Moreover, different *Bifidobacterium* species were found in mice fed HAM-RS2. *Bifidobacterium animalis* was specifically enriched in mice fed 36% HAM-RS2, and this species was previously shown to degrade certain RS formulations with greater efficiencies than species *pseudolongum* and *breve* (Wronkowska *et al.*, 2008). Overall, these results support the concept of concentration-dependent effects of dietary substrates on the gut microbiota and the importance of consuming RS and prebiotics in concentrations that induce optimal levels of intestinal fermentation and host-microbe cell signaling (Davis *et al.*, 2010). Further investigations are needed to understand the effects of different sources (e.g. potato, rice, maize), preparations (type 1–4), and doses of RS on the intestinal microbiota of aged individuals.

The process of aging is accompanied by disturbances in the endocrine and immune systems that can result in a decline in appetite and accompanying weight loss (Moss *et al.*, 2012). Sustained intake of dietary HAM-RS2 for 10 weeks can improve the physiological parameters associated with aging including the increased expression of hormones regulating the metabolism of glucose and lipids in visceral fat (Zhou *et al.*, 2012). Here, after confirming those changes in host physiology on a fraction of mice, they were correlated with the abundances of certain intestinal bacterial genera. Cecal proportions of *Akkermansia*, *Bifidobacterium*, and *Allobaculum* were directly related to digestive tract weight and to the quantity of food consumed after an overnight fast. This supports the hypothesis that those genera were the main HAM-RS2-fermenting bacteria and were associated with an improved appetite response after a mild fast. *Allobaculum* was previously shown to be one of the intestinal genera that are the most sensitive to changes in host diet (Ravussin *et al.*, 2011). Interestingly, *Allobaculum* abundance was strongly inversely correlated with the amounts of circulating leptin and expression of several genes correlated with energy expenditure homeostasis and inflammation (Ravussin *et al.*, 2011). *Akkermansia* and *Bifidobacterium* proportions were also correlated with the level of expression of proglucagon, the precursor of the potent anorexigenic peptide GLP-1 (Moss *et al.*, 2012), which is associated with the improvement of glycemic and insulin responses and reductions in fat mass of mice fed prebiotics (Delzenne *et al.*, 2011). One possible mechanism to explain these effects is that *Akkermansia* fermentation of mucin results in the production of propionate (Derrien, 2004), a SCFA known to stimulate the production of GLP-1 in rodents (Zhou *et al.*, 2008). Generally, *Akkermansia* is increasingly associated with improved gut health and ameliorations in body weight disorders (Zhang *et al.*, 2009; Png *et al.*, 2010; Santacruz *et al.*, 2010; Delzenne *et al.*, 2011).

Notably, certain mice did not respond to HAM-RS2 consumption and grouped with the Control mice (Fig. 1). Mouse 18%RS6 harbored very low proportions of *Bifidobacterium* and *Allobaculum*, high levels of *Turicibacter*, and low gastrointestinal weights compared to the other mice fed the diet containing 18% HAM-RS2. One possibility for this finding is that this mouse initially harbored a microbiota deprived in HAM-RS2-responsive genera and therefore was not able to respond to the carbohydrate. Nonresponders can be confounding to data analysis of dietary intervention and clinical studies (Reid *et al.*, 2010). Conversely, two mice from the Control group (5 and 6) were colonized by a gut microbiota most similar to the 18% HAM-RS2-fed mice and contained the highest proportions of *Bifidobacterium* and *Allobaculum* and fewest representatives of *Turicibacter* spp. among the Controls, although not at the same levels found in HAM-RS2-fed mice. Lastly, we observed that mouse 18%RS1 was colonized by a cecal microbiota that was most similar to the 36% HAM-RS2-fed mice. Like the 36% HAM-RS2-fed mice, high amounts of proglucagon gene transcripts were found in the intestine of this animal.

To address inter-individual differences, future studies on aging mice should include fecal analysis and quantification of noninvasive physiological parameters (e.g. weight, food intake, circulating cytokine, and hormone levels) of the animals prior to the introduction of RS into the diet to establish a baseline and control for inter-individual differences in the gut microbiota. In addition, comparison of plasma markers for inflammation among individuals receiving different amounts of RS would indicate whether microbial changes and their effects include lessening the inflammatory status typically observed in elderly (Biagi *et al.*, 2010).

These analyses combined with monitoring the mouse gut microbiota from adulthood to old age will help to identify microbial composition of a healthy aged gut and

to guide predictions on which individuals will be nonresponders to changes in diet. On the basis of these results, similar studies can be performed in humans to confirm that changes in the RS-induced intestinal microbiota and associated physiological responses are not affected by species-specific microbiota, anatomy, and feeding behavior differences, including coprophagy. Overall, this study suggests that *Allobaculum*, *Akkermansia*, and *Bifidobacterium* are associated with energy homeostasis in the body and the prevention of detrimental age-associated declines in food consumption. These findings support the concept that dietary changes can result in dramatic adjustments to the gut microbiota and that intestinal microorganisms contribute to the health status of the individual.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Average proportions of the most dominant Firmicutes (a) and Bacteroidetes (b) phylotypes identified by 16S rRNA sequencing of the microbiota in the cecum of aged mice fed 0% (Controls), 18% or 36% HAM-RS2.
Fig. S2. Amounts of total bacteria and *Clostridium* in the clusters IV and XIV present in the ceca of aged mice fed 0% (Controls), 18% or 36% HAM-RS2 as determined by real-time PCR.
Fig. S3. Radial cladogram of *Bifidobacterium* 16S rRNA gene sequences from aged mice ceca.
Table S1. Composition of the diets used in this study.

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