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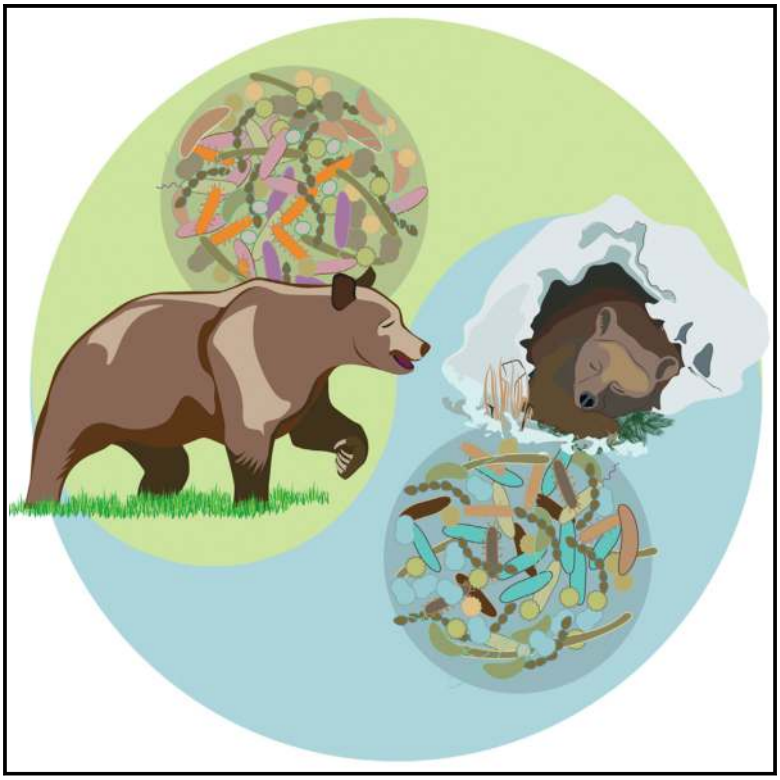
The Gut Microbiota Modulates Energy Metabolism in the Hibernating Brown Bear *Ursus arctos*

Graphical Abstract



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In Brief

Sommer et al. show that the microbiota and serum metabolites in brown bears differ seasonally between hibernation and active phase. Colonization of mice with a bear microbiota promoted increased adiposity. These findings suggest that seasonal microbiota variation may contribute to metabolism of the hibernating brown bear.

Highlights

- Bear microbiota composition differs seasonally between hibernation and active phase
- Blood metabolites differ seasonally in the brown bear
- The bear gut microbiota promote energy storage during summer

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Article

The Gut Microbiota Modulates Energy Metabolism in the Hibernating Brown Bear *Ursus arctos*

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SUMMARY

Hibernation is an adaptation that helps many animals to conserve energy during food shortage in winter. Brown bears double their fat depots during summer and use these stored lipids during hibernation. Although bears seasonally become obese, they remain metabolically healthy. We analyzed the microbiota of free-ranging brown bears during their active phase and hibernation. Compared to the active phase, hibernation microbiota had reduced diversity, reduced levels of Firmicutes and Actinobacteria, and increased levels of Bacteroidetes. Several metabolites involved in lipid metabolism, including triglycerides, cholesterol, and bile acids, were also affected by hibernation. Transplantation of the bear microbiota from summer and winter to germ-free mice transferred some of the seasonal metabolic features and demonstrated that the summer microbiota promoted adiposity without impairing glucose tolerance, suggesting that seasonal variation in the microbiota may contribute to host energy metabolism in the hibernating brown bear.

INTRODUCTION

Free-ranging brown bears (*Ursus arctos*) undergo cycles of intense eating and weight gain during the summer followed by prolonged dormant hypometabolic fasting for up to 6 months during the winter (Evans et al., 2012; Tøien et al., 2011). Despite the large fat accumulation before hibernation, bears remain metabolically healthy (Arinell et al., 2012; Nelson, 1973; Stenvinkel et al., 2013), which contrasts with the strong association between obesity and insulin resistance in humans. Thus, the brown bear may constitute a model for healthy obesity and studying hibernation might be a promising approach to develop novel therapies for obesity. The intestines of mammals harbor diverse microbial ecosystems that have profound effects on host physiology (Sommer and Bäckhed, 2013). The gut microbiota contributes to energy harvest from the diet (Bäckhed et al., 2004, 2007; Sommer and Bäckhed, 2013; Sommer et al., 2015) and is altered in obesity and type 2 diabetes (Khan et al., 2014). Furthermore, diet, which is seasonably variable in bears (Persson et al., 2001; Stenvinkel et al., 2013; Stofik et al., 2013), strongly affects the gut microbiota (Ley et al., 2008; Zoetendal and de Vos, 2014) and both fasting and hibernation alter the gut microbiota composition (Carey et al., 2013; Crawford et al., 2009; Dill-McFarland et al., 2014; Sonoyama et al., 2009).

Here, we investigated how hibernation in free-ranging brown bears affects the gut microbiota and plasma metabolites, and whether a seasonally altered microbiota contributes to the healthy obesity phenotype during summer. We used 16S rRNA profiling and next-generation sequencing to comprehensively analyze the fecal microbiota of free-ranging brown bears captured during hibernation (February) and during the active period (June) of the same year (Figure 1A). We showed that the winter microbiota comprised fewer bacterial taxa (Figure S1A) and was more homogenous than the summer microbiota (Figure S1B), which may reflect the varied diet among bears during the summer.

RESULTS AND DISCUSSION

Principal coordinate analysis of the overall composition of the bear fecal microbiota samples using unweighted UniFrac revealed a clear separation depending on the seasonal origin (Figures 1B and S2). We identified 24 bacterial phyla in the bear fecal microbiota (Table S1). The dominating bacterial phyla in the summer microbiota were Proteobacteria, Firmicutes, and Actinobacteria (Figure 1C). In the winter microbiota, Bacteroidetes increased in abundance, whereas Firmicutes and Actinobacteria were less abundant (Figure 1D). A number of low abundant phyla were only present in the summer microbiota (Figure S3A). At the species level, 199 of the 4,447 detected operational taxonomic units (OTUs) were significantly altered ($q < 0.05$; q = FDR

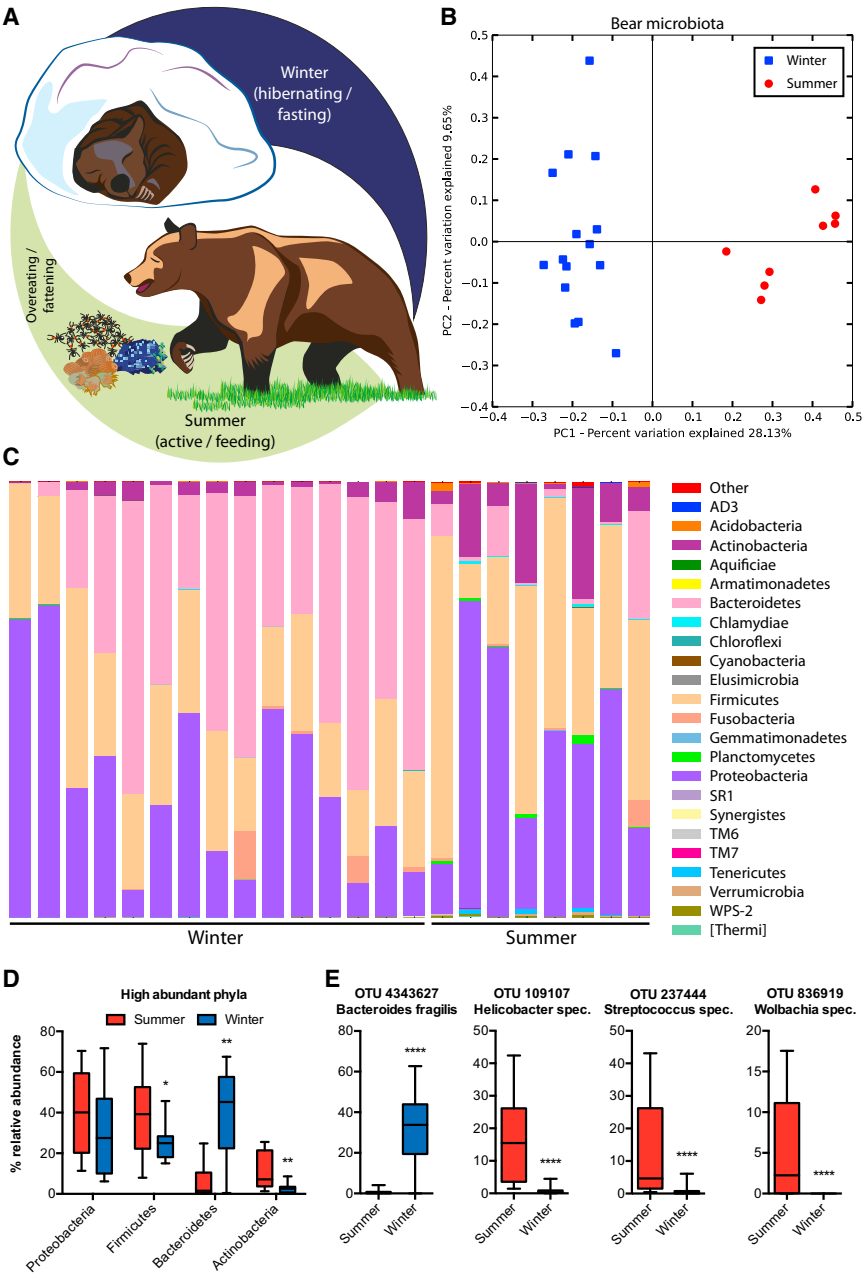

Figure 1. Seasonal Differences in the Bear Fecal Microbiota

(A) Seasonal cycle of the brown bear.

(B) Principal coordinate analysis of the bear fecal microbiota from summer and winter.

(C) Bacterial taxonomic representation in bear microbiota in summer and winter on phylum level.

(D) Significantly altered bacterial phyla between summer and winter in bear microbiota.

(E) Relative abundance (%) of high-abundant and season-dependent OTUs of the bear fecal microbiota.

Data are mean ± SEM n = 8 for summer and n = 15 for winter. *q < 0.05; **q < 0.01; ****q < 0.0001.

corrected p value) between winter and summer (Table S1). The most significant among these were OTU 4343627 (*Bacteroides fragilis*), which was enriched in the winter, and OTUs 109107 (*Helicobacter spec.*), 237444 (*Streptococcus spec.*), and 836919 (*Wolbachia spec.*), which were reduced in winter (Figure 1E). *Wolbachia* are symbionts of several insects (Hedges et al., 2008; Teixeira et al., 2008), and the increased abundance during summer presumably results from the intake of insects as part of the bear summer diet (Große et al., 2003). Furthermore, the winter microbiota had higher levels of several Enterobacteriaceae and lower levels of several Rhizobiales and Actinomycetales species (Figures S3B–S3D).

An enrichment of Bacteroidetes and lower relative abundance of Firmicutes has previously been observed in the microbiota of hibernating animals (Carey et al., 2013; Dill-McFarland et al., 2014; Sonoyama et al., 2009; Stevenson et al., 2014). The increase in Bacteroidetes may be explained by their capacity to switch their metabolism toward degradation of host glycans in the absence of dietary polysaccharides (Sonnenburg et al., 2005) or their capacity to metabolize protein and fat (Wu et al., 2011) putatively provided by the intestinal epithelium. In contrast, most Firmicutes taxa require dietary fiber. These changes in the microbiota phyla were accompanied by a loss of weight and body fat in the hibernating bear. Similar trends have been reported in studies comparing obese and lean subjects (Ley et al., 2005) or using calorie restriction in humans and mice (Crawford et al., 2009; Furet et al., 2010; Ley et al., 2006; Turnbaugh et al., 2009). Furthermore, two studies of calorie-restricted mice reported an increase in *Bacteroides fragilis* (Santacruz et al., 2009) but reduced Streptococcaceae and TM7 (Zhang et al., 2013). *Bacteroides fragilis* was the predominant bacterium in the microbiota from the hibernating bears, whereas both *Streptococcus* and TM7 were reduced during hibernation. Together these data indicate that many of the changes in the bear microbiota are associated with caloric restriction. In contrast to small hibernators, Verrucomicrobia including *Akkermansia muciniphila* were not increased during hibernation in free-ranging brown bears.

To identify metabolites that varied according to the activity status of the bears, we used targeted metabolomics to analyze blood samples taken from the jugular vein during winter and summer. Supporting previous publications (LeBlanc et al., 2001; Otis et al., 2011), we found that serum levels of cholesteryl esters, triglycerides, and free cholesterol were significantly

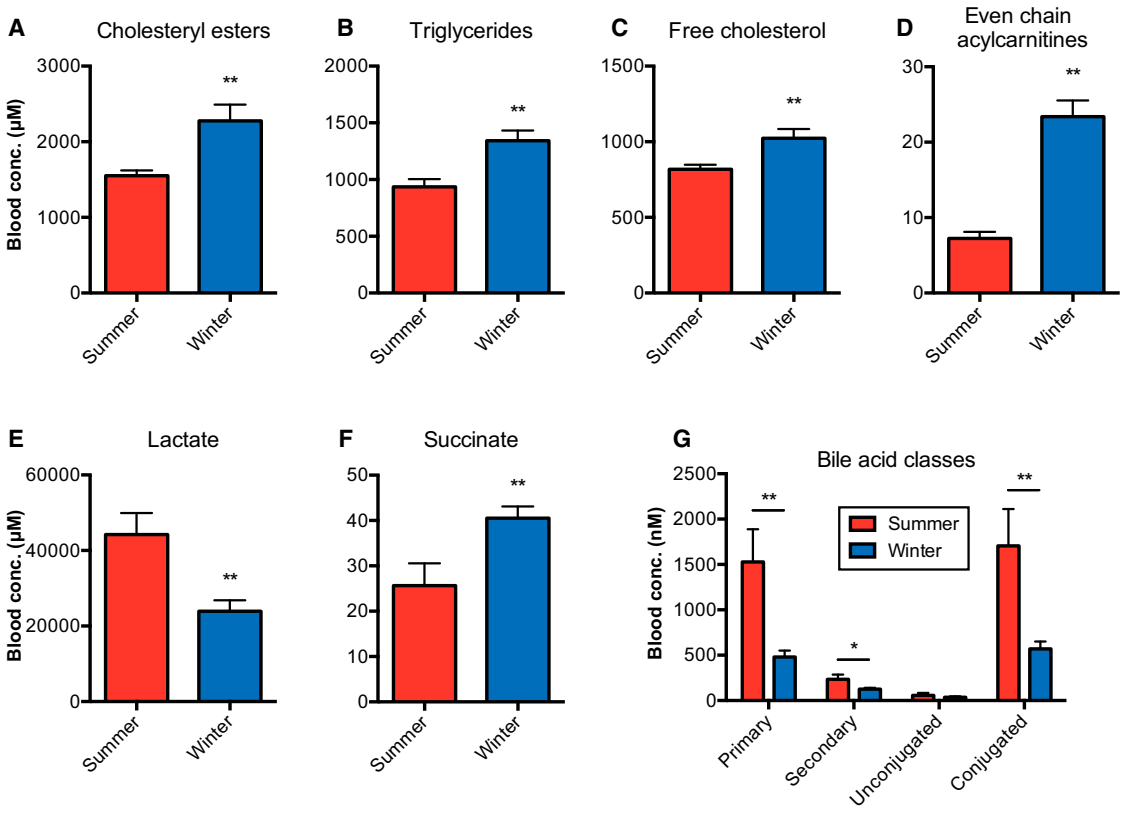

Figure 2. Seasonal Differences in Metabolites in Bear Blood

(A–G) Concentrations of the lipid classes (A) cholesteryl esters, (B) triglycerides, and (C) free cholesterol and of (D) even chain acylcarnitines, markers of FAO, and of the organic acids (E) lactate and (F) succinate, and of (G) bile acid classes in bear blood from summer and winter. Data are presented as mean ± SEM n = 12 for summer and n = 15 for winter. *q < 0.05; **q < 0.01.

higher in winter (Figures 2A–2C; Table S2). This finding is consistent with the fact that energy from fat stores is obtained by lipolysis during hibernation (Arinell et al., 2012; Nelson, 1973). Even chain acylcarnitines, which largely represent intermediates of mitochondrial fatty acid oxidation (FAO), were also higher in winter (Figure 2D), in agreement with high FAO activity during hibernation. In contrast, C3 and C5 acylcarnitines, markers of amino acid oxidation, were decreased during hibernation (Table S2). Finally lactate (Figure 2E) and the levels of several gluconeogenic amino acids (e.g., alanine, methionine, tyrosine) were reduced (Table S2) during winter compared to summer, whereas succinate was increased, which could indicate reduced glucose utilization and increased gluconeogenesis. Taken together, these data suggest that during hibernation bears mobilize and oxidize lipids as survival strategy, accompanied by reduced glucose utilization and increased utilization of amino acids for gluconeogenesis. The decreased lactate levels were also consistent with decreased abundance of lactate-producing bacteria during hibernation; e.g., the Firmicutes *Bacillus* or *Lactobacillus* and the Actinobacteria *Micrococcus* (Reddy et al., 2008). Similarly, the increased succinate levels during hibernation correlate with increased abundance of succinate-producing bacteria such as *Enterococcus* (Song and Lee, 2005) in the winter microbiota. However, the host can also produce lactate and succinate, and thus seasonal changes in host metabolism might also contribute to the differences in lactate and succinate.

We also observed that total bile acid levels in the serum were lower in the winter with large reductions in primary and conjugated bile acids (Figure 2G; Table S2). Notably, expression of the rate-limiting enzyme of bile acid production CYP7A1 is reduced in the liver of hibernating mammals (Fedorov et al., 2011; Otis et al., 2011), and the microbiota contributes to modifications of bile acids (Sayin et al., 2013). Bile acids promote lipid uptake and respond to food intake. Bears do not eat for up to 6 months during hibernation, which likely explains the reduced bile acid levels in the winter. Levels of deoxycholic acid and lithocholic acid, both of which are dependent on the microbiota, are known to have hemolytic activity (Oelberg et al., 1984; Schölmerich et al., 1984) and were reduced during hibernation (Table S2). Notably, several blood parameters that are linked to hemolysis and dehydration were also altered during hibernation (Table 1). For example, levels of red blood cells and hemoglobin were higher in the winter, whereas lactate dehydrogenase (marker of hemolysis) and bilirubin (used during recycling of hemoglobin) levels decreased during hibernation. Thus, microbiota-dependent changes in the bile acid profile might contribute to the reduced hemolysis during hibernation.

To test whether the seasonal differences in the bear microbiota affect host physiology, we colonized germ-free mice with a

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Table 1. Signs of Dehydration and Reduced Hemolysis in Blood of Hibernating Brown Bears

Parameter	Unit	Summer Mean (Range)	Winter Mean (Range)	Ratio W/S	p Value
Total bile acids	nM	1,762 (137–4,379)	606 (171–1,177)	0.3	<0.01
White blood cells	10^9/l	7.7 (3.4–15.7)	6.2 (3.8–15.6)	0.8	ns
Red blood cells	10^{12}/l	6.6 (6.2–7)	8.6 (7.7–9.4)	1.3	<0.001
Hemoglobin	g/l	161 (132–176)	203 (183–223)	1.3	<0.001
Hematocrit	%	42.6 (36.6–46.1)	54.2 (48–60)	1.3	<0.001
Platelets	10^9/l	310 (251–359)	184 (65–265)	0.6	<0.001
Neutrophils	10^9/l	3.3 (2.4–4.4)	3.5 (2.1–4.4)	1.1	ns
Lymphocytes	10^9/l	1.2 (0.8–1.9)	1.5 (0.9–2.7)	1.2	ns
Monocytes	10^9/l	0.3 (0.2–0.5)	0.4 (0.3–0.7)	1.3	ns
Eosinophils	10^9/l	0.003 (0–0.01)	0 (0–0)	0.0	<0.05
Basophils	10^9/l	0.01 (0–0.04)	0.001 (0–0.01)	0.2	ns
Alkaline phosphatase	U/l	134 (100–174)	19.1 (13–27)	0.1	<0.001
Alanine transaminase	U/l	36 (23–60)	11.4 (9–14)	0.3	<0.001
Aspartate transaminase	U/l	90 (57–148)	53.1 (39–85)	0.6	<0.05
Bilirubin	μM	18 (9.9–30.9)	10.6 (5–23)	0.6	0.08
Lactate dehydrogenase	μkat/l	13.3 (13.3–13.3)	9.1 (7.2–11.2)	0.7	<0.001
Gamma glutamyltransferase	μkat/l	0.5 (0.3–0.7)	0.3 (0.2–0.5)	0.5	<0.001
C-reactive protein	mg/l	0.003 (0–0.01)	0.014 (0–0.04)	5.1	ns

Hematology analysis was performed on blood samples from brown bears during summer and winter, and marker enzymes were measured. Data show mean and range with n = 11–15 for summer and n = 7 for winter. ns, nonsignificant.

summer or winter bear microbiota (Figure 3A). 16S rRNA profiling of the colonized mice confirmed successful colonization (Figures 3B and S4). There was no seasonal difference in alpha diversity, possibly because all mice received the same food. Mice colonized with a summer bear microbiota trended toward a greater weight ($p = 0.09$) and showed a greater fat gain than mice colonized with a winter bear microbiota (Figures 3C and 3D) but did not display a significant difference in epididymal white adipose tissue weight (Figure 3E). In humans, adiposity is associated with reduced insulin sensitivity (Shulman, 2014). In contrast, brown bears seem to become only temporarily insulin resistant with mild hyperglycemia during hibernation but remain insulin sensitive during the rest of the seasonal cycle independent of fat accumulation (L. Nelson, personal communication; Stenvinkel et al., 2013). The increased weight and adiposity of the mice colonized with a summer bear microbiota were not due to higher bacterial abundance as tested by 16S rDNA qPCR (summer $2.27 \pm 1.16 \times 10^{11}$ and winter $2.55 \pm 1.03 \times 10^{11}$ 16S rDNA copies/g cecal content, $p = 0.6$). Despite their increased fat mass, mice colonized with summer bear microbiota showed no differences or even a slight improvement in glucose metabolism compared to mice colonized with a winter microbiota (Figure 3F). By performing targeted metabolomics, we showed that the seasonal metabolic phenotype of the bears could be partially transferred to germ-free mice by colonization with a bear microbiota. For example, mice colonized with a winter bear microbiota trended toward slightly higher serum levels of cholesteryl esters (Table S2) and triglycerides (Figure 3G) compared with mice that were colonized with summer microbiota.

Conclusions

In conclusion, our data show that the seasonal lifestyle of the brown bear with phases of severe hyperphagia and fat accumulation in the summer and prolonged fasting and inactivity during hibernation is accompanied by seasonal changes in metabolism and microbiota. Furthermore, colonization with the seasonal bear microbiota was sufficient to transfer some of the seasonal metabolic features to germ-free mice. Together this might indicate that the seasonal differences in the bear microbiota contribute to the seasonal metabolic changes, presumably due to the different physiologic demands of phases of severe hyperphagia and hibernation with prolonged fasting. Thus, the microbiota may be linked to the healthy obesity phenotype in brown bears and as such not only yields insights into the physiology of hibernating mammals, but also further supports targeting the microbiota as potential treatment of obesity. However, studying free-ranging animals also limits our information regarding, e.g., seasonal food intake and the experimental procedures that can be performed. Although informative, our findings from the colonization of germ-free mice cannot adequately reflect the physiologic state in a hibernating bear. Thus, further mechanistic studies using, e.g., bears in captivity in which calorie content, food consumption, microbiota composition, and the animal's physiology can be controlled, are required to functionally validate and elucidate which components of the microbiota contribute to the seasonal metabolic differences and the involved molecular pathways.

EXPERIMENTAL PROCEDURES

Blood and fecal samples were taken from 16 free-ranging Eurasian brown bears (*Ursus arctos*) during hibernation (February or March) and during the active period (June) of the same year.

Germ-free mice were colonized with a winter or summer bear fecal microbiota by oral gavage. Body composition was analyzed before and 14 days after colonization by MRI (EchoMRI) according to the manufacturer's instructions. Intraperitoneal glucose tolerance test was performed on day 15 post-colonization.

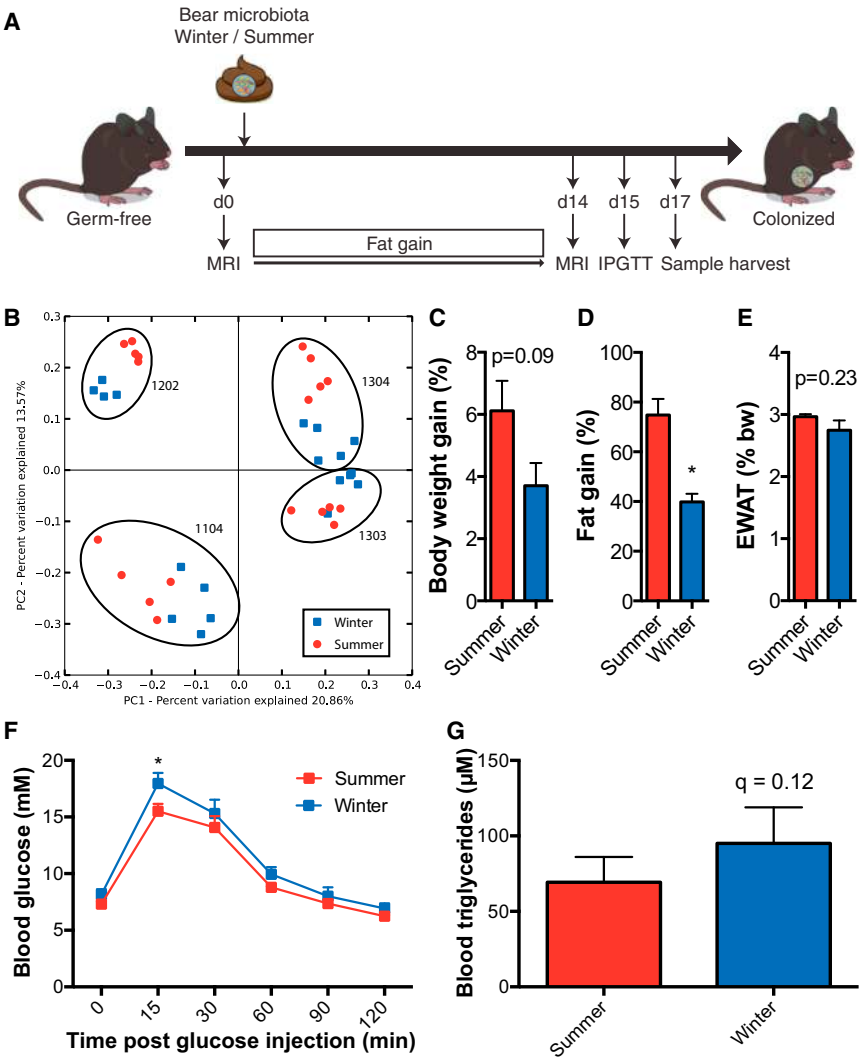

Figure 3. Metabolic Programming by the Seasonal Bear Microbiota

(A) Experimental scheme. Germ-free mice were colonized with a bear summer or winter microbiota and followed for 2 weeks.

(B) Principal coordinate analysis of the cecal microbiota of mice colonized with a bear fecal microbiota from summer or winter. 1104, 1202, 1303, and 1304 denote bear fecal donors.

(C–E) Weight gain (C), body-fat gain (D), and epididymal white adipose tissue (EWAT) (D) weight were determined.

(F) Glucose metabolism was assessed via intraperitoneal glucose tolerance test (IPGTT).

(G) Concentrations of triglycerides in blood of mice colonized with seasonal bear microbiota. Data are mean ± SEM of four experiments (n = 4) with each five animals per colonization. *p < 0.05.

DNA was isolated from bear feces and ceca of colonized mice and 16S rRNA profiling performed as described previously (Sommer et al., 2014) by MiSeq sequencing.

Metabolites were analyzed by mass spectrometry as described.

Data were analyzed by Student's t test, and the statistical p values were further corrected for multiple testing using Benjamini Hochberg method in R program. Data are presented as mean ± SEM.

For detailed description of all experimental procedures, see the Supplemental Information.

ACCESSION NUMBERS

The Miseq sequences derived from the 16S profiling have been deposited to the European Nucleotide Archive and are available under accession numbers ERS1023032–ERS1023094.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.026.

AUTHOR CONTRIBUTIONS

F.S. and F.B. conceived and designed the experiments; F.S., M.S., and O.I. performed the experiments: F.S., M.S., O.I., and F.B. analyzed the data; J.M.A., J.K., and J.J. contributed reagents/materials/analysis tools; F.S. and F.B. wrote the paper; and F.S., M.S., O.I., J.M.A., J.K., J.J., C.B.N., O.F., and F.B. commented on the manuscript.

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