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Diet-induced obesity, energy metabolism and gut microbiota in C57BL/6J mice fed Western diets based on lean seafood or lean meat mixtures

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Abstract

High protein diets may protect against diet-induced obesity, but little is known regarding the effects of different protein sources consumed at standard levels. We investigated how a mixture of lean seafood or lean meat in a Western background diet modulated diet-induced obesity, energy metabolism and gut microbiota. Male C57BL/6 J mice fed a Western diet (WD) containing a mixture of lean seafood (seafood WD) for 12 weeks accumulated less fat mass than mice fed a WD containing a mixture of lean meat (meat WD). Meat WD-fed mice exhibited increased fasting blood glucose, impaired glucose clearance, elevated fasting plasma insulin and increased plasma and liver lipid levels. We observed no first choice preference for either of the WDs, but over time, mice fed the seafood WD consumed less energy than mice fed the meat WD. Mice fed the seafood WD exhibited higher spontaneous locomotor activity and a lower respiratory exchange ratio (RER) than mice fed the meat WD. Thus, higher activity together with the decreased energy intake contributed to the different phenotypes observed in mice fed the seafood WD compared to mice fed the meat WD. Comparison of the gut microbiomes of mice fed the two WDs revealed significant differences in the relative abundance of operational taxonomic units (OTUs) belonging to the orders *Bacteroidales* and *Clostridiales*, with genes involved in metabolism of aromatic amino acids exhibiting higher relative abundance in the microbiomes of mice fed the seafood WD.

Keywords: Dietary protein; Obesity; Seafood; Meat; Western diet; Microbiota; Protein source

1. Background

Human and animal studies have shown that increasing the amount of protein at the expense of carbohydrate and/or fat is an effective strategy for weight reduction, short-term weight maintenance and protection against diet-induced obesity [1–3]. Furthermore, protein intake has been reported to augment diet-induced thermogenesis [4–8]. Still, systematic studies investigating how different protein sources modulate energy metabolism are limited in number.

Dietary whey protein, relative to casein, reduces diet-induced obesity in high fat-fed mice [9,10] and induces a higher postprandial thermic effect in humans after ingestion of test meals [11]. Inclusion of a fish meal three times a week combined with a caloric-restricted diet was associated with greater weight loss compared to control diets without fish in young adults [12]. Further, it was shown that exchange of the dietary protein source from casein to fish protein hydrolysates prevented adiposity in rats [13,14]. Similarly, a Western diet containing hydrolyzed compared to intact casein was able to reduce diet-induced obesity in mice [15,16], indicating that not only the source but also processing of the protein may modulate whole body energy metabolism.

The gut microbiota has within recent years been established as an important factor modulating energy harvest from the food and obesity

Abbreviations: BCAA, Branched-chain amino acids; DAUC, Decremental area under the curve; DAA, Dispensable amino acids; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; EE, Energy expenditure; eWAT, Epididymal white adipose tissue; ENA, *European* Nucleotide Archive; FFA, Free fatty acids; Gly, Glycerol; HDL, High-density lipoprotein; HUMAnN, HMP Unified Metabolic Analysis Network; OH-but, Hydroxybutyrate; IL-6, Interleukin-6; IAUC, Incremental area under the curve; IAA, Indispensable amino acids; iWAT, Inguinal white adipose tissue; ITT, Insulin tolerance test; LA, Linoleic acid; ALA, α-linoleic acid; LDL, Low-density lipoprotein; LF, Low fat; MUFA, Monounsaturated fatty acids; NMDS, Nonmetric multidimensional scaling; OGTT, Oral glucose tolerance test; OTU, Operational taxonomic unit; PRCF, Percent relative cumulative frequency; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; RER, Respiratory exchange ratio; rWAT, Retroperitoneal white adipose tissue; revised QUICKI, Revised quantitative insulin sensitivity check index; SFA, Saturated fatty acids; TC, Total cholesterol; TG, Triglycerides; WD, Western diet.

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development [17–19]. The microbiota of lean or obese subjects generally clusters separately, and a few studies using fecal transplantation or transfer of single bacteria have pointed to a causal relationship between the gut microbiota and obesity development [20,17,19]. In addition, diet interventions have shown that changes in the macronutrient composition of the diet are associated with changes in the composition of the gut microbiota [21,22] and that these changes can be observed already after 1 day [20]. The effect of different protein sources on the gut microbiota composition is not fully elucidated, but one study has demonstrated that high-protein diet-induced weight loss in humans was accompanied by changes in the gut microbiota associated with a potentially detrimental metabolic profile in the gut [23]. Furthermore, feeding rats meat, dairy or plant proteins altered the cecal microbiota composition [24].

To date, only few studies have investigated if lean seafood and lean meat, when consumed as the protein source in a Western diet, could affect obesity development and associated metabolic changes.

Based on previous results, we hypothesized that a Western diet with lean seafood as the protein source would lead to less weight gain compared to a diet with lean meat as the protein source. Further, we hypothesized that the differences in protein source could drive changes in the composition of the gut microbiota, which potentially could affect the host's metabolism. Hence, we here investigated how a mixture of lean seafood and a mixture of lean meat modulated energy metabolism and thereby obesity development using diets with a macronutrient composition that reflects human consumption in Western societies.

2. Methods

2.1. Ethical statement

The animal experiments were approved by the National Animal Health Authority (Norwegian approval identification 4057 and 5358, and Danish approval identification 2014–15-2934-0127). Adverse events were not observed.

2.2. Experimental diets

The seafood and meat diets were based on a Western diet (5TJN, Western diet for rodents, TestDiet; energy percent: 16% protein, 40% fat, 44% carbohydrates) and made by completely exchanging casein with either lean seafood powder (seafood WD) or lean meat powder (meat WD). The lean seafood powder consisted of equal amounts (1/5 of each species by dry weight) of cooked and freeze-dried skinless filets from ling, rosefish, cod, wolf fish and muscle from Canadian scallop. The meat powder consisted of equal amounts (1/3 of each species by dry weight) of cooked and freeze-dried skinless chicken breast, pork tenderloin and beef sirloin. We aimed to compare the mixture of lean seafood with the mixture of lean meat as food items without any chemical modifications such as removal of endogenous fat. Therefore, we chose to compensate for the slightly higher endogenous fat content in the lean seafood mixture by reducing the amount of lard added to the seafood WD. One group of mice was fed a casein-based LF reference diet (5TJS, TestDiet; energy percent: 16% protein, 12% fat, 72% carbohydrates). The diets were provided in pellet form, prepared by ssniff Spezialdiäten GmbH (Soest, Germany). The seafood WD and meat WD were equal in gross energy content and comparable in sum amino acids (3% lower in meat WD) and sum fatty acids (8% higher in meat WD). The composition of the diets is shown in Tables S1, S2 and S3.

2.3. Animals and housing

In all studies, male C57BL/6 J Bom Tac mice 7 weeks of age were obtained from Taconic Europe (Ejby, Denmark). The mice were housed individually and kept on a 12/12-h light/dark cycle at thermoneutrality, 28 ± 2 °C. Before starting the experiments, the mice were given the LF diet for 1 week to acclimate. After the acclimatization period, the mice were divided into experimental groups in a manner to equalize group means of lean, fat and body mass.

2.4. Twelve weeks feeding study

Fresh water and feed were provided three times per week, and body mass was recorded once per week. The feeding trial lasted for 12 weeks and consisted of 30 mice (n = 10/group). The mice were given free access to the diets, and feed leftovers were collected and weighted. Data on energy intake, body composition and body mass are based on the first 9 weeks of the study, prior to the oral glucose tolerance (week 10) and insulin tolerance (week 11) testing. Fat mass and lean mass were determined before study start and in weeks 5 and 9 using quantitative magnetic resonance (Minispec mq 7.5, NMR analyzer, Bruker, Germany). In week 12, feed-deprived mice (6 h) were anesthetized with isoflurane (Isoba-vet, Schering-Plow, Denmark) and euthanized by cardiac puncture. Liver, kidneys, heart, cecum content and epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT) and retroperitoneal white adipose tissue (rWAT) were quickly dissected out, weighed, snap frozen and stored at −80 °C. Whole blood drawn from the heart by cardiac puncture was collected in EDTA tubes and immediately centrifuged at 4 °C at 2500 g for 5 min. EDTA plasma was separated and stored at −80 °C.

2.5. Analyses of diet compositions

Gross energy in the diets was measured using a bomb calorimeter following the manufacturer's instructions (Parr calorimeter 6300, Parr Instruments, USA). Total fat, fatty acid and amino acid composition were analyzed using the protocol described in detail in Tastesen *et al.* (2014) [25].

Total fat content was determined gravimetrically after organic extraction. Fatty acids were quantified using a gas chromatograph (GLC Trace GC 2000, Thermo Scientific, USA) and detected with a flame ionization detector (Thermo Scientific, USA). Amino acids were separated and detected on the ACQUITY UPLC System (Waters, USA).

2.6. Glucose and insulin tolerance tests

In week 10 and 11 an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT), respectively, were performed on conscious mice feed-deprived for 6 h. For the OGTT, mice were given 3 mg glucose/g lean mass. Blood samples were collected from tail vein before (0) and at 15, 30, 60 and 120 min after glucose administration and analyzed with a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway). For the ITT, mice were given 0.75 units insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) per kg lean mass, intraperitoneally. Blood samples were collected from the tail vein before (0) and at 15 and 30 min after insulin injection and analyzed with a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway). Incremental area under the curve (IAUC) for the OGTT and decremental area under the curve (DAUC) for the ITT were calculated using the following formulas: IAUC = AUC-(basal glucose*120 min). DAUC = (basal glucose*30 min)-AUC.

2.7. Plasma analyses

MaxMat PL II analyzer (MAXMAT S.A., Montpellier, France) and conventional kits were used to measure plasma levels of HDL-cholesterol, glycerol, FFA (Dialab, Austria), LDL-cholesterol, triglyceride, total cholesterol (MaxMat, France) and D-3 Hydroxybutyrate (Randox, United Kingdom) from 6 h feed-deprived mice. Insulin mouse ELISA kit (EIA-3439) (DRG Diagnostics GmbH) and mouse IL-6 ELISA kit (KMC0061) (Invitrogen, US) were used according to producers' manuals to quantify plasma insulin and IL-6 levels. Revised quantitative insulin sensitivity check index (revised QUICKI) was calculated for all animals using the formula: 1/(log(fasting glucose as mg/dl) + log (fasting insulin as μU/ml) + log (fasting plasma FFA as mM)) [26].

2.8. Liver triglycerides (TG) determination

Lipids were extracted from liver samples with chloroform : methanol, 2:1 (v/v). Lipid classes were analyzed using an automated high performance thin layer chromatography (HPTLC) system (Camaq, Switzerland) and separated on HPTLC plates coated with silica gel 60 F [27].

2.9. 16S ribosomal-RNA gene-based sequencing

Cecal content was collected right after the mice were euthanized and the samples were immediately frozen in liquid nitrogen. Bacterial DNA extraction (NucleoSpin soil kit, Macherey-Nagel), PCR-based library formation and sequencing (Illumina MiSeq) were performed as described previously [28].

2.10. Bioinformatics

PCR-based library formation, sequencing (Illumina MiSeq) and taxonomy assignment were performed as described in Holm *et al.* [28]. Subsequent analyses were performed in R v3.1.2 using the metagenomeSeq [29], PhyloSeq [30], Vegan [31] and GGplot2 [32] packages. Data were filtered for low-abundance operational taxonomic units (OTUs) by removal of OTUs present in fewer than 3 of the 30 samples and with a relative abundance across all samples ≤0.005%. Analyses in R were performed with an average of 18,413 ± 6866 (SD) sequences per sample after filtering. Alpha diversity was estimated using unfiltered data. Beta diversity was calculated for each group using Vegan. Read counts were normalized with metagenomeSeq [29] that uses a cumulative sum scaling in which raw counts are divided by the cumulative sum of counts up to a particular quantile. Statistical analyses using metagenomeSeq were performed based on effective sample sizes: taxa/OTUs were not included if they had fewer than X effective number of positive samples, where X is the median of estimated effective samples per feature calculated using metagenomeSeq. For PICRUSt [33] analysis, the data were preprocessed as recommended on the PICRUSt Website using the pick_closed_reference_otus.py QIIME script [34] against the Greengenes database

Version 13.5 [35]. Using PICRUSt, the OTU table was normalized by 16S rRNA gene copy number and the functional metagenome was predicted using default settings. HUMAnN2 [36] was subsequently used to calculate the relative abundance of KEGG [37] modules. The data were normalized to sequencing depth, and modules exhibiting differences in abundance were identified using LefSe Version 1.0 [38] with an alpha value on 0.05 and an LDA score threshold on 2.0.

All sequence data are available from the European Nucleotide Archive (ENA) with study accession number: PRJEB9856.

2.11. Indirect calorimetry and determination of spontaneous locomotor activity

A separate set of 15 mice was used for the assessment of energy metabolism in young mice at the transition from LF to the two Western diets before the onset of obesity. Based on body mass and body composition data (EchoMRI quantitative magnetic resonance whole-body composition analyzer Echo Medical Systems), mice were divided into either the seafood group ($n=8$) or the meat group ($n=7$). Prior to measurements in the CaloCages, mice were placed in training cages for 3 days with free access to the LF diet. Following the acclimatization period, the mice were transferred to CaloCages fitted with infrared light-beam frames (ActiMot2, TSE Systems, Bad Homburg, Germany), where O_2 consumption, CO_2 production and spontaneous locomotor activity were measured using the PhenoMaster open-circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany). In total, the mice were kept in the CaloCages for 6 days. For the first 3 days the mice had free access to an LF diet, on day 4 the animals were given free access to the experimental diets for another 3 days. Based on the two last consecutive light (06.00–17.30 h) and dark (18.00–05.30 h) phases on either LF or WD, the respiratory exchange ratio (RER) was calculated from VO_2 and VCO_2, and *spontaneous locomotor activity* was defined as total counts of beam breaks. Energy expenditure (EE) was calculated using the following equation: 3.9 cal/L × L VO_2 + 1.1 cal/L × L VCO_2 [39]. The changes in RER and EE between the last 48 h on LF and the last 48 h on WD were analyzed by generating percent relative cumulative frequency (PRCF) curves as described by Riachi *et al.* [40]. As no significant difference in body composition (fat/lean ratio) was observed between the mice used for calorimetry, we chose to normalize VO_2, VCO_2 and EE to body weight.

2.12. Meal response test

After 1 week on the LF diet, a separate set of 26 mice was divided into a seafood WD or a meat WD group ($n=13$/diet) based on their body mass (24.4 ± 0.4 and 24.7 ± 0.3 g) and composition (10.29 ± 0.01 and 10.32 ± 0.01 fat%). Prior to the meal response test, mice were feed-deprived for 16 h, and a blood sample for basal measurements of glucose and C-peptide was obtained (time point − 10 min). For the meal response test, the feed-deprived mice consumed 0.15 g of the seafood WD or meat WD within 10 min. Thereafter (time point 0), blood was collected at 0, 10, 20 and 30 min for determination of plasma C-peptide and blood glucose using a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway). The blood for plasma C-peptide analyses was collected using EDTA-coated micro tubes (Minivette® POCT, SARSTEDT AG & Co, Nümbrecht, Germany) before transferring to tubes containing a mixture of proteases, esterases and DPP-IV inhibitors optimized for blood (BD P800, Puls, Oslo, Norway). After plasma separation, samples were stored at − 80 °C. Plasma C-peptide was determined using a commercial ELISA kit in accordance with the manufacturer's instructions (Mouse C-Peptide ELISA, Crystal Chem, IL, USA).

2.13. Diet preference test

After 1 week on the LF diet, 40 mice were feed-deprived for 16 h and subjected to a diet preference test. During the test, mice in individual cages were simultaneously offered both the seafood WD and the meat WD. The first choice of diet and the amount eaten was registered during a 6 h period with free access to both diets.

2.14. Statistical analyses

The data represent group means ± S.E.M. The data were subjected to ANOVA analyses followed by Tukey's multiple comparisons, and group means were considered statistically different at $P<.05$. Data that were repeatedly measured, that is, data from measurements of body mass, energy intake, OGTT, ITT and glucose and c-peptide from the meal response test were analyzed by repeated measurements ANOVA followed by Tukey's post hoc. Mean EC50 and Hill slope values were analyzed with two-tailed *t* test. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software INC., La Jolla, CA, USA) and Statistica (StatSoft).

3. Results

3.1. Reduced energy intake and fat mass in mice fed lean seafood compared to mice fed lean meat

To test the hypothesis that effects on weight gain and body composition differed in relation to protein source, Western diets containing either lean seafood (seafood WD) or lean meat (meat WD) were fed to obesity-prone male C57BL/6 J mice.

Mice fed the seafood WD had a lower energy intake than mice fed the meat diet, and this difference was significant from week 3 and throughout the study (Fig. 1A).

To investigate the possibility that the difference in feed intake was due to the mice being more attracted to the meat WD, we performed a diet preference test. No difference was observed in first choice preference between the diets (Fig. 1B), but during the following 6 h the mice ate significantly more of the meat WD (Fig. 1C).

In addition to lower energy consumption, feed efficiency (body mass gain per energy intake) was significantly lower in mice fed seafood WD compared to mice fed the meat WD (Fig. 1D).

After 9 weeks, mice receiving the seafood WD had gained less weight than mice receiving the meat WD, and as expected, mice receiving the WDs had gained more weight during the feeding experiment than mice fed the LF diet (Fig. 1E). Body composition measurements showed that differences in body mass were due to a higher accumulation of fat mass in mice receiving meat WD than in mice receiving seafood WD (Fig. 1E). The tissue masses of epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT) and retroperitoneal white adipose tissue (rWAT) at week 12 confirmed this difference (Fig. 1F). Total lean mass (Fig. 1E) and tissue masses of liver, kidney and heart were similar in meat- and seafood-fed mice (Fig. 1G). Both liver and kidney masses were higher in mice fed the WDs than in mice fed the LF diet (Fig. 1G). However, as body mass was higher for the WD-fed mice the relative tissue masses (*i.e.*, tissue mass normalized to body mass) were equal for the LF and WD-fed mice.

3.2. Mice fed a Western diet with lean seafood have increased spontaneous locomotor activity and decreased respiratory exchange ratio

Meat WD-fed mice gained more weight than the seafood WD-fed mice. Likewise they had higher energy intake which would contribute to the observed differences in body weight gain. Based on this, we decided to investigate the metabolic effects of lean seafood and meat consumption using indirect calorimetry combined with measurements of spontaneous locomotor activity. Indirect calorimetry was performed prior to the onset of obesity to eliminate differences in body weight which could affect the results, and furthermore, this protocol allowed analysis of the change between being fed LF and the experimental diets in each individual mouse to compensate for the natural variation between the mice. As expected, mice given the meat WD ate significantly more than mice given the seafood WD (Fig. 2A). Interestingly, the mice that switched from the LF diet to the meat WD decreased spontaneous locomotor activity, whereas those switching to the seafood WD remained active (Fig. 2B). Despite the difference in spontaneous locomotor activity, no difference was found in energy expenditure (Fig. 2C and D). The RER was decreased by the change from LF to seafood WD, but no effect was detected when the diet was changed from LF to meat WD (Fig. 2E and F). In addition, the ΔRER Hill slopes indicated a slight reduction in the metabolic flexibility of the seafood WD-fed mice (Fig. 2F).

3.3. Mice fed a Western diet with seafood have improved plasma lipid profiles

To examine if the meat and seafood WDs modulated plasma parameters associated with fat and carbohydrate metabolism differently, blood was collected after 6 h of food deprivation at the end of the study. Plasma levels of LDL-, HDL- and total cholesterol were higher in meat WD-fed mice than in seafood WD-fed mice (Fig. 3A). Moreover, as compared to the LF-fed mice, the seafood WD-fed mice

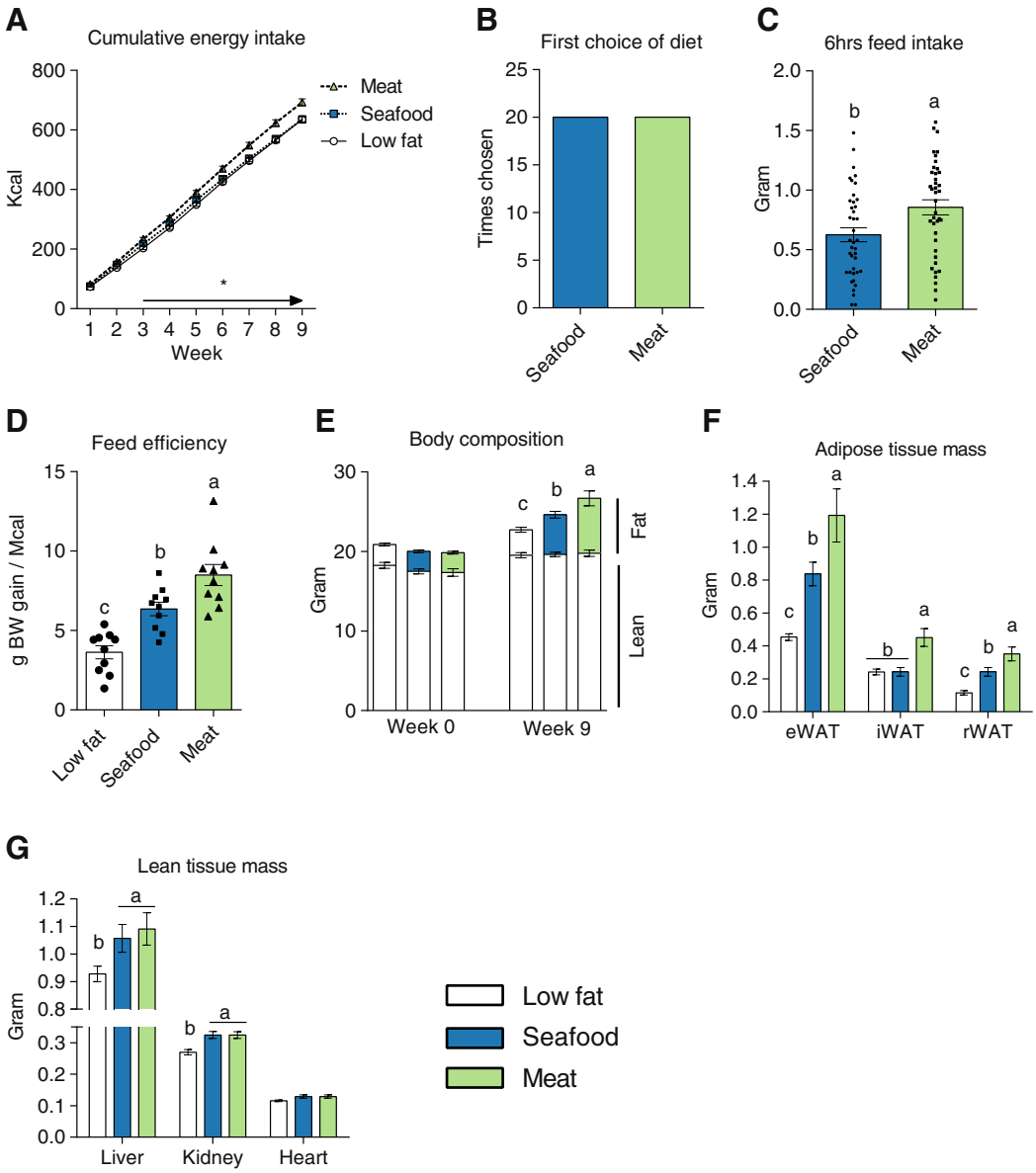

Fig. 1. C57BL/6 male mice fed a diet with lean meat as the protein source consumed more energy and gained more body fat than mice fed a seafood-based diet. (A) Cumulative energy intake. Arrow indicates significant ($P<.05$) increased energy intake of meat WD-fed mice. (B) Choice of diet represents which diet was first ingested when mice had a choice of both diets at the same time. (C) Amount eaten represents amount of diet eaten during the course of 6 h with access to both diets ($n=40$). (D) Feed efficiency at week 9. (E) Body composition at week 0 and 9; noted significant differences are for fat mass. (F) Adipose tissue mass after 12 weeks of feeding, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), retroperitoneal white adipose tissue (rWAT). (G) Lean tissue dissected out in week 12. The data represent group means ($n=10$/group) ±S.E.M. Different letters denote statistical significance ($P<.05$).

had reduced concentration of plasma free fatty acids (FFA) (Fig. 3B). Mice fed WD had higher levels of plasma triglycerides (TG) than mice fed the LF diet, whereas no significant differences were found for hydroxybutyrate (OH-but) and glycerol (Gly) between the treatment groups (Fig. 3B).

3.4. Mice fed a Western diet with meat have impaired glucose tolerance and indications of mild insulin resistance

To examine if the increased accumulation of fat mass seen in meat-fed mice was accompanied by reduced glucose tolerance, we performed an OGTT in week 10. Mice fed the meat WD had significantly higher 6 h fasting blood glucose concentration, relative to the LF-fed mice (Fig. 4A). In addition, mice fed the meat WD, but not those fed the seafood WD, had significantly higher glucose levels following the glucose administration as compared to the LF-fed mice (repeated ANOVA, $P=.0043$ and $P=.057$ for the LF *vs.* meat WD and LF *vs.* seafood WD, respectively) (Fig. 4B). However, there were no differences in IAUC (Fig. 4C). To investigate if variations in glucose levels during the OGTT were due to reduced whole-body insulin sensitivity, an insulin tolerance test was performed during week 11. Again, 6 h fasting blood glucose seemed to be higher in the meat-fed mice, even though the difference did not reach statistical significance (univariate ANOVA, P for diet $=0.19$) (Fig. 4D). No significant differences were found during the ITT (repeated ANOVA, P for diet $=0.13$) (Fig. 4E) or for the DAUC (Fig. 4F), suggesting that whole-body insulin sensitivity was not altered by the treatments. However, at termination, the fasting plasma insulin levels in meat WD-fed mice were higher than the levels in the LF and seafood WD-fed mice (Fig. 4G), and the

revised QUICKI [26] was found to be lower in the meat WD-fed than in the LF and seafood WD-fed mice (Fig. 4H) indicating mild insulin-resistance in the meat WD-fed mice. As low-grade inflammation is associated with impaired insulin sensitivity, we measured plasma interleukin-6 (IL-6) concentration. Compared to the LF and seafood WD-fed mice, the meat WD-fed mice had a higher plasma IL-6 level (Fig. 4I). In keeping with the observation that increased IL-6 is associated with increased hepatic TG concentration [41], we found hepatic TG content to be elevated in meat WD-fed mice as compared to the LF-fed mice (Fig 4J).

3.5. Lean seafood and lean meat diets have similar acute effects on blood glucose and plasma C-peptide concentrations

To elucidate whether the diets had acute effects on postprandial plasma glucose concentration and insulin secretion in the mice, we performed a meal response test. Blood glucose levels were similar at

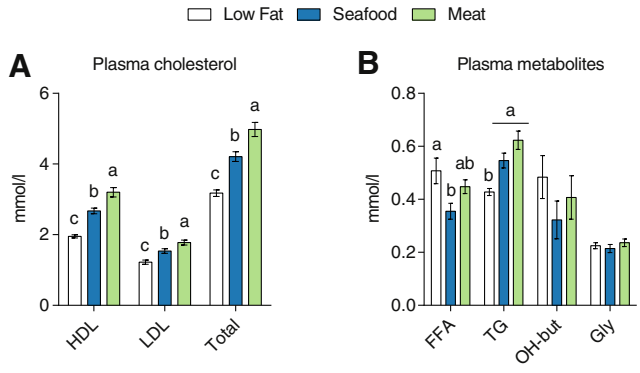

Fig. 3. Plasma metabolite concentrations in C57BL/6 J male mice feed-deprived for 6 h after 12 weeks on experimental diets. (A) High-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol (TC). (B) Free fatty acids (FFA), triglycerides (TG), D-3 hydroxybutyrate (OH-but), glycerol (Gly). The data represent group means ± S.E.M. Different letters denote statistical significance ($P<.05$).

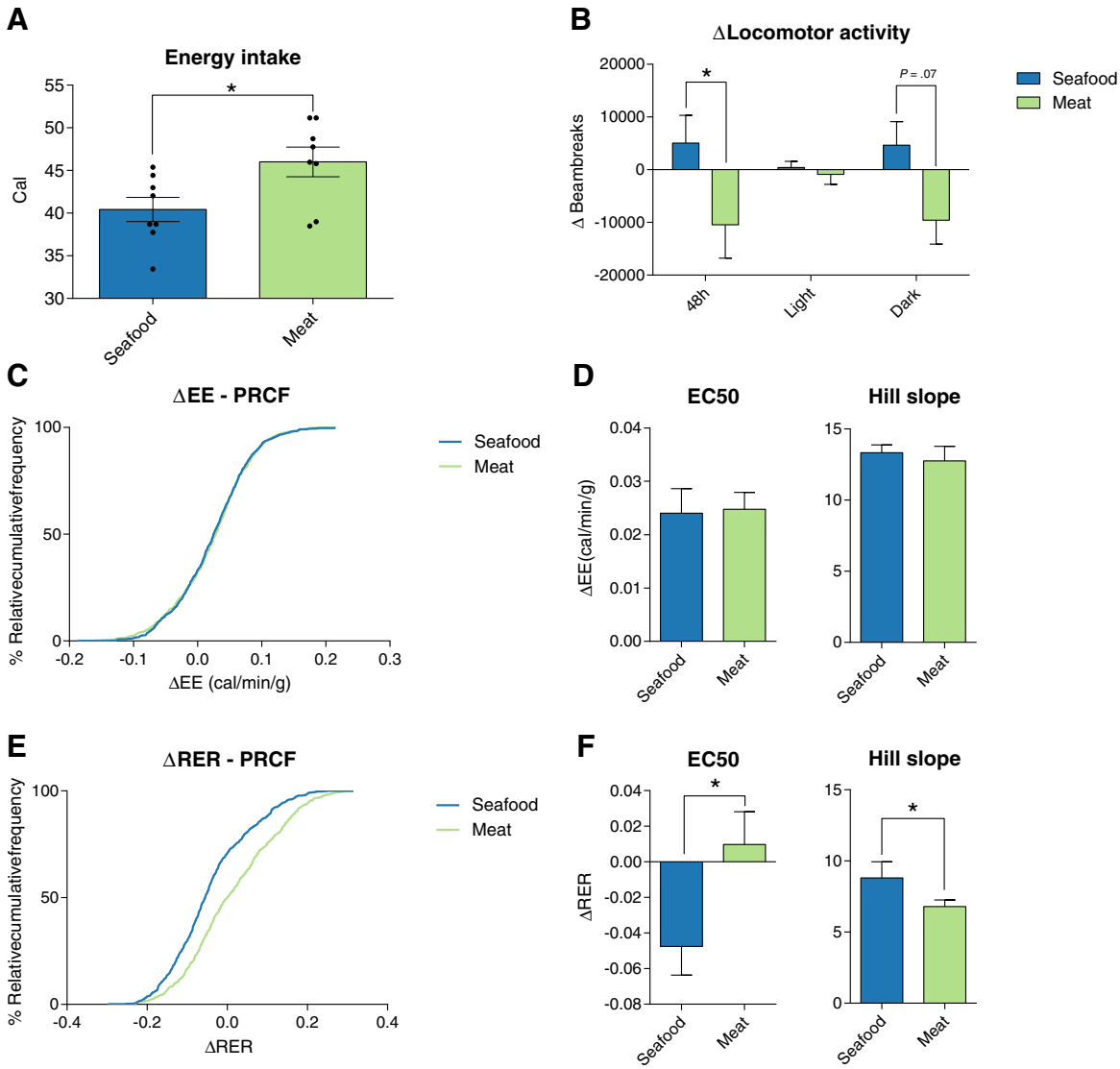

Fig. 2. Indirect calorimetry and spontaneous locomotor activity measured in a separate set of C57BL/6 J male mice before the onset of obesity. (A) Energy intake during the 3 days on experimental diet. (B–F) Changes between the last 2 days on LF and the last 2 days on experimental diets for: (B) spontaneous locomotor activity, (C) mean percent relative cumulative frequency (PRCF) of energy expenditure (EE), (D and F) EC50 and Hill slope calculated using nonlinear regression of sigmoidal dose–response (variable slope) curve, (E) mean PRCF curve of respiratory exchange ratio (RER). The data represent group means ± S.E.M. Number of mice: Seafood WD, $n=8$; meat WD, $n=7$. * Indicates statistical significance ($P<.05$).

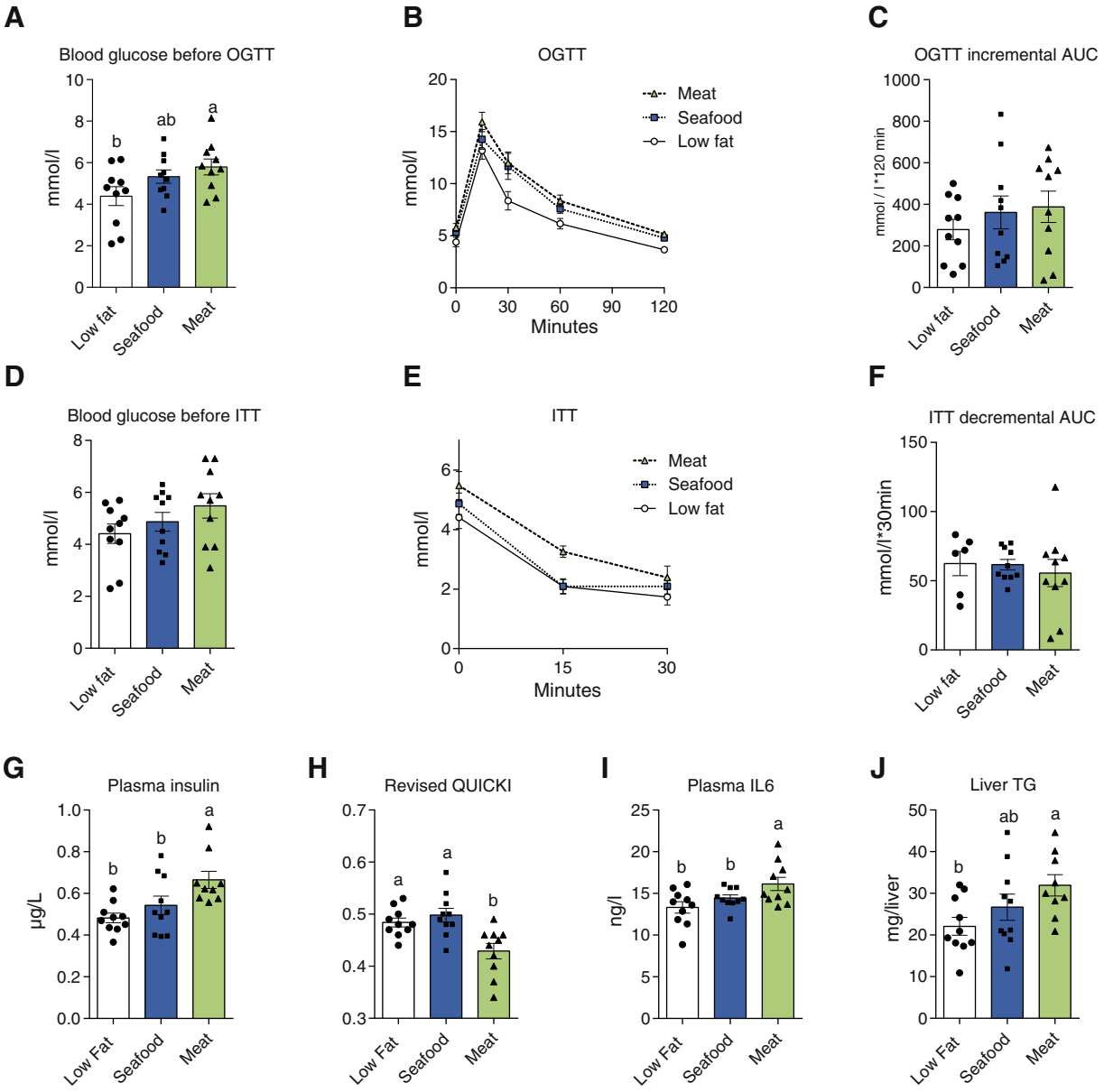

Fig. 4. Glucose and insulin tolerance tests performed on C57BL/6 J male mice feed-deprived for 6 h after 10 and 11 weeks, respectively. (A) Feed-deprived (6 h) blood glucose levels before starting the oral glucose tolerance test ($n=10$/group). (B) Glucose response curve after administration of 3-mg glucose/g lean mass by gavage ($n=10$/group). (C) Incremental area under the curve (IAUC) ($n=10$/group). A single data point from low fat and one from seafood are not shown in the plot as they are below zero. (D) Feed-deprived (6 h) glucose levels before starting the insulin tolerance test ($n=10$/group). (E) Glucose response curve after administration of 0.75 units insulin/kg lean mass, $n=10$/per group except in LF where $n=8$ at time point 0 and 15 min and $n=6$ at 30 min (4 mice were treated with glucose due to low blood glucose levels and data points after treatment were therefore not included). (F) Decremental area under the curve (DAUC), $n=10$ for the WD groups and $n=6$ in LF. (G) Feed-deprived (6 h) plasma insulin levels (H) revised QUICKI and (I) plasma IL-6 levels after 12 weeks. (J) Total liver TG after 12 weeks. The data represent group means ± S.E.M. Different letters denote statistical significance ($P<.05$).

all time points (Fig. 5A). Likewise, postprandial C-peptide levels were not statistically ($P=.28$) different (Fig. 5B).

3.6. Analysis of the cecal microbiota revealed differences in relative abundance of OTUs belonging to the Bacteroidales and Clostridiales order

As alterations in the gut microbiota have been shown to affect host energy metabolism, we searched for potential effects of the diets on the gut microbiota. Cecal samples were collected at the end of the experiment, and the bacterial composition of the microbiota was analyzed using 16S ribosomal-RNA gene-based sequencing. We found no differences in alpha (within sample) or beta (between samples) diversity between the three treatments (Fig. 6E and F). Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances revealed a clear separation between mice fed the LF diet and mice fed the two WDs (Fig. 6D; adonis statistics: $P=.001$), whereas, no clear separation between the gut microbiomes of the mice fed the WDs was observed (adonis statistics: $P=.064$). However, analyses based on metagenomeSeq [29] revealed that the relative abundance of OTUs belonging to the orders *Bacteroidales* (families: *Bacteroidaceae* and *Porphyromonadaceae*; Fig. 6B) and *Clostridiales* (families: *Lachnospiraceae* and *Ruminococcaceae*; Fig. 6B) differed between mice fed the

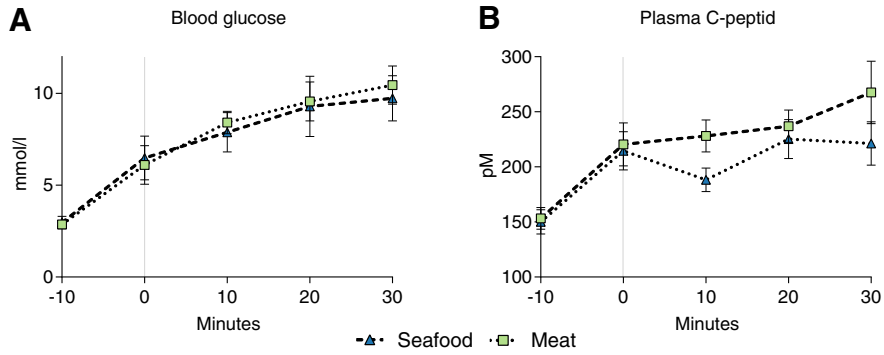

Fig. 5. Meal response test in a separate set of C57BL/6J male mice. (A) Blood glucose and (B) plasma C-peptide concentrations in 16h feed-deprived mice (-10 min) and after consumption of 0.15 g of experimental diets (0, 10, 20 and 30 min). The data represent group means ± S.E.M. Different letters denote statistical significance ($P<.05$). $n=13$/group.

two WDs (Table S4). Comparing mice fed the two WDs, we found that the relative abundance of two OTUs belonging to the genus *Bacteroides* was higher in the mice fed the meat WD than in mice fed the seafood WD, whereas the relative abundance of one OTU belonging to the genus *Robinsoniella* was higher in mice fed the seafood WD than in mice fed the meat WD (Fig. 6G). The OTUs identified as statistically significantly affected represented 22 ± 3% and 93 ± 3% of the total abundance of the OTUs classified as *Bacteroides* and *Robinsoniella*, respectively (Fig. 6G). To predict possible functional consequences of the observed differences between the microbiota composition of the meat and seafood WD-fed mice, we used PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [33] and HUMAnN (HMP Unified Metabolic Analysis Network) [36] to predict the relative abundance of bacterial genes and pathways. Based on these data four KEGG modules in the seafood WD-fed mice were enriched including amino acid transport and tyrosine — and phenylalanine biosynthesis (Fig. S1). The meat WD-fed mice were enriched in genes involved in lysine degradation and the pentose phosphate and the glucoronate pathway.

4. Discussion

A number of studies have been conducted to elucidate the effects of dietary protein in relation to energy metabolism and body weight management. However, the knowledge regarding how different protein sources, consumed at normal dietary levels, influence energy balance is scarce. Furthermore, several studies investigating the effects of different protein sources on energy balance have been of rather short duration [42]. Using obesity-prone C57BL/6 J mice as a model, we have compared the effects of 12 weeks intake of a mixture of lean seafood with that of a mixture of lean meat on energy metabolism, diet-induced obesity and gut microbiota.

Meat WD-fed mice ate more and gained significantly more weight compared to seafood WD-fed mice. Body composition revealed that lean body mass did not differ. Whereas fat mass increased in both WD-treated groups, it increased significantly more in meat WD- than seafood WD-fed mice. Both WD-fed groups had higher absolute liver and kidney masses. However, as body mass also was higher in the WD-fed mice, the relative kidney and liver weights (tissue mass/body mass) were equal in all treatment groups. Calculation of feed efficiency revealed that meat WD-fed mice gained more weight per calorie consumed compared to mice fed seafood WD. This suggests that the meat diet was more obesogenic independently of energy intake.

During indirect calorimetry measurements we found that the meat WD-fed mice decreased spontaneous locomotor activity, while seafood WD-fed mice remained active. A similar observation was done by Tastesen *et al.* (2014), where mice showed a tendency ($P=.06$) to remain active after changing from LF to a high-fat diet containing a mixture of cod and scallop compared to mice given a high-fat diet based on either casein or chicken meat [43]. Despite the difference in activity, we detected no difference in energy expenditure between the two groups. In line with this, Lillefosse *et al.* (2013) detected higher spontaneous locomotor activity, but no difference in energy expenditure, when male C57BL/6 J mice were fed a WD containing hydrolyzed casein compared to a WD containing intact casein [16]. We cannot exclude that minor differences in energy expenditure, undetectable by the given calorimetry setup and time frame, over time would accumulate and contribute to the total fat gain in the present study.

In accordance with the increased obesity development after the meat WD intake, these mice also had indications of impaired glucose, clearance impaired insulin sensitivity, inflammation, and dyslipidemia as compared to the LF-fed mice. These phenotypes were less profound or not observed in the seafood WD-fed mice. The seafood WD was particularly rich in taurine and glycine and had also slightly higher content of arginine and lysine relative to the meat WD. Diets rich in taurine have been shown to prevent high-fat, high-sucrose-induced obesity and improve plasma lipid profiles in mice [25] and to be associated with decreased energy intake and satiety in both mice and human studies [44,45,25]. Likewise, intake of diets with high content of glycine has been associated with decreased accumulation of fat mass in rodent studies [46,25]. A higher dietary content of the amino acids arginine, glycine, taurine and lysine has previously been associated with antiinflammatory effects in rats [47]. In the present study, plasma IL-6 was elevated in meat WD- but not in seafood WD-fed mice as compared to the LF-fed mice, suggesting different inflammatory status. In addition, plasma free fatty acids were lower in seafood WD but not meat WD-fed mice as compared to the LF-fed mice. Recently, hepatic TG synthesis was shown to be dependent on plasma-free fatty acids [48]. Moreover, elevated plasma IL-6 concentration was shown to be an underlying factor leading to insulin resistance in adipose tissue and liver and hyperglycemia [41]. In the present study, we observed no difference in whole body insulin sensitivity, mainly reflecting skeletal muscle insulin sensitivity. By contrast, the revised QUICKI was significantly lower in meat WD-fed mice relative to the LF- and seafood WD-fed mice, suggesting altered insulin sensitivity in adipose tissue and liver. A higher inflammatory status in the meat WD-fed mice might explain the differences in glucose and lipid metabolism observed in the present study. However, we cannot exclude a potential contribution from dietary amino acids in modulating energy balance, inflammation and plasma and liver lipid profiles.

In the present study, we aimed to compare a mixture of lean seafood with a mixture of lean meat as food items without any chemical modifications such as removal of endogenous fat. Therefore,

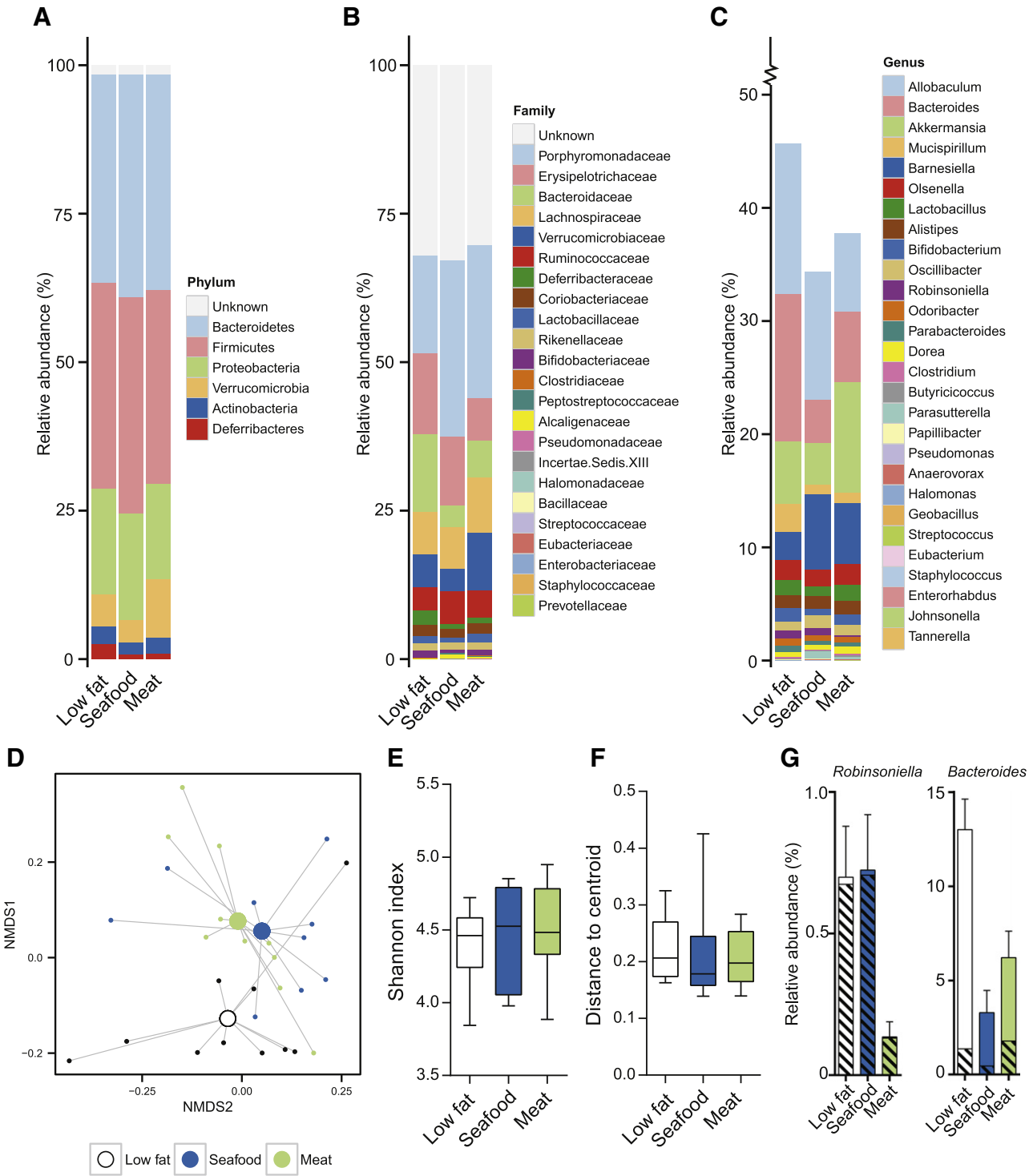

Fig. 6. Cecal microbiota composition of C57BL/6 J male mice after 12 weeks on LF, seafood WD or meat WD. Taxa summary plots based on average relative abundance at (A) phylum-, (B) family- or (C) genus level. "Unknown" refers to OTUs that we were unable to classify. Data represent mean relative abundance. (D) Nonmetric Multidimensional Scaling (NMDS) plot using Bray–Curtis dissimilarity indices. (E) Median alpha diversity based on Shannon index of unfiltered microbiota data. (F) Median beta diversity based on Sørensen index. (G) Bar plot of the mean relative abundance of the genera *Robinsoniella* and *Bacteroides*. The fraction of the bars marked with stripped pattern indicates the fraction of OTUs that differed significantly (adj. $P<.05$) in relative abundance between seafood WD- and meat WD-fed mice.

the nutrient contribution from the mixtures was not equal, and the seafood WD was richer in n-3 polyunsaturated fatty acids, whereas the meat WD had a higher level of linoleic acid. Intake of n-3 polyunsaturated fatty acids has been shown to be less obesogenic compared to consumption of linoleic acid in rodent models under *iso*-energetic conditions [49,50]. This might further contribute to the increased feed efficiency observed in the meat WD-fed mice. In addition, intake of n-3 polyunsaturated fatty acids has been suggested to increase fatty acid oxidation [51] which could contribute to the lower RER observed in the seafood WD-fed mice. Moreover, in WD-fed

mice, a high dietary n-3/n-6 ratio has previously been associated with prevention of insulin resistance and hepatic TG accumulation [51–53] and with a n-3 dominated oxylipin and ceramide pattern, which possibly may prevent inflammation [53]. Thus, the higher dietary n-3/n-6 ratio in the seafood WD as compared to in the meat WD (0.3 *vs.* 0.12) might have contributed to the metabolic differences observed between the treatment groups.

We identified only minor differences in the gut microbiota between the meat and seafood WD-fed mice. However, in the two WD-fed groups OTUs belonging to order *Clostridiales* and *Bacteroidales* differed in relative abundance. Thus, we observed that the relative abundance of one OTU from the genus *Robinsoniella* was significantly higher in mice fed the seafood WD than in mice fed the meat WD. Conversely, the relative abundance of two OTUs from the genus *Bacteroides* was higher in mice fed the meat WD compared to mice fed the seafood WD. *Bacteroides* has previously been correlated with the intake of animal proteins and fat in humans [20]. The differential effects of lean seafood and lean meat on the relative abundance of *Bacteroides* suggest that this correlation may be influenced by dietary protein source. *Robinsoniella* has previously been found to be more resistant to bile acids and inflammation in IL-10$^{-/-}$ mice [54]. Based on the current data, we cannot exclude that the difference in energy intake and lipid profile of the diets, apart from protein source, have an effect on the gut microbiota composition, and the functional consequences of such changes in the gut microbiota require further investigation to be fully explained. However, predictive functional profiling of the microbiota composition indicated differences in metabolic pathways including amino acid transport and the biosynthesis of the aromatic amino acids tyrosine and phenylalanine. Changes in microbiota tyrosine and phenylalanine metabolism might be of relevance, as increased fasting plasma concentrations of the aromatic amino acids are associated with disturbed glucose metabolism and insulin resistance [55–57].

5. Conclusions

Mice fed with lean seafood WD consumed less energy and gained less fat compared to mice fed lean meat WD. In addition, feed efficiency and RER were higher in the meat WD-fed mice than in the seafood WD-fed mice. While mice fed meat WD had reduced spontaneous locomotor activity, mice fed seafood WD remained active, which together with the decreased feed intake would contribute to the diminished weight gain in lean seafood WD-fed mice. Comparison between the gut microbiomes of mice fed the two WDs revealed a higher relative abundance of one OTU of the genus *Robinsoniella* in mice fed the seafood WD, whereas the relative abundance of two OTUs belonging to the genus *Bacteroides* was higher in meat WD-fed mice than in seafood WD-fed mice. Further studies are warranted in order to decipher the underlying mechanisms behind the differences in energy intake and metabolic alterations observed in response to intake of meat and seafood.

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Competing interests

The authors have no conflicting interest, financial or otherwise.

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