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Response of Gut Microbiota to Fasting and Hibernation in Syrian Hamsters

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Running title: Gut microbiota in hamsters

ABSTRACT

Although hibernating mammals wake occasionally to eat during torpor, this period represents a state of fasting. Fasting is known to alter the gut microbiota in non-hibernating mammals; therefore, hibernation may also affect the gut microbiota. However, there are few reports of gut microbiota in hibernating mammals. The present study aimed to compare the gut microbiota in hibernating torpid Syrian hamsters with that in active counterparts using culture-independent analyses. Hamsters were allocated to either torpid, fed active, or fasted active groups. Hibernation was successfully induced by maintaining darkness at 4˚C. Flow cytometry analysis of cecal bacteria showed that 96-h-fasting reduced the total gut bacteria. This period of fasting also reduced the concentrations of short chain fatty acids in the cecal contents. In contrast, total bacterial numbers and concentrations of short chain fatty acids were unaffected by hibernation. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments indicated that fasting and hibernation modulated the cecal microbiota. Analysis of 16S rRNA clone library and species-specific real-time quantitative PCR showed that the class *Clostridia* predominated in both active and torpid hamsters and that the populations of *Akkermansia muciniphila*, a mucin degrader, were increased by fasting, but not by hibernation. From these results, we conclude that gut microbiota responds differently to fasting and hibernation in Syrian hamsters.

INTRODUCTION

Some mammalian species have evolved the physiological phenomenon of hibernation to survive unfavorably winter environments (9). Hibernation is realized by entering torpor in order to eliminate the need to maintain a constant, high body temperature. During torpor, typical hibernating mammals, such as hamsters and ground squirrels, lower their body temperature to only a few degrees above ambient temperatures to reduce energy expenditure. Torpor is interrupted by periods of intense metabolic activity. During these inter-bout arousals, physiological parameters are restored rapidly to near-normal levels. Thus, hibernators alternate between hypothermic and euthermic states during hibernation.

Some hibernating mammals awake to forage during torpor, while food-storing hibernators such as hamsters eat cached food during inter-bout arousals. However, hibernation essentially involves periods of fasting. Fasting is known to affect the gut microbiota in non-hibernating mammals such as mice (12); therefore, it is possible that hibernation also influences the gut microbiota. Given that gut microbiota play important roles in mammalian tissue development and homeostasis (28), it is of interest to investigate the changes in the gut microbiota that may take place during hibernation. To date, this issue has received little attention; to our knowledge, there are only two reports on the gut microbiota in hibernating mammals. Schmidt *et al.* showed that although the total count of coliforms, streptococci, and psychrophilic organisms in the feces of arctic ground squirrels held in a cold room at 3˚C remained constant, the composition changed with a decrease in coliform count and a 1,000-fold increase in the numbers of aerobic psychrophilic gram-negative bacteria (31). Barned and Burtone reported that although there was some reduction in total numbers of viable bacteria in the cecum during hibernation, composition of the microbiota remained stable (6). In terms of amphibians, Banas *et al.* and Gossling *et al.* reported the reduction and compositional changes of the gut microbiota in hibernating leopard frogs (4, 5, 18, 19).

Only 20 to 40% of bacterial species from the mammalian intestinal tract can be cultured and identified using classical culture methods (22, 34, 36). In contrast, culture-independent methods based on the amplification of bacterial 16S rRNA genes by PCR have revealed a great diversity of microbiota in environmental samples (3, 37). The present study compared the gut microbiota in hibernating torpid Syrian hamsters with that in active counterparts using culture-independent analyses.

MATERIALS AND METHODS

Animals and diets

The following study was approved by the Hokkaido University Animal Use Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals. Male Syrian hamsters (aged 10 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed individually in standard plastic cages with nesting material in a temperature-controlled (23 ± 2˚C) room with a dark period from 20:00 to 8:00 h. They were allowed free access to chow (Labo H Standard, Nosan Corporation, Yokohama, Japan) and tap water. Hamsters were kept in these conditions until 12 weeks of age.

Experimental design

Hamsters were divided into a fed active, non-hibernating group (n = 6), a fasted active, non-hibernating group (n = 6) and a hibernating group (n = 6). The two active groups continued to be housed in the conditions described above while the latter were housed individually in constant darkness at 4˚C in order to induce hibernation (27). All hamsters were allowed free access to chow and tap water. In the hibernating group, a temperature sensor (coupled with a data logger) (RTR-52A, T & D Corporation, Nagano, Japan) was attached to each cage to monitor the duration of each hibernation bout. Of the hibernating group, 4 of the 6 hamsters experienced 9 to 10 hibernation cycles and were killed by exsanguination from the abdominal aorta. Six hamsters from the fasted active group were killed by exsanguination from abdominal aorta under anesthesia by inhalation with diethyl ether after fasting for 96 h, and the remaining 6 fed active hamsters were killed without fasting. The cecal contents of all animals were excised and subjected to analyses of microbiota.

Flow cytometry analysis of viable, injured, and dead bacterial cells in cecal

contents

Population and viability of bacteria were analyzed by flow cytometry (FCM) according to Ben-Amor *et al.* (7). In brief, a portion (≈ 100 mg) of the cecal contents was suspended in 1 ml of anaerobic phosphate-buffered saline (PBS) containing 1 mM dithiothreitol and 0.01% (w/v) Tween 20 and homogenized by vortexing for 3 min. After centrifugation at 700 x g for 1 min, the supernatant was carefully recovered and centrifuged at 6,000 x g for 3 min. The pellet was washed twice, resuspended in anaerobic PBS, and then serially diluted. Thereafter, the diluted samples were incubated for 15 min at room temperature in anaerobic PBS supplemented with 10^4 particles/ml fluorospheres (Flow-Check fluorospheres, Beckman Coulter, Tokyo, Japan), 1 μg/ml propidium iodide (PI, Wako Pure Chemical Industries, Osaka, Japan), and 5 nM SYTO-BC (Molecular Probes, Eugene, OR). Samples were analyzed by flow cytometry (Epics XL, Beckman Coulter).

Profile analysis of cecal microbiota by PCR-denaturing gradient gel electrophoresis

DNA was extracted from cecal contents using a fecal DNA isolation kit (MO Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA samples were used as a template to amplify the fragments of the 16S rRNA gene with universal primers, U968-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (CGG TGT GTA CAA GAC CC) (37), and denaturing gradient gel electrophoresis (DGGE) analysis of the amplicon was carried out as previously described (15). Quantity One software (version 4.6.0, Bio-Rad, Hercules, CA) was used for band identification and normalization of band patterns from DGGE gels. A dendrogram showing the similarity of band patterns was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) clustering method in Quantity One software as previously described (15).

Analysis of the 16S rRNA gene sequences in cecal bacteria

Cecal DNA samples were pooled in each group and used as a template to amplify the fragments of the 16S rRNA gene with universal primers, U968 (AAC GCG AAG AAC CTT AC) and L1401. PCR was performed in a reaction volume of 25 μl that contained 500 nM each of primers, 1 × PCR buffer, 0.2 mM each of dNTPs, and 1.25 U of *Taq*-HS polymerase (Takara, Ohtsu, Japan). The reaction conditions were 94˚C for 5 min, followed by 20 cycles of 94˚C for 30 s, 56˚C for 20 s, and 68˚C for 40 s, and final extension at 68˚C for 7 min. The amplicons were purified by a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and cloned into pGEM-Easy T vectors (Promega, Madison, WI). Transformation was performed with competent *Escherichia coli* XL-1 Blue cells, and the transformants were spread on Luria-Bertani agar plates supplemented with 25 μg/ml ampicillin, 30 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 20 μg/ml isopropyl-β-D-thiogalactopyranoside, and incubated overnight at 37˚C. White colonies were randomly picked from each sample and grown on Luria-Bertani agar. Clones carrying inserts were identified by colony PCR using a Colony PCR M13 set (Nippongene, Tokyo, Japan). Plasmid DNAs in the positive clones were amplified for sequencing with an Illustra TempliPhi DNA amplification Kit (GE Healthcare Bioscience, Tokyo, Japan) according to the manufacture's instructions. Resultant amplicons were sequenced by using an ABI3730XL or ABI3730 (Applied Biosystems, Carlsbad, CA) with M13-F (GTT TTC CCA GTC ACG ACG TT) as a sequencing primer. Artifact chimeric sequences were detected using the CHECK_CHIMERA program of the Ribosomal Database Project (RDPII) (10) and omitted from following analysis. Sequence data were aligned with the Clustal X multiple sequence alignment program (35) and corrected by manual inspection. The sequences were compared to the database of RDPII. Naïve Bayesian rRNA classifier version 2.0 from RDPII was used to

assign 16S rRNA gene sequences to the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology, release 6.0 (10), with a setting threshold value of 90%. The sequences were assigned to individual operational taxonomy units (OTUs) based on a sequence similarity of at least 98% (33). Phylogenetic tree was constructed by the neighbor-joining method (29) in the Clustal X program with 1,000 bootstrap replicates. Analysis of the 16S rRNA gene sequences was also performed using the DDBJ/EMBL/GenBank DNA database and the BLAST algorithm.

Real-time quantitative PCR for *Akkermansia muciniphila* in cecal contents

Populations of *A. muciniphila* were determined by real-time quantitative PCR (RT-qPCR) according to Collado *et al.* (11). In brief, amplification and detection of cecal DNA were performed with the Thermal Cycler Dice Real Time System (Takara). *A. muciniphila* species-specific primer pair (AM1, CAG CAC GTG AAG GTG GGG AC; AM2, CCT TGC GGT TGG CTT CAG AT) was used. RT-qPCR was performed in a reaction volume of 25 μl, containing 12.5 μl SYBR Premix Ex *Taq* (Takara), 200 nM each of the forward and reverse primers, and 1 μl of cecal DNA samples. The reaction conditions were 95˚C for 5 min, followed by 40 cycles at 95˚C for 15 s, 60˚C for 40 s, and 72˚C for 30 s, and a final extension at 72˚C for 5 min. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product. All samples were analyzed in duplicate.

Using a cecal DNA sample from a fasted active hamster as a template, a fragment of 16S rDNA was amplified by PCR with the *A. muciniphila* species-specific primer pair (AM1 and AM2 as described above). The size of the amplicon, estimated by agarose gel electrophoresis, was identical to the expected size (327 bp) (11), and the sequence was completely identical to *A. muciniphila* (data not shown). The amplicon was purified and subcloned into a bacterial plasmid as described above. The plasmid

DNA was extracted with QIAprep Spin Miniprep kit (Qiagen) and used as a standard for RT-qPCR. Additionally, the plasmid DNA was added to each cecal DNA sample in order to check for the presence of PCR inhibitors in the samples, and we confirmed no PCR inhibitors in all samples.

Measurement of short chain fatty acid concentrations in cecal contents

Concentrations of short chain fatty acids (SCFA) in the cecal contents of hamsters were determined using HPLC by the internal standard method (20). A portion of the cecal contents (≈ 100 mg) was homogenized in 2 ml of 10 mM sodium hydroxide solution containing 0.5 g/l crotonic acid (Wako Pure Chemical Industries) as an internal standard, then centrifuged at 10,000 × g for 15 min. Concentrations of individual SCFA (acetate, propionate, *i*-butyrate, *n*-butyrate, *i*-valerate, *n*-valerate, succinate, and lactate) in the supernatant were measured using an HPLC system (LC-6A, Shimadzu, Kyoto, Japan) equipped with a Shim-pack SCR-102H column (inner diameter, 8 mm; length, 30 cm; Shimadzu) and a CDD-6A electroconductivity detector (Shimadzu).

Statistical analysis

Results are presented as mean ± SEM or as individual values for each hamster. Tukey-Kramer's test following one-way ANOVA was used to analyze the differences among the means of the experimental groups. StatView for Macintosh (version 5.0, SAS institute Inc., Cary, NC) was used for the analysis. Differences were considered significant at $p<0.05$.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained from cloning experiments have been deposited in DDBJ with accession numbers AB511067 to AB511276.

RESULTS AND DISCUSSION

Hibernation in mature hamsters can be induced at any time of the year by subjecting them to a reduced ambient temperature of 4˚C and constant darkness for about 45 days (27). In the present study, hibernation was successfully initiated in 4 out of 6 hamsters. Representative time course of housing temperature exhibits a clear hibernation rhythm with individual hibernation bout and inter-bout arousal (**Supplemental Fig. 1**). Thus, monitoring the housing temperature alerted us to the time course of hibernation rhythms for each hamster. Four torpid hamsters that had experienced 9 to 10 hibernation cycles were killed just prior to the start of the next inter-bout arousal. The duration of the average individual hibernation bout in 4 hibernating hamsters was 92.3 ± 7.1 h.

FCM analysis was performed to determine total population and viability of bacteria in cecal contents (**Supplemental Fig. 2**), and the results were summarized in **Table 1**. Wet weights of cecal contents were significantly higher in torpid hamsters than in active hamsters and there was no significant difference between fed and fasted active hamsters. The cecal contents in fasted active hamsters appeared much more fluidal when compared to fed active hamsters and torpid hamsters. Total bacterial populations were significantly lower in fasted active hamsters than in fed active and torpid hamsters, and there was no significant difference between fed active and torpid hamsters. Viabilities of cecal bacteria were significantly higher in torpid hamsters than in active hamsters, although there was no significant difference between fed and fasted hamsters. The proportions of injured bacterial cells were significantly higher in fasted active hamsters than in fed active hamsters and torpid hamsters. Consequently, the proportions of dead bacterial cells were significantly higher in fed active hamsters than in fasted active hamsters and torpid hamsters and tended to be lower in torpid hamsters than in fasted active hamsters. Additionally, the concentrations of SCFA, predominant fermentation products of bacteria, in the cecum were shown in **Table 2**. The concentrations of total SCFA and acetic acid were significantly lower in fasted active

hamsters than in fed active hamsters and torpid hamsters. A similar tendency was shown in the concentrations of propionic, succinic, and lactic acids, although there were no significant differences among the groups. Fasted active hamsters showed no detectable levels of *n*-butyric acid. As the concentrations of SCFA represent the difference between production and absorption in the cecum, the lower concentrations of cecal SCFA in fasted active hamsters appear to reflect a decrease in the production of SCFA caused by a reduction in bacterial populations and viabilities. Regarding SCFA in the hibernating animals, Hume *et al*. reported that the concentrations in the cecal contents of free-living alpine marmot were significantly lower in emergence from hibernation in April as compared to active animals in summer (21). The authors described that the lower concentrations of cecal SCFA in emergence might reflect the resting state of the gut microbiota, due to low body temperatures in hibernating animals (21). Although the reduced metabolic activity of gut microbiota due to low temperatures is also presumably the case in the present study, no reduction in the concentrations of SCFA was observed in the cecal contents of torpid hamsters. This might reflect a lower ability to absorb SCFA in the cecum, due to reduced metabolic activity during hibernation bouts. Additionally, periodic consumption of cached food during inter-bout arousal might supply a substantial amount of substrate for fermentation in the cecum of hibernating hamsters.

The 16S rRNA gene profiles of the bacterial collections in the cecal samples were generated by PCR coupled with DGGE. The DGGE band profile is shown in **Figure 1A**. The intensity and position of detected bands were subjected to cluster analysis. The dendrogram shows three large clusters of fed active hamsters, fasted active hamsters, and torpid hamsters, except one fed active hamster belonging to the cluster of torpid hamsters (**Fig. 1B**). The results suggest that the gut microbiota is modulated by fasting and by hibernation in hamsters. To further investigate the difference, 16S rRNA clone libraries were constructed from DNA samples pooled in

each group. The results of the phylogenetic analysis are shown in **Table 3**. For fed active, fasted active, and torpid hamsters, 95, 93, and 91 sequences were analyzed. The class *Clostridia* in the phylum *Firmicutes* was the most abundant taxonomic group in all treatment groups. However, the frequency of the class *Clostridia* in fasted active hamsters (37.6%) tended to be lower than that in fed active hamsters and torpid hamsters (82.1 and 81.3%, respectively). Comparatively, in fasted active hamsters, the phylum *Verrucomicrobia* was the second most abundant taxonomic group (24.7%), and the third was the phylum *Proteobacteria* (20.4%). Based on the 98% sequence similarity criterion (33), 56, 49, and 44 OTUs were found in the libraries from fed active, fasted active, and torpid hamsters, respectively. According to Good's method (17), coverage of the diversity in the 16S rRNA gene sequences was 41, 47, and 52% in fed active, fasted active, and torpid hamsters, respectively. A phylogenetic reconstruction of the relationships between the OTUs is presented in **Supplemental Figure 3**. The phylogenetic tree shows six distinctive clusters of the phylum *Bacteroidetes*, the phylum *Verrucomicrobia*, the class *Bacilli*, the class *Alphaproteobacteria*, the class *Deltaproteobacteria*, the family *Ruminococcaceae* and other families of *Clostridia*. Although the DGGE band profile shows three distinct clusters of fed active, fasted active, and torpid hamsters (**Fig. 1**), the phylogenetic tree shows no distinct clusters in association with these animal groups.

It is noteworthy that all sequences in the phylum *Verrucomicrobia* detected in fasted active hamsters were classified to the genus *Akkermansia* (similarity 100%). Derrien *et al.* (14) isolated *Akkermansia muciniphila* as a mucin-degrading bacterium in the human intestine. RT-qPCR using a species-specific primer pair for *A. muciniphila* revealed that the bacterial populations corresponding to *A. muciniphila* were significantly higher in fasted active hamsters than in fed active hamsters, and those in torpid hamsters were intermediate (**Fig. 2**). Mucin-degrading bacteria have a competitive advantage during nutrient deprivation, such as during malnutrition, or total

parenteral nutrition (TPN) (13, 25). Therefore, increased populations of *A. muciniphila* in the cecal contents of fasted active hamsters suggest that a lack of food-derived enteral nutrients encourages the growth of mucin-degrading bacteria such as *A. muciniphila*. However, the populations of *A. muciniphila* in the cecal contents of torpid hamsters were comparable to those of fed active hamsters, despite fasting during hibernation bouts. Because the growth of *A. muciniphila* reportedly occurs at 20-40 ˚C, with optimum growth at 37 ˚C (14), lower temperatures might prevent the growth of *A. muciniphila* in the cecum of torpid hamsters.

Because mucins are highly sulfated acidic mucopolysaccharides, degradation of mucins by intestinal bacteria release sulfate, which in turn stimulates sulfate-reducing bacteria such as *Desulfovibrio* spp. (16). In the present study, the family *Desulfovibrionaceae* was the most common family in the phylum *Proteobacteria*, the third most abundant taxonomic group in fasted active hamsters, and the frequency of the family *Desulfovibrionaceae* was higher in fasted active hamsters than in fed active and torpid hamsters (**Table 3**). These findings suggest that fasting stimulates the growth of sulfate-reducing bacteria such as *Desulfovibrio* spp. through increased degradation of mucins by *A. muciniphila* in the cecum of active hamsters. Considering the toxicity of hydrogen sulfide produced from reduction of sulfate by sulfate-reducing bacteria, abundance of the family *Desulfovibrionaceae* might be associated with higher proportions of injured bacteria in the cecum of fasted active hamsters.

Mucins are the main constituents of the mucus covering the epithelial surface of the gastrointestinal tract, and thus contribute to the epithelial protective barrier against pathogenic microorganisms, as well as chemical, physical or enzymatic damage. Therefore, the degradation of intestinal mucins possibly results in reduced barrier function, which in turn becomes pathologic. As mucin-degrading bacteria have a competitive advantage during nutrient deprivation (13, 25), the gut mucosal barrier may be disrupted by fasting and/or TPN. Indeed, TPN reportedly reduced the absolute

quantity of mucus gel in rat ileum (30), and also induced intestinal bacterial translocation (BT) in rats (1, 2, 24, 32). In contrast, BT was not induced by up to 72 h of fasting in rats (8, 12), although increased mucosal permeability was reported in fasted rats (8). Nevertheless, fasting can exacerbate the risk of morbidity and mortality for BT (23). The present study showed that prolonged fasting stimulated the growth of *A. muciniphila* in the cecum of active hamsters. In this context, *A. muciniphila* may be involved in disruption of the gut mucosal barrier, possibly followed by BT under certain circumstances, during deprivation of enteral nutrients.

To our knowledge, the present study is the first investigating the gut microbiota in hibernating animals by culture-independent methods. The results suggest that gut microbiota responds differently to fasting and hibernation in Syrian hamsters. Fasting, in particular, stimulated the growth of the mucin degrader *A. muciniphila*. However, coverage of the diversity in the 16S rRNA gene sequences of gut microbiota was about 50% in the present study. Additionally, the present study made no attempt to assess the dominant species of the gut microbiota by identification of bacterial origins of 16S rRNA gene sequences obtained from DGGE gels and to compare to those from 16S rRNA gene clone library. Therefore, further studies are required to illustrate more precisely the gut microbiota alterations in response to fasting and hibernation.

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STATEMENT

We declare all authors (Kei Sonoyama, Reiko Fujiwara, Naoki Takemura, Toru Ogasawara, Jun Watanabe, Hiroyuki Ito, and Tatsuya Morita) have no conflicts of interest.

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Table 1 Flow cytometry counting of cecal bacteria in Syrian hamsters[1]

	Fed	Fasted	Torpid
Cecal contents (g)	3.8 ± 0.5 [a]	3.3 ± 0.3 [a]	7.6 ± 0.4 [b]
Total bacterial number			
Log count/cecum	10.6 ± 0.1 [a]	9.4 ± 0.1 [b]	10.9 ± 0.1 [a]
Log count/g	10.0 ± 0.1 [a]	8.8 ± 0.1 [b]	10.0 ± 0.1 [a]
Viability			
Viable %	81.3 ± 1.7 [a]	77.7 ± 3.1 [a]	90.9 ± 0.9 [b]
Injured %	9.2 ± 0.6 [a]	15.9 ± 2.6 [b]	5.7 ± 0.7 [a]
Dead %	9.5 ± 1.3 [a]	6.4 ± 1.0 [b]	3.4 ± 0.2 [b]

[1] Data are mean ± SEM of six, six, and four hamsters in fed, fasted, and torpid groups, respectively. Mean values with different letters were significantly different ($p<0.05$) as estimated by Tukey-Kramer's test following one-way ANOVA.

Table 2 Short chain fatty acids in cecal contents of Syrian hamsters[1]

	Fed	Fasted	Torpid
		μmol/g contents	
Acetic acid	71.8 ± 11.1 [a]	18.5 ± 1.9 [b]	56.1 ± 6.8 [a]
Propionic acid	15.6 ± 5.6	3.5 ± 0.3	9.0 ± 1.1
n-Butyric acid	21.1 ± 5.8 [a]	n.d. [b]	11.4 ± 0.8 [ab]
Succinic acid	3.26 ± 1.69	0.22 ± 0.07	1.66 ± 0.38
Lactic acid	4.38 ± 3.01	0.16 ± 0.04	0.34 ± 0.18
Total short chain fatty acids	116.2 ± 22.9 [a]	22.4 ± 2.2 [b]	78.5 ± 8.6 [a]

[1] Data are mean ± SEM of six, six, and four hamsters in fed, fasted, and torpid groups, respectively. Mean values with different letters were significantly different ($p<0.05$) as estimated by Tukey-Kramer's test following one-way ANOVA. Isobutyric, *n*-valeric, and *i*-valeric acids were not detected.

Table 3 Taxonomic distribution of 16S rRNA gene sequences in cecal contents of Syrian hamsters

	Fed		Fasted		Torpid	
	Number (%) of sequences					
Phylum *Bacteroidetes*	**0**	**(0.0)**	**1**	**(1.1)**	**2**	**(2.2)**
Class *Bacteroidetes*	0	(0.0)	1	(1.1)	2	(2.2)
Order Bacteroidales	0	(0.0)	1	(1.1)	2	(2.2)
Unclassified Bacteroidales	0	(0.0)	1	(1.1)	2	(2.2)
Phylum *Firmicutes*	**86**	**(90.5)**	**37**	**(39.8)**	**81**	**(89.0)**
Class *Bacilli*	0	(0.0)	0	(0.0)	1	(1.1)
Order *Lactobacillales*	0	(0.0)	0	(0.0)	1	(1.1)
Family *Lactobacillaceae*	0	(0.0)	0	(0.0)	1	(1.1)
Class *Clostridia*	78	(82.1)	35	(37.6)	74	(81.3)
Order *Clostridiales*	76	(80.0)	34	(37.6)	74	(81.3)
Family *Veillonellaceae*	0	(0.0)	0	(0.0)	1	(1.1)
Family *Lachnospiraceae*	50	(52.6)	13	(14.0)	55	(60.4)
Family *Ruminococcaceae*	21	(22.1)	18	(19.4)	13	(14.3)
Unclassified *Clostridiales*	5	(5.3)	3	(3.2)	5	(5.4)
Unclassified *Clostridia*	2	(2.2)	1	(1.1)	0	(0.0)
Unclassified *Firmicutes*	8	(8.4)	2	(2.2)	6	(6.6)
Phylum *Proteobacteria*	**2**	**(2.2)**	**19**	**(20.4)**	**1**	**(1.1)**
Class *Alphaproteobacteria*	1	(1.1)	2	(2.2)	0	(0.0)
Unclassified *Alphaproteobacteria*	1	(1.1)	2	(2.2)	0	(0.0)
Class *Deltaproteobacteria*	1	(1.1)	12	(12.8)	1	(1.1)
Order *Desulfovibrionales*	1	(1.1)	12	(12.8)	1	(1.1)
Family *Desulfovibrionaceae*	1	(1.1)	12	(12.8)	1	(1.1)
Unclassified *Proteobacteria*	0	(0.0)	5	(5.4)	0	(0.0)
Phylum *Verrucomicrobia*	**0**	**(0.0)**	**23**	**(24.7)**	**0**	**(0.0)**
Class *Verrucomicrobiae*	0	(0.0)	23	(24.7)	0	(0.0)
Order *Verrucomicrobiales*	0	(0.0)	23	(24.7)	0	(0.0)
Family *Verrucomicrobiaceae*	0	(0.0)	23	(24.7)	0	(0.0)
Unclassified *Bacteria*	**7**	**(7.3)**	**13**	**(14.0)**	**7**	**(7.7)**
Total	95	(100.0)	93	(100.0)	91	(100.0)

FIGURE LEGENDS

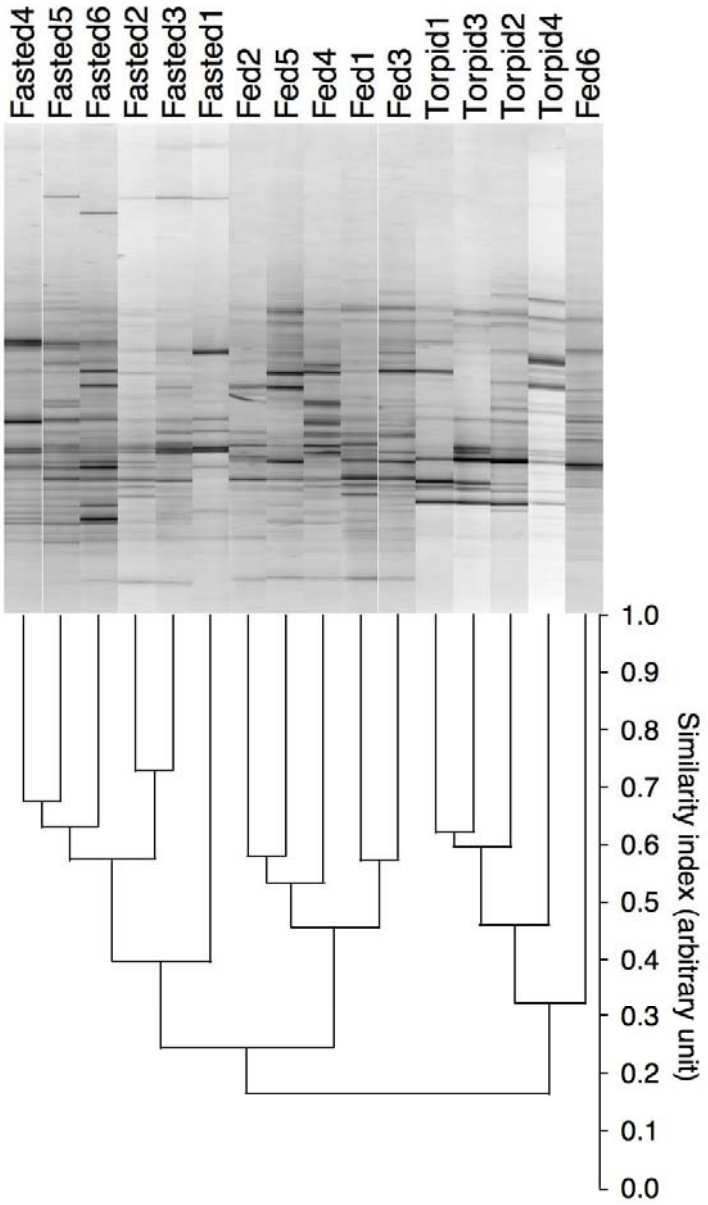
Figure 1 PCR-DGGE analysis of the cecal microbiota based on 16S rRNA gene sequences in hamsters. DGGE gel image stained with SYBR green is shown in chart A. Similarities among DGGE band profiles of cecal bacteria of hamsters were calculated based on the position and intensity of bands, and the dendrogram of DGGE band profiles constructed by the UPGMA clustering method is shown in chart B. Each lane in gel and each line in dendrogram represent individual hamsters. Distances are measured in arbitrary units.

Figure 2 Populations of *Akkermansia muciniphila* in cecal contents of hamsters. RT-qPCR with a species-specific primer pair was performed to estimate the populations of *A. muciniphila*. Each closed circle represents the value of individual hamsters, and horizontal bars represent mean values. Mean values with different letters were significantly different ($p<0.05$) estimated by Tukey-Kramer's test following one-way ANOVA.

Supplemental Figure 1 A representative presentation of housing temperature time course in hibernating hamsters. A temperature sensor coupled with data logger was attached to each cage to monitor the hibernation cycle.

Supplemental Figure 2 Flow cytometry analysis of bacterial cells in cecal contents of hamsters. Chart A shows a representative presentation of dual-parameter (side and forward scatters) dot plot of a cecal sample. Total bacterial population was calculated from the counts in the bacteria region and those in the fluorospheres region. Chart B shows a representative cytogram of a cecal sample stained with SYTO-BC and PI. The two-color dot plot discriminated between SYTO-BC-stained viable cells, double-stained injured cells, and PI-stained dead cells.

Supplemental Figure 3 Phylogenetic tree showing relationship of 16S rRNA gene sequences found in retrieved clones from cecal contents of hamsters with their closely related bacteria retrieved from DDBJ/EMBL/GenBank database. 16S rRNA sequences (about 400 bp long) were used to draw the tree, which was constructed using the neighbor-joining analysis. The number shown at the nodes of the tree indicate bootstrap values for each node out of 1,000 bootstrap resamplings(values < 700 are not shown). *Deferribacter thermophilus* (T)(U75602) was used as the outgroup to root the tree. The scale bar represents 0.02 substitutions per nucleotide position. Clone names starting with FE, FA, and TO represent the clones retrieved from fed active, fasted active, and torpid hamsters, respectively.



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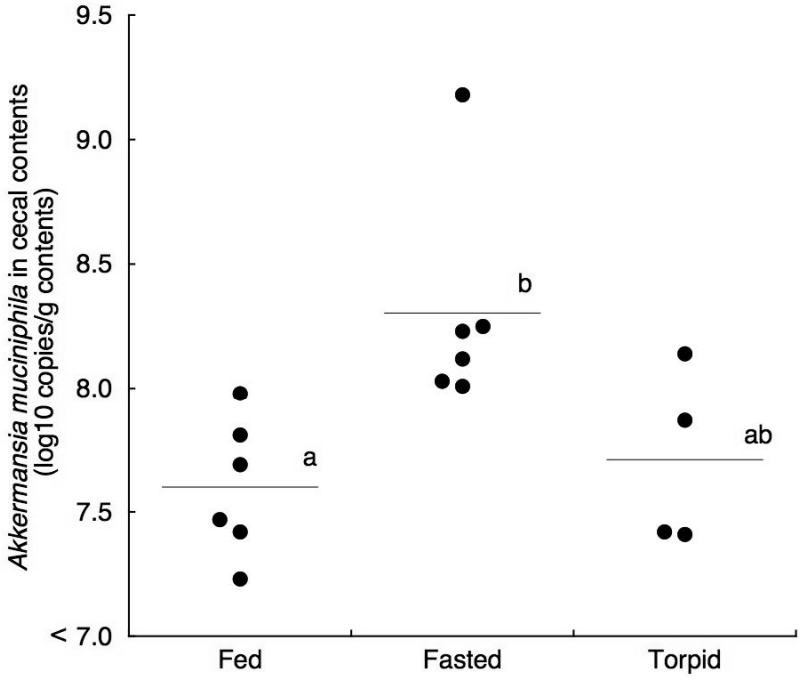
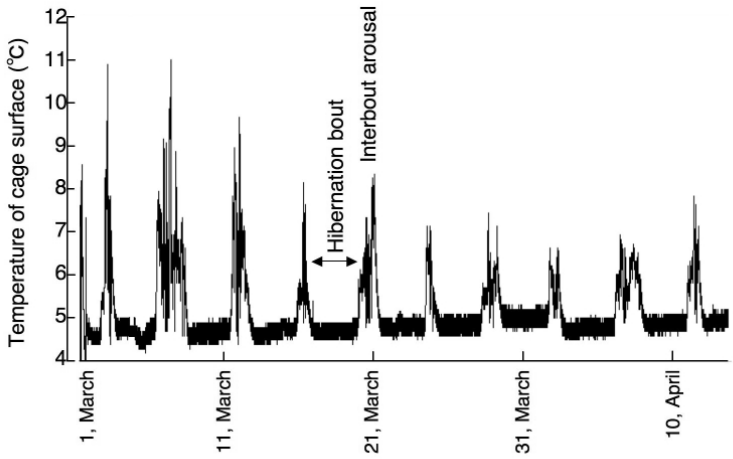

9.5
9.0
8.5
8.0
7.5
< 7.0
Akkermansia muciniphila in cecal contents
(log10 copies/g contents)
a
b
ab
Fed
Fasted
Torpid



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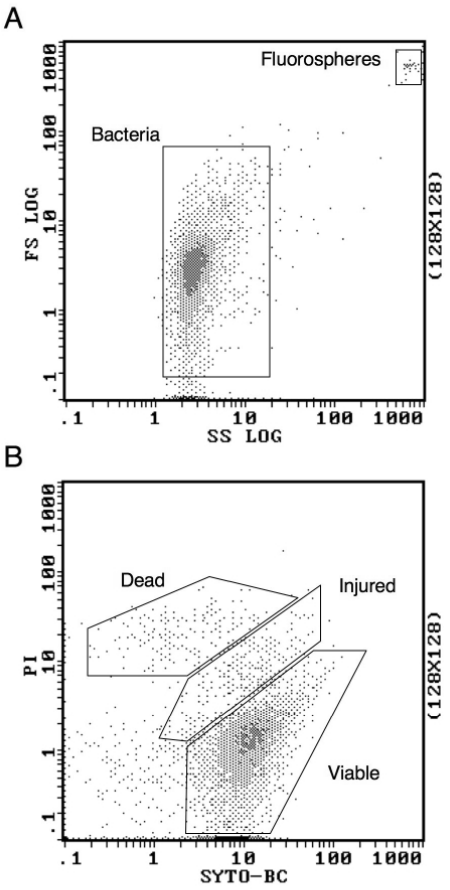

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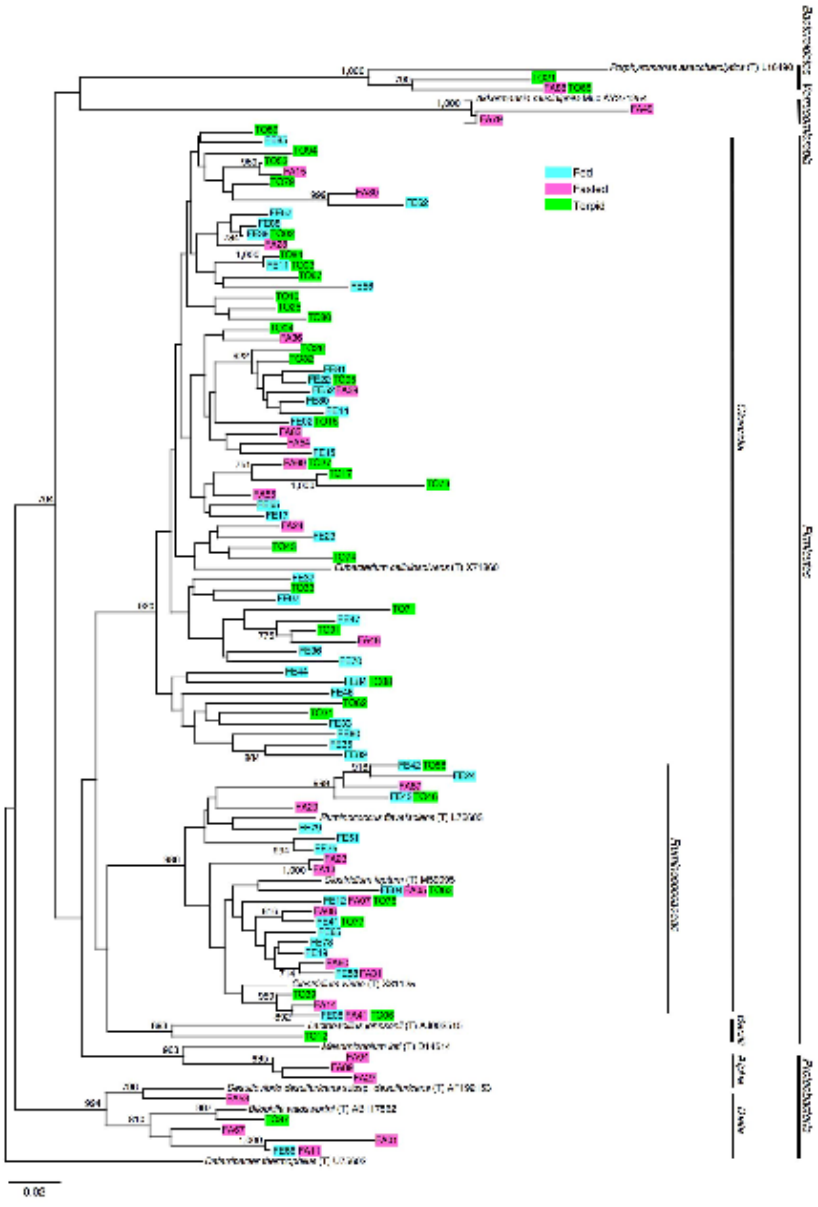

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