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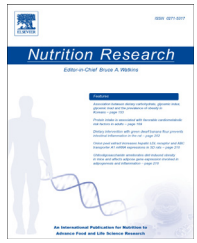

Obesity-induced alterations in the gut microbiome in female mice fed a high-fat diet are antagonized by dietary supplementation with a novel, wax ester–rich, marine oil

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ARTICLE INFO

Article history:
Received 9 April 2020
Revised 1 September 2020
Accepted 2 September 2020

Keywords:
C57Bl/6J female mice
High-fat diet
Calanus oil
Omega-3 fatty acids
Intestinal bacteria composition

ABSTRACT

Dietary supplementation with calanus oil, a novel wax ester–rich marine oil, has been shown to reduce adiposity in high-fat diet (HFD)–induced obese mice. Current evidence suggests that obesity and its comorbidities are intrinsically linked with unfavorable changes in the intestinal microbiome. Thus, in line with its antiobesity effect, we hypothesized that dietary supplementation with calanus oil should counteract the obesity-related deleterious changes in the gut microbiota. Seven-week-old female C57bl/6J mice received an HFD for 12 weeks to induce obesity followed by 8-week supplementation with 2% calanus oil. For comparative reasons, another group of mice was treated with exenatide, an antiobesogenic glucagon-like peptide-1 receptor agonist. Mice fed normal chow diet or nonsupplemented HFD for 20 weeks served as lean and obese controls, respectively. 16S rRNA gene sequencing was performed on fecal samples from the colon. HFD increased the abundance of the *Lactococcus* and *Leuconostoc* genera relative to normal chow diet, whereas abundances of *Allobaculum* and *Oscillospira* were decreased. Supplementation with calanus oil led to an apparent overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Exenatide prevented the HFD-induced increase in *Lactococcus* and caused a decrease in the abundance of *Streptococcus* compared to the HFD group. Thus, HFD altered the gut microbiota composition in an unhealthy direction by increasing the abundance of proinflammatory genera while reducing those considered health-promoting. These obesity-induced changes were antagonized by both calanus oil and exenatide.

Abbreviations: Cal, calanus oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Ex, exenatide; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; HFD + Cal, HFD supplemented with 2% (w/w) Calanus Oil; HFD + Ex, HFD plus treatment with exenatide; NCD, normal chow diet; PUFA, polyunsaturated fatty acids; SDA, stearidonic acid; WAT, white adipose tissue.

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https://doi.org/10.1016/j.nutres.2020.09.002

1. Introduction

The role of gut microbiota in human health has received significant interest in recent years. Alterations in the composition of the gut microbiome have been associated with obesity and metabolic disorders, such as insulin resistance and type 2 diabetes mellitus, thereby increasing the risk for cardiometabolic disease [1-5]. The causal relationship between alterations in the gut microbiota and disease development is, however, still unclear, and it is not fully understood if changes in the microbial composition occur before or after disease onset [6]. However, it is known that diet is an important driver behind changes in the gut microbiota [7-9].

Humans have low ability to synthesize the long-chain omega-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). It is therefore necessary to obtain these essential fatty acids via the diet. Studies describing the impact of EPA and DHA on the gut microbiota in humans are relatively sparse. Rajkumar et al. [10] were the first to study the impact of EPA and DHA on human gut microbiota but failed to detect any major effects on its composition. Later studies providing omega-3 PUFA supplements to both healthy and obese individuals revealed similar changes in both groups, such as a decrease in *Faecalibacterium*, often associated with an increase in the Bacteroidetes and butyrate-producing bacteria of the Lachnospiraceae family [11-13]. Animal studies have also reported alterations in gut microbiota following dietary supplementation with marine oils, and interestingly, these alterations in the microbiome were associated with reductions in body weight gain and white adipose tissue (WAT) inflammation [14] as well as a reduced adiposity index [15].

Calanus oil is a novel marine oil extracted from the copepod *Calanus finmarchicus*. The oil consists mainly of wax esters, a lipid class where fatty acids are esterified to long-chain fatty alcohols [16]. This novel marine oil is, compared to other marine oils, relatively low in EPA and DHA (6% and 4%, respectively) but relatively rich in stearidonic acid (18:4n-3, SDA, 7%), the precursor of EPA and DHA [17,18]. In addition, about 11% of the fatty acids present in the oil are (long-chain) monounsaturated fatty acids. The main fatty alcohols are represented by the equivalents of the dominant monounsaturated fatty acids, namely, eicosenol (20:1n-9) and docosenol (22:1n-11) [18].

Digestion and utilization of wax esters are poorly understood [19], although it is evident that humans are able, to some extent, to hydrolyze waxes and absorb the liberated fatty acids and alcohols [20]. Furthermore, previous studies in mice have shown that the wax esters are hydrolyzed and absorbed in the intestine and that the wax ester–derived fatty acids are incorporated in liver and WAT [18]. Dietary lipids, such as triacylglycerol and phospholipids, are quickly digested and absorbed in the upper parts of the gastrointestinal tract and will normally not reach the lower part [21]. Wax esters are hydrolyzed by a bile salt–dependent pancreatic carboxyl esterase [20]. They are relatively hydrophobic and therefore more difficult to emulsify, and as a result, wax esters may exhibit a longer retention time [22,23], allowing release of the liberated fatty acid and fatty alcohols in the distal part of the intestine.

The mouse model of diet-induced obesity shares many of the same characteristics as human obesity [24] and has become an important tool for understanding the pathological mechanisms involved in obesity-related diseases, such as insulin resistance, oxidative stress, and liver steatosis [25]. In particular, the development of a low-grade inflammatory state in obese adipose tissue and altered adipose tissue metabolism with increased release of fatty acids are believed to play a central role in the development of obesity-related pathology.

Previous studies reported that dietary supplementation with a small amount (2%) of oil from *C finmarchicus* significantly reduced intra-abdominal and ectopic fat deposition in male mice during high-fat feeding [26,27]. Because obesity is characterized by unfavorable alterations in the composition and function of the gut microbiome [4], we hypothesized that reduced obesity following intake of calanus oil could prevent or antagonize such alterations of the bacterial composition in the gut. To test this hypothesis, normal mice were made obese during a 12-week period on high-fat diet (HFD). This was followed by 8 weeks of feeding on HFD with or without 2% calanus oil. For comparative reasons, we also included a group of HFD mice that were treated with the antidiabetic compound exenatide. This glucagon-like peptide-1 (GLP-1) receptor agonist also reduces fat deposition in mice during high-fat feeding [28].

2. Methods and materials

2.1. Study design and animals

All animal experiments were approved by the local authority of the National Animal Research Authority in Norway (FOTS id 8430). All mice were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes. The animals were housed at 21°C, 3 animals per cage, under a reversed light/dark cycle (12-hour dark/12-hour light). The animals had ad libitum access to food and drinking water, and body weight and food intake were recorded once a week.

In this study, 4 groups of 5- to 6-week-old C57Bl/6J female mice (Charles River, Sulzfeld, Germany) were studied (5 mice per group). The first group served as the lean control group and was fed a normal chow diet (NCD) containing 10% of energy from fat (NCD, no. 58Y2, Test Diet; IPS Limited, Richmond, Indiana, USA) for 20 weeks. The other groups were diet-induced obese by feeding a lard-based high-fat diet containing 46% of energy from fat (HFD, no. 58V8, Test Diet; IPS Limited). The second group received the HFD for 20 weeks. The third and fourth groups received the HFD for 12 weeks followed by 8 weeks of treatment. The third group received HFD supplemented with 2% (wt/wt) commercial Calanus Oil, supplied by Calanus AS, Tromsø, Norway (HFD + Cal). The addition of the oil was compensated for by removal of 2 g lard/100 g diet, making the total fat and energy content of the HFD and the HFD + Cal similar. See Table 1 for a list of ingredients of the different diets and Table 2 for a list of fatty acid composition of Calanus Oil, the HFD, and the HFD + Cal. The nutritional profiles of the diets are shown in the supplementary data. The fourth group was fed HFD and treated with exenatide, 10 μg/kg/d, via miniosmotic pumps implanted subcutaneously at the back of the animals (HFD + Ex). After

Table 1 – Ingredient composition in g/kg of the 3 experimental diets

Ingredients	NCD	HFD	HFD + Calanus
Sucrose	331.29	200.92	200.92
Dextrin	298.56	84.83	84.83
Casein, vitamin tested	189.56	233.06	233.06
Powdered cellulose	47.39	58.27	58.27
Maltodextrin	33.17	116.53	116.53
Soybean oil	23.7	29.13	29.13
Lard	18.96	206.84	186.84
Calanus Oil-841	0	0	20
Potassium citrate, tribasic monohydrate	15.64	19.23	19.23
Calcium phosphate	12.32	15.15	15.15
DIO mineral mix	9.48	11.65	11.65
AIN-76A vitamin mix	9.48	11.65	11.65
Calcium carbonate	5.21	6.41	6.41
L-Cystine	2.84	3.5	3.5
Choline bitartrate	1.9	2.33	2.33
FD&C yellow no. 5	0.5	0	0
FD&C red 40 lake	0	0.5	0
Green dye	0	0	0.5
Total g/kg	1000	1000	1000
Total energy in kcal/g (kJ/g)	3.76 (15.73)	4.6 (19.25)	4.6 (19.25)

NCD, normal control diet (TestDiet 58Y2 with 10% energy from fat); HFD, high-fat diet (TestDiet 58V8 with 45% energy from fat); HFD + Cal, high-fat diet with 2% Calanus Oil (TestDiet 58V8 with 2% Calanus Oil). See supplementary materials for details.

implantation of the miniosmotic pumps, the mice were single housed to avoid animal interaction, which could damage the surgical wound. Mice that did not receive pumps were also single housed to control for possible effects of single housing.

Table 2 – Fatty acid composition (mg/g lipid) of Calanus Oil and experimental diets

Fatty acids	Calanus Oil	HFD	HFD + Cal
14:0	64.42	10.41	11.95
16:0	45.05	173.65	149.83
18:0	2.42	106.63	92.16
20:0	0.40	1.70	1.37
16:1*n-7*	17.17	10.95	9.98
18:1*n-7*	1.53	15.84	13.52
18:1*n-9*	15.54	243.82	208.42
20:1*n-9*	24.01	4.74	5.64
20:1*n-11*	3.90	ND	ND
22:1*n-9*	2.63	ND	ND
22:1*n-11*	43.33	ND	2.20
24:1*n-9*	2.81	ND	ND
18:2*n-6*	6.64	133.04	116.06
18:3*n-3*	13.72	12.49	11.67
18:4*n-3*	69.58	ND	4.54
20:2*n-6*	0.71	3.15	2.69
20:4*n-6*	1.39	0.48	1.15
20:5*n-3*	54.73	ND	3.35
22:5*n-3*	2.96	ND	ND
22:6*n-3*	39.35	ND	2.81

Modified from Pedersen et al. [18]. ND, not detected.

At the end of the feeding experiment, the mice were killed with an overdose of pentobarbital (100 mg/kg, 300 μL intraperitoneally). Organs were carefully dissected out and stored at –80 °C until further analysis. Feces samples were collected from the colon for microbiota analysis, after which the gastrointestinal tract was rinsed with saline before freezing.

2.2. *Fatty acid analysis of the colon wall and abdominal fat*

Fatty acids were methylated by dissolving 60 mg tissue sample in 2 mol/L HCl in methanol with 0.05% BHT and heating for 2 hours at 100 °C, and the fatty acid composition was determined by gas chromatography using an Agilent 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a Varian CP7419 capillary column (Varian Inc, Palo Alto, CA, USA). The content of the individual fatty acids in the samples was expressed in percent of the total fatty acid content.

2.3. *Quantitative real-time polymerase chain reaction*

The expression of mRNA of genes related to inflammation and fat metabolism in the colon and omental WAT was determined using quantitative reversed real-time polymerase chain reaction (PCR). Samples of the intestinal wall (colon, n = 6-8 per group) were immersed in RNA later (Qiagen, Hilden, Germany), and WAT samples (n = 6-8 per group) were immersed in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and thawed overnight at 4°C. Thirty to 40 mg colon tissue and 50-100 mg WAT tissue were used for RNA extraction according to the Rneasy Lipid Tissue kit Protocol (Qiagen). RNA concentrations were measured spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80 °C before use for cDNA. cDNA was subsequently made from 2 μg total RNA according to High Capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was stored at –20 °C until real-time PCR (quantitative PCR) was performed in a Roche LightCycler 96 using a 1:4 dilution of the cDNA and the fast-start essential DNA green master (Roche, Basel, Switzerland). Five housekeeping genes were analyzed to normalize the expression of the target genes to the geometric mean of the 2 best housekeeping genes, which were selected on the basis of the average expression stability values determined with geNorm. For quantification of the gene expression in the colon, the housekeeping genes *sdha* (succinate dehydrogenase complex subunit A) and *hprt* (hypoxanthine-guanine phosphoribosyl transferase) were used, and for the WAT, *sdha* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were used. Forward and reverse primers of the target genes analyzed in the colon and omental fat are shown in Table 3.

2.4. *Bacterial DNA isolation*

Total microbial DNA was isolated from feces samples with a QIAamp PowerFecal DNA kit (Qiagen) according to the manufacturer's protocol, with the following exceptions. The disintegration of the cells was done with a FastPrep device at a vortexing speed of 6.5 m/s for 30 seconds followed by

Table 3 – Forward and reverse primers of the target genes analyzed in the intestinal (colon) wall and omental fat

Gene	Accession number	Primer	Melting temperature (°C)	Product size
SDHA	NM_023281	F: TGT TCA GTT CCA CCC CAC A R: CAC GAC ACC CTT CTG TGA TG	59.0 59.0	62 bp
GAPDH	NM_001289726.1	F: TCA CCA CCA TGG AGA AGG C R: GCT AAG CAG TTG GTG GTG CA	59.3 61.2	169 bp
HPRT	NM_013556.2	F: TCC TCC TCA GAC CGC TTT T R: CCT GGT TCA TCA TCG CTA ATC	58.2 56.6	90 bp
NFkB	NM_008689.2	F: ATG GCA GAC GAT GAT CCC TAC R: CGG AAT CGA AAT CCC CTC TGT T	59.4 60.4	167 bp
GPR 120	NM_181748.2	F: GTG CCG GGA CTG GTC ATT GTG R: TTG TTG GGA CAC TCG GAT CTG G	63.8 62.3	123 bp
EMR1	NM001355722.1	F: TTG TAC GTG CAA CTC AGG ACT R: GAT CCC AGA GTG TTGATG CAA	59.6 58.3	144 bp
CD36	NM_001159558.1	F: TTG TAC CTA TAC TGT GGC TAA ATG AGA R: CTT GTG TTT TGA ACA TTT CTG CTT	59.7 57.4	72 bp
MCP1	NM_011333.3	F: TTA AAA ACC TGG ATC GGA ACC R: GCA TTA GCT TCA GAT TTA CGG)	56.2 55.1	121 bp
TLR4	NM_ 021297.3	F: TTC TTC TCC TGC CTG ACA CC R: CTT TGC TGA GTT TCT GAT CCA T	59.3 56.7	94 bp
Adiponectin	NM_009605.5	F: CCT GGC CAC AAT GGC ACA CCA R: GTG ACG CGG GTC TCC AGC C	65.8 65.5	233 bp
PPARα	NM_011144.6	F: ACG ATG CTG TCC TCC TTG ATG R: GTG TGA TAA AGC CAT TGC CGT	60.1 59.5	67 bp
PPARδ	NM_011145.3	F: GCT GCT GCA GAA GAT GGC A R: CAC TGC ATC ATC TGG GCA TG	61.0 59.3	63 bp
PPARγ	NM_001127330.2	F: CCA TTC TGG CCC ACC AAC R: AAT GCG AGT GGT CTT CCA TCA	58.3 59.7	67 bp
FIAF	NM_020581.2	F: GCC ACC AAT GTT TCC CCC AAT G R: TAC CAA ACC ACC AGC CAC CAG AGA	62.6 65.7	118 bp
IL-1β	NM_008361.4	F: TGT AAT GAA AGA CGG CAC ACC R: TCT TCT TTG GGT ATT GCT TGG	58.9 56.1	68 bp
MUC2	NM_023566.4	F: ATG CCC ACC TCC TCA AAG AC R: GTA GTT TCC GTT GGA ACA GTG AA	59.7 59.1	101 bp
GLP2R	NM_ 175 681.3	F: TCA TCT CCC TCT TCT TGG CTC TTA C R: TCT GAC AGA TAT GAC ATC CAT CCAC	61.6 60.0	196 bp
IL-18	NM_008360.2	F: CAT GTA CAA AGA CAG TGA AGT AAG AGG R: TTT CAG GTG GAT CCA TTT CC	59.9 55.3	122 bp
ZO-1	NM_009386.2	F: GAG CGG GCT ACC TTA CTG AAC R: GTC ATC TCT TTC CGA GGC ATT AG	60.5 59.0	75 bp
IFNγ	NM_008337.4	F: TTG GCT TTG CAG CTC TTC CT R: TGA CTG TGC CGT GGC AGT A	60.2 61.5	58 bp
Occludin	NM_008756.2	F: TTG AAA GTC CAC CTC CTT ACA GA R: CCG GAT AAA AAG AGT ACG CTG G	59.0 59.1	129 bp

incubation at 70 °C for 5 minutes. The vortexing and incubation were repeated once. The elution was done with 100 μL of elution buffer and stored at –20 °C until used.

2.5. *16S rDNA amplification*

Twenty nanograms of DNA was used to prepare PCR amplicons of the V4-V5 region of 16S rRNA according to Fliegerova et al. [29]. The mixture contained OneTaq DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA), and the thermal profile consisted of initial denaturation for 5 minutes at 95 °C followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 57 °C, and 30 seconds at 72 °C and a final elongation for 5 minutes at 72 °C. The PCR amplicons were checked by electrophoresis in 1.5% agarose (25 minutes at 90 V), purified with a QIAquick PCR Purification Kit (QIAGEN) according to the protocol, and quantified by Nanodrop OneC.

2.6. *Next-generation sequencing*

The obtained PCR products were used to prepare amplicon libraries for diversity analyses by a next-generation sequencing approach on a Personal Genome Machine (Life Technologies, Carlsbad, California, USA) according to Milani et al. [30]. Two hundred nanograms of DNA from each sample was used to prepare sequencing libraries with a NEBNext Fast DNA Library Prep Set kit (New England Biolabs) according to the manufacturer's protocol. Ion Xpress Barcode adapters (Thermo Fisher Scientific) were used to label each sample. The obtained libraries were used to prepare a sequencing template by using an Ion PGMTM Hi-QTM View OT2 Kit (Thermo Fisher Scientific). The template was then sequenced on an Ion 316TM Chip Kit v2 (Thermo Fisher Scientific) by using an Ion PGMTM Hi-QTM View Sequencing kit (Thermo Fisher Scientific). Two independent sequencing runs were performed.

2.7. Next-generation sequencing data analyses

The sequences were obtained in the form of the FASTQ format and further processed by the QIIME2 analyses pipeline [31]. The sequences were demultiplexed and dereplicated using the versatile and open-source tool VSEARCH [32]. The bioinformatics software PICRUSt v 1.1.3 was applied for both the taxonomical and functional analyses [33]. The sequences were clustered and identified by performing closed-reference operational taxonomic unit (OTU) picking selecting a cutoff at 90% sequence identity against the Greengene reference OTUs (gg_13_5_otus). The resulting OTU table was normalized using the *normalize_by_copy_number.py* script. Functional trait abundance was predicted using the *predict_metagenomes.py* script. The predictions were collapsed into Kyoto Encyclopedia of Genes and Genomes pathways level 3 using the *categorize_by_function.py* script, and the resulting abundance table was imported in the bioinformatics software STAMP for statistical analysis [34].

Diversity index analysis and unweighted UniFrac distance metrics analysis [35] were carried out by exporting the QIIME2 biom file to MicrobiomeAnalyst. The Web site microbiomeanalyst.ca was used for visualization of the α diversity index and the heat map. The absolute and relative abundance tables were exported and used in STAMP for statistical analysis of the taxonomy data, in GraphPad prism 8.2.1 for the (stacked) bar graphs, and in MEGAN Community Edition (version 6.15.2) for visualization of the principal component analysis.

2.8. Statistical analyses

Data are presented as the means with their standard errors. Statistical analysis of the functional and taxonomic data was done using STAMP [34]. Noncorrected 2-sided Student *t* test between the HFD group and the other three groups was done to check for significant differences vs the HFD group (n = 5 for each group). Mean differences were considered significant at $P < .05$. Statistical analysis on the gene sequencing was done in GraphPad prism 8.2.1. Differences between treatment groups were assessed by 1-way analysis of variance followed by Dunnet post hoc test where the groups were compared to the HFD group (n = 6-8 for each group). Sample sizes were determined based on availability of the samples because this study was ran in parallel to another study using the same mice [36].

3. Results

3.1. Body weight gain and fatty acid composition of the diets, and abdominal fat and colon wall

The present microbiota data were obtained on a subgroup of mice (n = 5) used in a recent publication by Jansen et al. [36]. Details about body weight development, weight of adipose tissue, and food intake are given in that publication. Here it is just pointed out that supplementation of the HFD with oil from *C finmarchicus* for the last 8 weeks did not affect body weight gain (Fig. 1), although it did result in a lower adiposity index (total weight of intra-abdominal fat depots) relative to the nonsupplemented HFD group. Treatment with exenatide, however, led to both lower weight gain (Fig. 1) and lower adiposity index. Of note, both treatments recovered the capacity of the heart to oxidize glucose, which was otherwise impaired in response to high-fat feeding [36], demonstrating that both the wax ester–rich oil and exenatide recovered metabolic flexibility of hearts from diet-induced obese mice. There were no differences in food intake between the diet groups during the treatment period [36]. The inclusion of 2% calanus oil in the HFD resulted in clearly detectable amounts of SDA, EPA, and DHA in this diet (Table 2). Analysis of the fatty acid composition of abdominal fat showed a statistically significant increase in the content of n-3 PUFA (SDA, DHA, and EPA), whereas the content of arachidonic acid (n-6 PUFA) was significantly reduced (Table 4). In the colon wall, the content of SDA was significantly increased ($P < .05$) in the HFD + Cal group relative to that of the HFD group (0.89% ± 0.23% vs 0.63% ± 0.07%), but for the other PUFA, the variability was too high to reach firm conclusions (data not shown).

3.2. Microbiota composition of feces samples from colon

The average sequence count over all 20 samples was 6761 sequence reads per sample. The average species diversity, or α diversity, within the 4 diet groups was measured using the Shannon Phylogenetic Diversity index on the OTU level (Fig. 2). No statistically significant differences were observed between the groups ($P = .0596$, Kruskal-Wallis statistics: 7.4229).

The β diversity, the difference between the diet groups, is presented through a principal coordinate analysis based on unweighted UniFrac distances (Fig. 3). The first and second principal coordinates are given. Axis 1 explains 43.7% of the variation, and axis 2 explains 22.6%. Distinct clusters separate the NCD and HFD + Ex groups. The HFD and HFD + Cal groups overlap, with the HFD + Cal group having a larger cluster than the HFD group.

Fig. 4A illustrates the relative abundance of the different bacterial phyla for each diet group. Firmicutes was the most abundant phylum in all 4 groups, followed by Bacteroidetes. There were no apparent differences in the relative abundance on the phylum level. But there were significant differences on the genus level as highlighted in Fig. 4B. It is obvious that the HFD and HFD + Cal groups share a similar bacterial profile, whereas the profiles of the HFD + Ex and NCD groups are different compared to HFD. To illustrate the relative abundance of the various genera in more detail, we also generated bar graphs ± standard error of the mean in Fig. 5. The most abundant genera in the feces samples were *Allobaculum*, *Lactobacillus*, *Lactococcus*, *Turicibacter*, and *Parabacteroides*. On first sight, it seemed that the main effect of HFD is a marked decline in *Allobaculum* and the presence of *Lactococcus* relative to the NCD group. Dietary supplementation with calanus oil did not seem to influence these HFD-induced changes in the microbiota composition, except for an apparently further decline in *Allobaculum* and an increased abundance of *Lactobacillus*. Treatment with exenatide reduced the high abundance in *Lactococcus* present in the HFD group. *Turicibacter* was abundant in all diet groups but mostly in the NCD and HFD + Ex group, whereas the presence of *Parabacteroides* seemed to be independent of diet.

A noncorrected 2-sided Student *t* test (comparing HFD vs NCD, HFD vs HFD + Cal, and HFD vs HFD + Ex) revealed that,

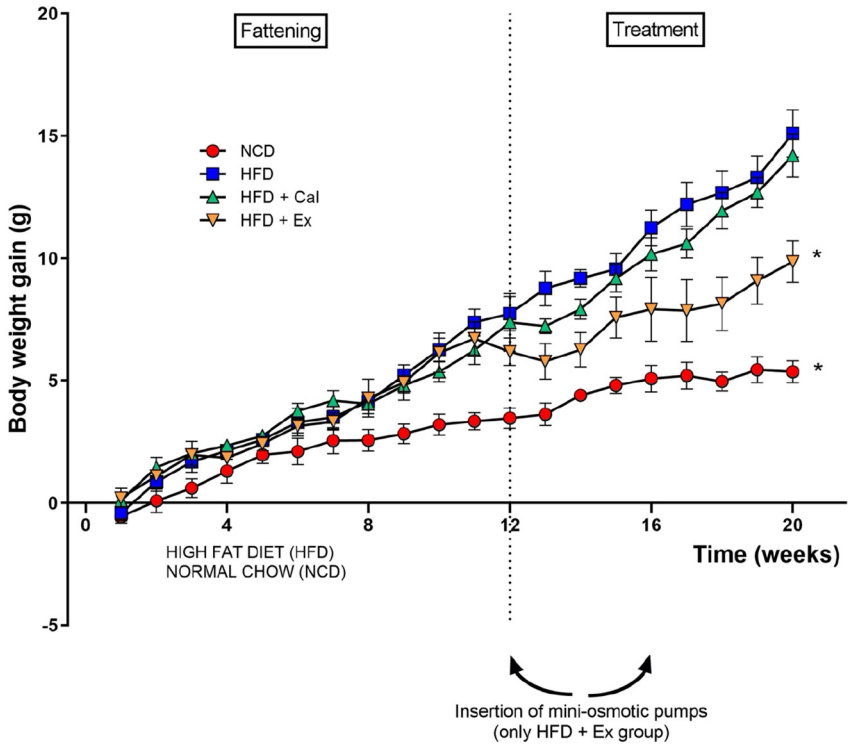

Fig. 1 – Body weight gain for the four different diet groups. Data are shown as means ± SEM. After 8 weeks of treatment, the weight gain of NCD and HFD + Ex groups was significantly different from HFD ($P < .05$).

indeed, the decrease in *Allobaculum* and increase in *Lactococcus* in the HFD compared to the NCD are significantly different, as was the increase in *Leuconostoc* (Fig. 6A). There were no significant differences between the HFD and HFD + Cal group. The apparent decrease in *Lactococcus* in the exenatide-treated group compared to the HFD group was significantly different, as well as the decrease in *Streptococcus* (Fig. 6B).

It is currently unknown at what abundance threshold certain gut bacteria lead to an increased disease risk. A statistical difference might therefore not necessarily lead to pathology or health improvement. Therefore, we visualize in Fig. 7 the differences of the various genera for each individual fecal sample in more detail with a heat map. Difference in colored tiles represents the multiplication of abundance of the genera, per sample, compared to the average abundance of those genera of all 20 fecal samples.

Table 4 – Content of PUFA in % of total fatty acid content in intra-abdominal fat from the various groups of mice

	SDA	DHA	EPA	AA
NCD	0.78 ± 0.15	0.26 ± 0.17	ND	0.42 ± 0.12
HFD	0.65 ± 0.03	0.19 ± 0.03	ND	0.40 ± 0.04
HFD + Cal	1.09 ± 0.12[a]	0.74 ± 0.10[a]	0.19 ± 0.04[a]	0.30 ± 0.04
HFD + Ex	0.71 ± 0.09	0.23 ± 0.04	ND	0.44 ± 0.09

Results are mean ± SD (n = 5 in each group).
[a] $P < .05$ vs HFD.

Some genera showed large variation in abundance within the diet groups (*Bacteroides*, *Parabacteroides*, *Lactobacillus*, *Akkermansia*, *Adlercreutzia*, *Coprococus*, *Blautia*, *Turicibacter*, *Bilophila*), whereas the abundance was more stable for other genera (*Lactococcus*, *Lauconostoc*, *Allobaculum*, *Ruminococcus*, *Streptococcus*, *Dorea*, *Oscillospira*). Thus, in line with the relative abundance shown in Fig. 5, the heat map shows that *Lactococcus*, *Leuconostoc*, and *Streptococcus* were above average present in the HFD and HFD + Cal groups, whereas *Dorea* and *Ruminococcus* were below average present in these groups. *Allobaculum* and *Oscillospira* were below average present in the 3 groups feeding on an HFD. Moreover, feces samples from the majority of mice in the HFD + Cal group were characterized by overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. In the HFD + Ex group, overrepresentation of *Ruminococcus* was particularly evident.

3.3. *Metagenomic functional prediction*

The 16S rRNA gene was used as a marker gene to extrapolate the taxonomic findings into functional predictions. The taxonomic information from screening samples against the Greengenes reference database was used to extract information from the complete genome sequences of the identified species. Combining whole genome information together with abundance measures for each species allowed for the prediction and quantification of functional pathways. Statistical analysis of the Kyoto Encyclopedia of Genes and Genomes data identified in total 328 genes related to

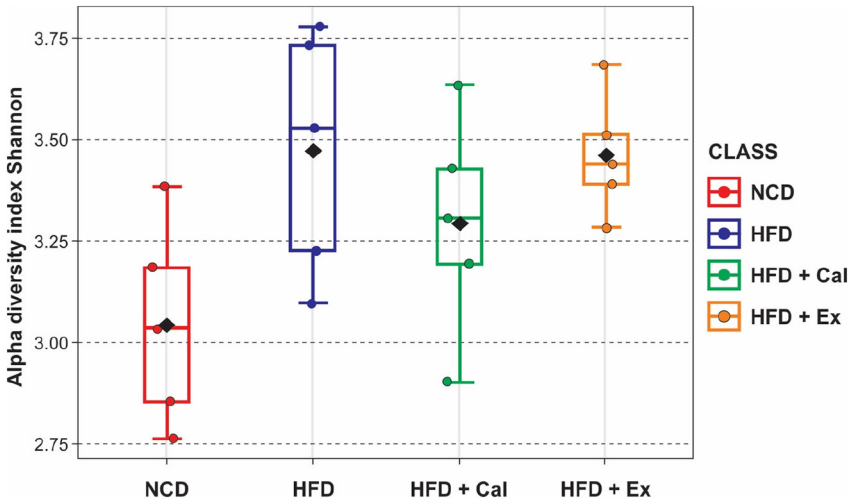

Fig. 2 – *α* diversity measured as Shannon Phylogenetic Index on OUT level for the 4 different diet groups. There were no statistically significant changes in the *α* diversity between the groups. (P = .0596, Kruskal-Wallis statistics: 7.4229).

functional categories (proteins) (Supplementary Table S1). Significant differences in the relative abundance of the predicted microbial genes (based on the noncorrected 2-sided Student t test) revealed the difference in the mean proportions of these genes between the HFD and NCD groups, the HFD and HFD + Cal groups, and the HFD and the HFD + Ex groups (Figs. 8A-C). As can be seen in Fig. 8A, 38 categories were differently expressed in the HFD vs NCD groups. Relevant for the current study was the overexpression of lipid and fatty acid biosynthesis proteins in the gut microbiota of the HFD-fed mice. Fig. 8B gives the 7 functional categories that were differently present in the colonic microbiota in the HFD and HFD + Cal groups. The novel marine oil upregulated 4 of the 7 categories in the high-fat–fed mice, which included proteins related to glycolysis, gluconeogenesis, and phosphate transferase systems (Fig. 8B). Ten categories where differently expressed in the microbiota of the HFD vs the HFD + Ex group (Fig. 8C). With respect to metabolic control, we note that proteins involved in glycine, serine, and threonine metabolism were overexpressed in the HFD + Ex group.

3.4. *Gene expression*

The gene expression of several markers for inflammation, fat storage regulation, and permeability of the intestinal wall

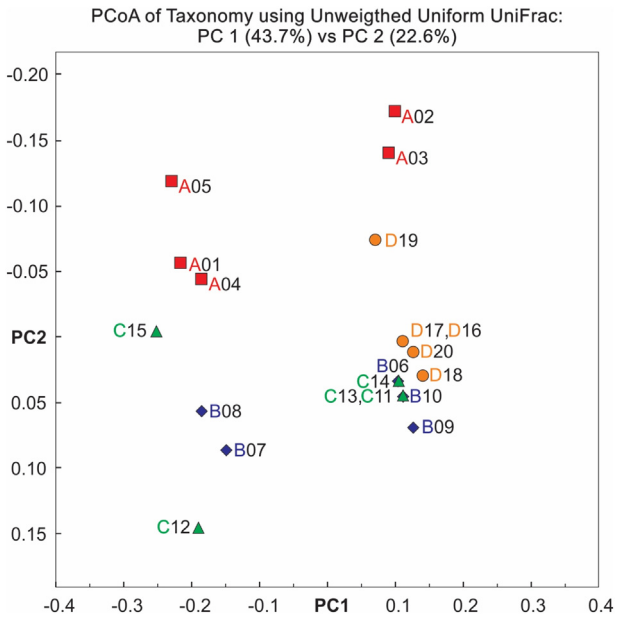

Fig. 3 – Principle coordinate analysis based on unweighted UniFrac in mice fed different diets. The symbols indicate values for individual mice. Percent variation explained by the principal coordinates PC 1 and PC 2 was 43.7% and 22.6%, respectively. Red squares, NCD; blue diamonds, HFD; green triangles, HFD + Cal; orange circles, HFD + Ex.

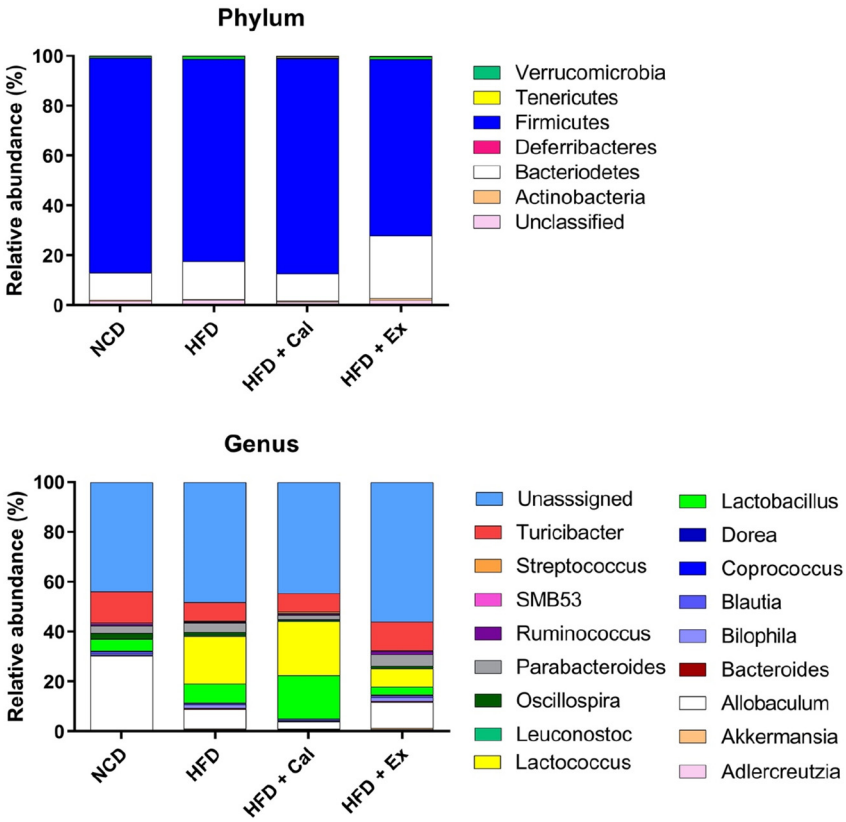

Fig. 4 – Relative abundance at the phyla (A) and genera levels (B) in the 4 diet groups.

were measured in the omental WAT (GPR120, IL-1β, IL-6, IL-10, TNF-α, CD36, EMR1a, adiponectin TLR4, MCP1, NF-κB, FIAF, PPARα, PPARβ, PPARγ, and GLP2 R) and the colon (GPR41, GPR43, GPR120, TNF-α, MUC2, GLP2 R, IL-1β, Il-10, IL-18, IFN-γ, FIAF, ZO-1, occluding, TLR4, and NF-κB). None of the analyzed genes were up- or downregulated depending on diet in the 2 tissues, with the exception of adiponectin (see also Supplementary Figs. S1 and S2).

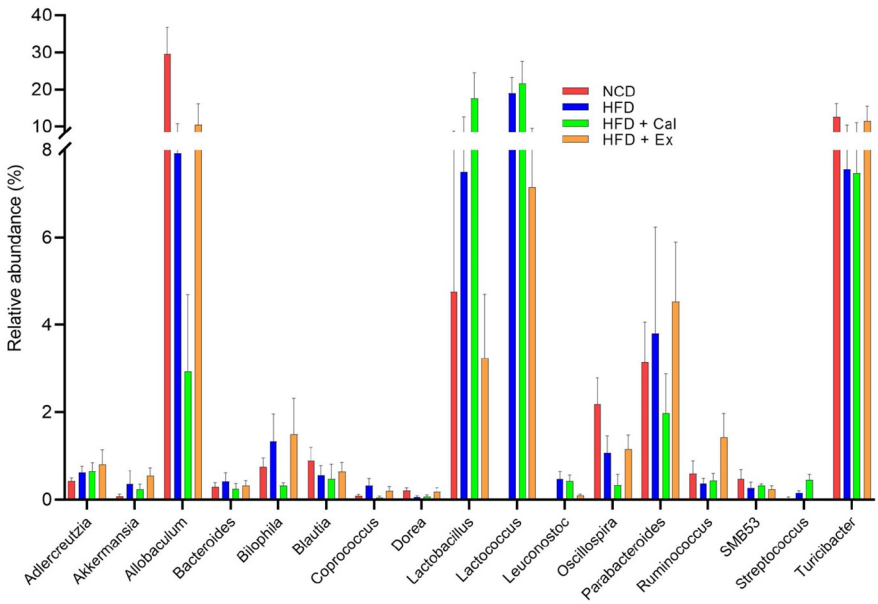

Fig. 5 – Relative abundance of the different genera present in colon feces samples from the 4 diet groups. Data shown as means ± SEM.

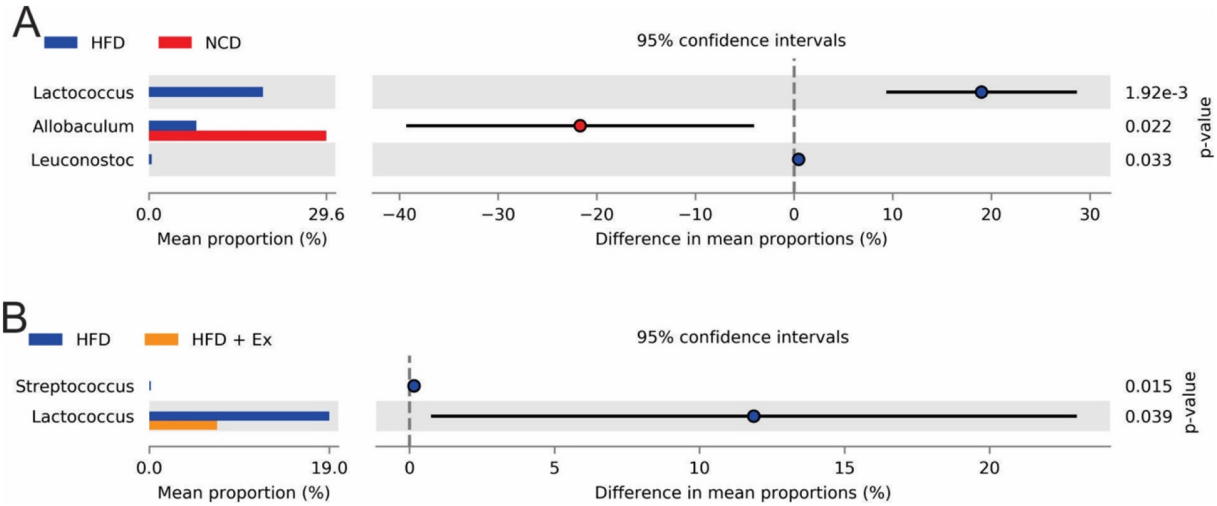

Fig. 6 – Comparison of mean proportion of bacterial genera. (A) HFD vs NCD groups. (B) HFD vs HFD + Ex groups. Only statistically significant differences ($P < .05$, noncorrected 2-sided Student t test) are shown.

4. Discussion

Diet has long been considered the most important driver behind changes in the intestinal microbiota [7-9]. In this study, we show that long-term feeding on a HFD led to an increase in the intestinal abundance of the bacterial genera *Lactococcus* and *Leuconostoc*, whereas the abundance of *Allobaculum* and *Oscillospira* was decreased. Supplementation of the HFD with 2% of wax ester–containing oil from *C*

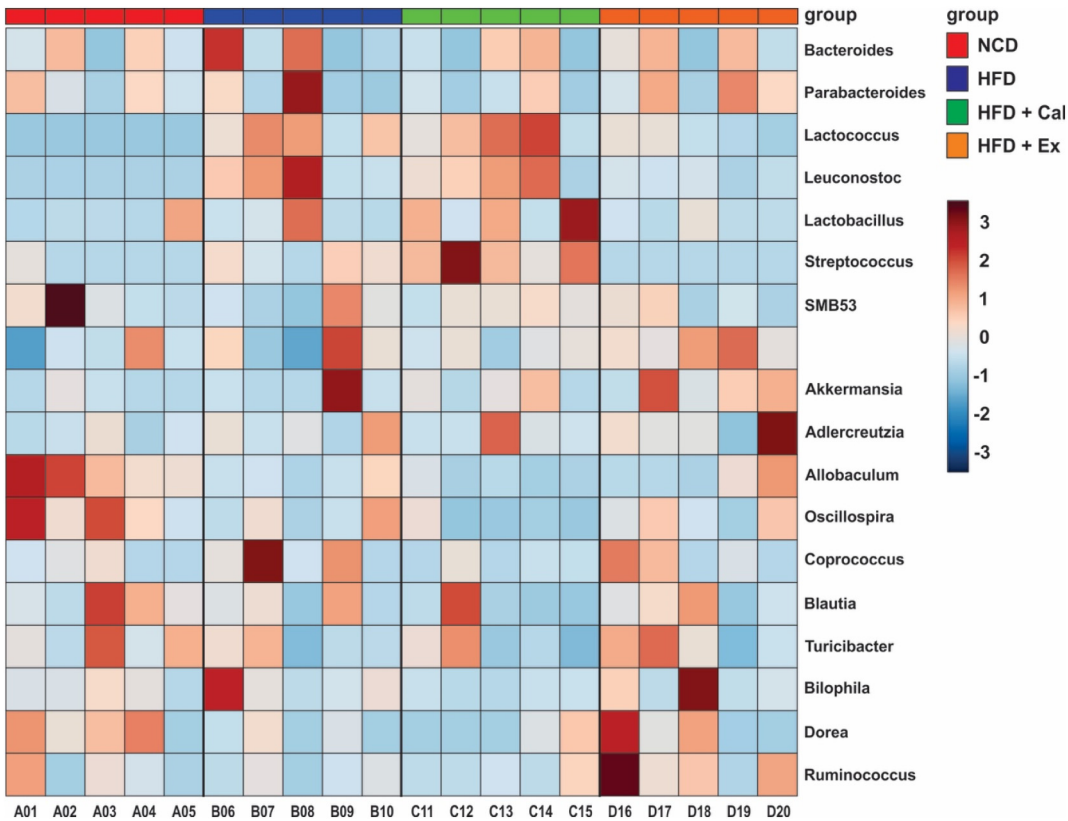

Fig. 7 – Heat map showing the representation of the different genera in each individual fecal sample compared to the average abundance of those genera. The multicolored bar at the top delineates the 4 diet groups, whereas the individual samples are indicated at the bottom of the heat map. The colored tiles indicate a positive (light to dark brown) or negative multiplication (light to dark blue) of the genus average across all individual samples.

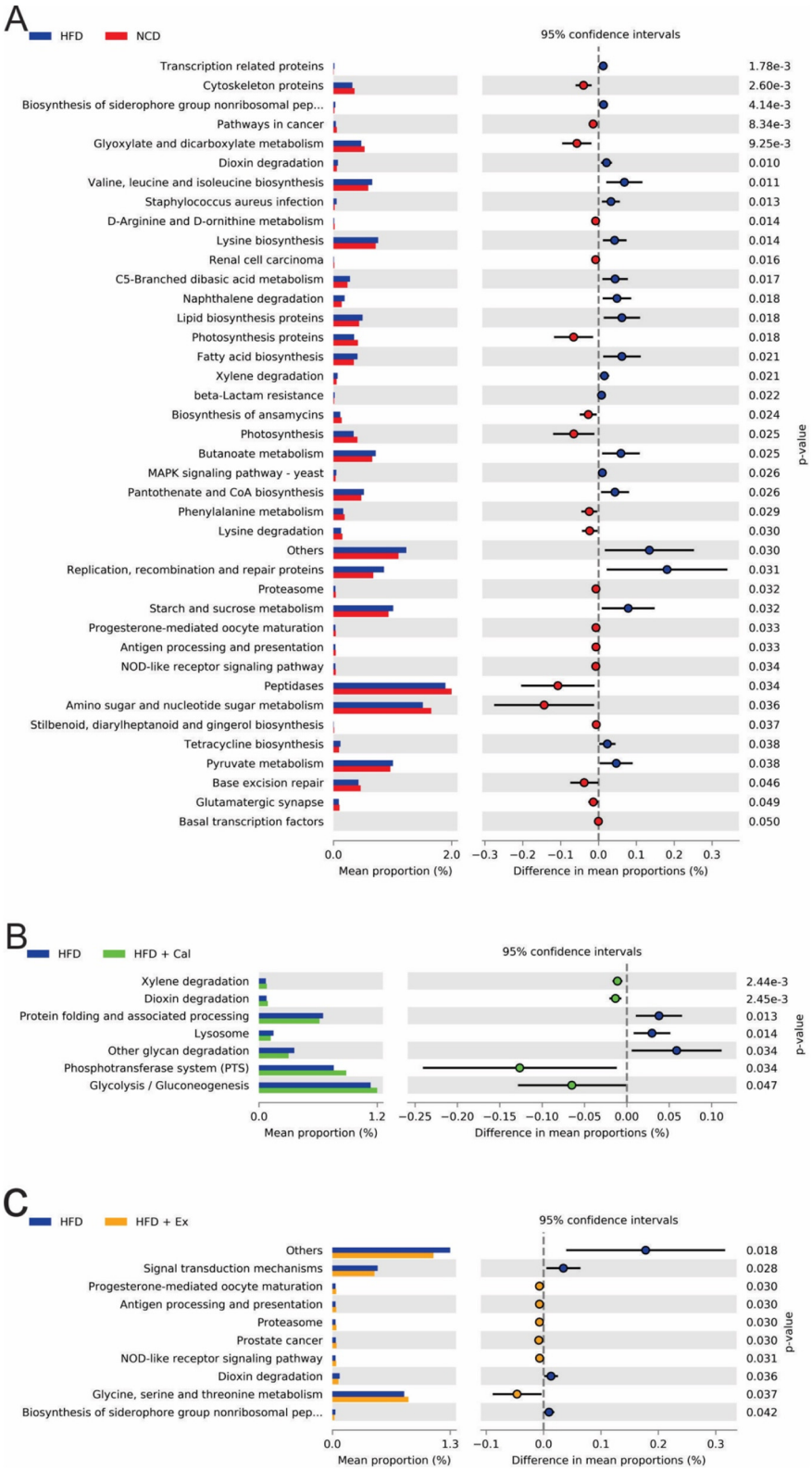

Fig. 8 – Comparison of mean proportion of predicted microbial genes present in colonic samples related to functional categories of (A) HFD vs NCD, (B) HFD vs HFD + Cal, and (C) HFD vs HFD + Ex. Only significant ($P < .05$) functional pathways are shown based on a noncorrected 2-sided Student t test. The colored circles represent the difference in mean proportions of the various functional categories with the 95% confidence interval, as calculated using bootstrap method.

finmarchicus did not alter this pattern significantly, although it led to an overrepresentation of *Lactobacillus* and *Streptococcus* and underrepresentation of *Bilophila*. Administration of the GLP-1 analog exenatide prevented the HFD-induced increase in *Lactococcus*, and in addition, it caused a decrease in the abundance of *Streptococcus*.

The high relative abundance of *Lactococcus* and the near depletion of *Allobaculum* in the HFD group are in agreement with previous animal studies [8,9]. *Lactococcus* is associated with proinflammatory markers [8,13,15], whereas *Allobaculum* is a short-chain fatty acid–producing bacterium which is considered health-promoting. Therefore, HFD used in the current study seems to change the microbiota composition in an unhealthy direction. This view was further supported by the finding that *Oscillospira*, a genus of importance for human health [37] and which has been reported to correlate negatively with body mass index and paracellular permeability in humans [38], was depleted in nearly all HFD mice.

Dietary fat has been shown to modulate gut microbiota [39], and to our knowledge, this is the first study investigating the effect of the oil from *C finmarchicus* on gut microbiota. As mentioned in the introduction, this marine oil contains more than 85% wax esters, consisting of saturated, monounsaturated, and polyunsaturated fatty acids bound to long-chain fatty alcohols. The general view is that the omega-3 fatty acids EPA and DHA represent the active substance in marine oil preparations, but the high content of SDA (7%) and fatty alcohols (about 350 mg/g), which is unique for this novel marine oil, could also be beneficial for human health, [40]. To which extent this fatty acid and fatty alcohols affect the composition of the gut microbiota is, to our knowledge, not known.

Supplementing HFD with 2% calanus oil was not able to restore the microbiota composition to that of the NCD group. Nevertheless, the relative abundance of *Lactobacillus* and *Streptococcus* was above average in the majority of the mice receiving the marine oil. This observation is in line with results by Caesar et al. [14], who reported increased abundance of *Lactobacillus* and *Streptococcus* when they replaced the lard in the high-fat diet with fish oil (Menhaden oil). Our results are also in line with findings by Mujico et al [41], who observed an increase in *Lactobacillus* following supplementation of HFD with EPA and DHA in female mice. Caesar et al. [14] reported reduced body weight gain and reduced inflammation due to an interaction between dietary lipids and the microbiota, which reduced toll-like receptor 4 (TLR4)–induced secretion of monocyte chemoattractant protein-1 (MCP-1/CCL2). Unlike the study by Caesar et al. (2015), we did not detect any differences between the groups with respect to WAT or colon gene expression of MCP-1, TLR4, and other inflammatory markers, which could be due to a relatively short treatment period with the marine calanus oil used in the present study. We observed an antiobesogenic effect of the wax ester–rich oil in terms of a reduced adiposity index. The mice did not, however, show a clear reduction in body weight development, as seen in previous experiments [26,27], and this could be due to a relatively short treatment period (8 vs 27 weeks). In addition, it should be noted that we used female mice in the current experiment and therefore cannot exclude gender differences with respect to the response of the marine oil supplementation. We did however observe an increased level of n-3 PUFA (and decline in n-6 PUFA) in intra-abdominal fat of mice receiving HFD supplemented with calanus oil, which is in line with previous results [18] and demonstrates very clearly that the wax ester in the oil is digested in the intestine is taken up, and thereby contributes to a healthier adipose tissue. In light of the role of omega-3 fatty acids as PPAR ligands [42] and the consequent activation of gene transcription, it was a bit surprising that the reduced adiposity index following calanus oil supplementation was not reflected in any significant change in PPAR expression in adipose tissue. One possibility could be that PPARα was upregulated primarily in metabolically active tissues, such as liver, heart, and skeletal muscle, leading to increased fatty acid oxidation and thereby draining of fatty acids from WAT.

The increased abundance of *Lactobacillus* might be of biological relevance because it is considered a health-promoting, short-chain fatty acid–producing probiotic strain that is often associated with weight loss, regulation of fat metabolism, and anti-inflammation (as reviewed in [13] and [43]). Most species of *Streptococcus* are considered pathogenic, but, for example, *Streptococcus thermophilus* is a probiotic. Unfortunately, our analysis was not capable of distinguishing between the different species of this genus, so the impact of the increased abundance of *Streptococcus* is not clear. Caesar et al. [14] also found a lower abundance of *Bilophila* in the fish oil–fed mice compared to the lard-fed mice. Interestingly, all 5 animals fed HFD + Cal in the current study also showed a below average abundance of *Bilophila*. This genus is shown to increase upon consumption of diets rich in saturated animal fats, and increased abundance of *Bilophila wadsworthia* is associated with inflammatory bowel disease [44]. To find out if the decreased adiposity index in response to dietary oil, as observed in the present study, was due to an enrichment of *Lactobacillus* and/or depletion of *Bilophila*, a larger study needs to be done.

In recent years, the effect of antidiabetic drugs on the gut microbiota has received more and more attention both in humans and in animal models. For example, it is known that the most used antidiabetic drug, metformin, as well as different α-glucosidase inhibitors (acarbose, miglitol, and voglibose), alters the composition of the gut microbiota [45,46]. A few studies examined the effect of liraglutide which, like exenatide, is a GLP-1 receptor agonist [47-49]. Treatment with liraglutide was associated with an increase in *Blautia* and *Turicibacter*, whereas the effect on *Allobaculum* and *Lactobacillus* was less clear and dependent on the animal model in question [47,48]. Wang et al. [47] found that liraglutide-induced weight loss was associated with, among others, *Lactobacillus*, *Turicibacter*, *Coprococcus*, and *Blautia*. The exenatide-treated mice in our study showed decreased body weight and fat mass compared to the HFD mice, and in line with Wang et al. [47], we observed a slight increase in the abundance of *Turicibacter* in this group. However, we did not observe any effect of exenatide on the other "weight reducing" bacteria. Interestingly, exenatide seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are considered pathogenic, while increasing the abundance of *Ruminococcus* and *Dorea*. The latter 2 genera are generally related to insulin resistance and disturbed metabolic health [50,51]. In addition, De Filippis et al. [52] found a correlation between *Ruminococcus* and trimethylamine N-oxide levels, a microbiota-dependent metabolite derived

from trimethylamine-containing nutrients that are abundant in a Western diet and has been associated with obesity, insulin resistance, and increased thrombosis potential in animal and clinical studies [53]. Thus, the previously reported improvement in insulin sensitivity following exenatide treatment [28] could imply the effect of reduced abundance of *Streptococcus* and *Lactococcus* overrides that of the increase in *Ruminococcus* and *Dorea*.

It is currently unknown how GLP-1 receptor agonists induce alterations in the microbiota profile, but changes in pH and nutrient composition in the gut, as well as delay in gut transit time and gastric emptying rate, could be involved [47]. Another proposed mechanism is via the interplay with bile acids [51]. GLP-1 receptor agonists are suggested to induce weight loss via reduced food intake due to inhibition of appetite and reduced gastric emptying. In our study however, we could not detect any difference in food intake between the HFD-fed groups. We therefore suggest that weight loss in response to exenatide treatment could be due to a re-establishment of the abundance of *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Turicibacter*, *Dorea*, and *Ruminococcus* which characterized the lean NCD group.

Functional analysis of the gut microbiota revealed significant changes between the NCD- and the HFD-fed mice. Mice fed an HFD had increased abundance of bacteria with capacity for lipid and fatty acid biosynthesis, that is, substrates which could become available as nutrients or bioactive compounds for the host and explain the observed increase in body weight and adiposity index in this group. The microbial composition of the HFD + Cal group was associated with increased activities of the phosphotransferase system, glycolysis, and gluconeogenesis compared to the HFD mice. These 2 functions enable the bacteria to take up and metabolize carbohydrates, producing metabolites which could potentially be used by the host. To determine if this is related to the reduced adiposity index in the mice fed oil from *C finmarchicus*, a further in-depth study is needed. Functional analysis of the microbiota from HFD + Ex mice showed upregulation of signal transduction pathways, but the implication of this observation also needs further studies.

This is the first study examining the effect of calanus oil, a wax ester–containing oil derived from *C finmarchicus*, on the gut microbiota composition. Obesity in female mice was associated with an enrichment of the proinflammatory *Lactococcus* and a depletion of the anti-inflammatory and health-promoting *Allobaculum* and *Oscillospira*, changing the microbiota composition in an unhealthy direction. Although dietary oil from *C finmarchicus* was not able to restore the microbiota composition to that of the lean control group, it resulted in a relatively high abundance of *Lactobacillus*, a health-promoting genus, which is often related to weight loss. It also reduced the abundance of the pathogenic *Bilophila* genus. Treatment with exenatide partly restored the bacterial profile found in the lean control group and seemed to reduce the abundance of *Streptococcus* and *Lactococcus*, which are both considered pathogenic. These data confirm our hypothesis, namely, that dietary supplementation with the antiobesogenic calanus oil is able to antagonize the unfavorable changes in the gut microbiota induced by high-fat feeding.

Differences in sample preservation and DNA extraction, library preparation, as well as use of different reference databases or software programs make it difficult to compare results from one laboratory to another [54-56]. In this study, the number of animals in each group was relatively small (n = 5), and hence, the power of the statistical analysis was relatively low. In addition, determination of the microbiota composition was based only on feces samples from the colon.

Acknowledgment

This work was supported by UiT The Arctic University of Norway, the Northern Norway Regional Health Authority (HNF-1341-17), and Calanus AS (Tromso, Norway). Calanus AS provided the oil that was used in the study, and one of the employees contributed with the investigation, resources, writing, reviewing, and editing the manuscript. TSL has a small position as scientific advisor in Calanus AS. AMP is employed as product manager in Calanus AS. PCS, KMJ, RLO, and JM declare no conflict of interest. We thank Erik Hjerde and Espen Åberg from the Department of Chemistry at UiT the Arctic University of Norway for their help regarding bioinformatics and translating the sequence data to taxonomic and functional data.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TSL has a small position as scientific advisor in Calanus AS. AMP is employed as product manager in Calanus AS. PCS, KMJ, RLO, and JM declare no conflict of interest.

Supplemental materials

Supplemental materials were provided that include diet information; Table S1, Overview of all identified functional categories; Figure S1, Gene expression of markers related to inflammation, intestine permeability, and fat metabolism in the colon of female mice; and Figure S2, Gene expression of markers related to inflammation and fat metabolism in omental WAT of female mice. Supplementary data to this article can be found online at https://doi.org/10.1016/j.nutres.2020.09.002.

REFERENCES

[1] Tindall AM, Petersen KS, Kris-Etherton PM. Dietary patterns affect the gut microbiome—the link to risk of cardiometabolic diseases. J Nutr. 2018;148:1402–7. https://doi.org/10.1093/jn/nxy141.

[2] Boulangé CL, Neves AL, Chilloux J, Nicholson JK, Dumas M-E. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Med. 2016;8:42. https://doi.org/10.1186/s13073-016-0303-2.

[3] Winer DA, Luck H, Tsai S, Winer S. The intestinal immune system in obesity and insulin resistance. Cell Metab. 2016;23: 413–26. https://doi.org/10.1016/j.cmet.2016.01.003.
[4] Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxima-induced inflammation in high-fat diet–induced obesity and diabetes in mice. Diabetes. 2008;57:1470–81. https://doi.org/10.2337/db07-1403.Additional.
[5] Graham C, Mullen A, Whelan K. Obesity and the gastrointestinal microbiota: a review of associations and mechanisms. Nutr Rev. 2015;73:376–85. https://doi.org/10.1093/nutrit/nuv004.
[6] Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. Br J Nutr. 2014;111: 387–402. https://doi.org/10.1017/S0007114513002560.
[7] Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen Y, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137:1716–24 e2 https://doi.org/10.1053/J.GASTRO.2009.08.042.
[8] Ravussin Y, Koren O, Spor A, Leduc C, Gutman R, Stombaugh J, et al. Responses of gut microbiota to diet composition and weight loss in lean and obese mice. Obesity. 2012;20:738–47. https://doi.org/10.1038/oby.2011.111.
[9] Hamilton MK, N MK, Boudry G, Lemay DG, Raybould HE. Changes in intestinal barrier function and gut microbiota in high-fat diet–fed rats are dynamic and region dependent. Am J Physiol Liver Physiol. 2015;308:G840–51. https://doi.org/10.1152/ajpgi.00029.2015.
[10] Rajkumar H, Mahmood N, Kumar M, Varikuti SR, Challa HR, Myakala SP. Effect of probiotic (VSL#3) and omega-3 on lipid profile, insulin sensitivity, inflammatory markers, and gut colonization in overweight adults: a randomized, controlled trial. Mediators Inflamm. 2014;2014. https://doi.org/10.1155/2014/348959.
[11] Pu S, Khazanehei H, Jones PJ, Khafipour E. Interactions between obesity status and dietary intake of monounsaturated and polyunsaturated oils on human gut microbiome profiles in the canola oil multicenter intervention trial (COMIT). Front Microbiol. 2016;7:1–14. https://doi.org/10.3389/fmicb.2016.01612.
[12] Watson H, Mitra S, Croden FC, Taylor M, Wood HM, Perry SL, et al. A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. Gut. 2018;67:1974–83. https://doi.org/10.1136/gutjnl-2017-314968.
[13] Costantini L, Molinari R, Farinon B, Merendino N. Impact of omega-3 fatty acids on the gut microbiota. Int J Mol Sci. 2017; 18:2645. https://doi.org/10.3390/ijms18122645.
[14] Caesar R, Tremaroli V, Kovatcheva-Datchary P, Cani PD, Bäckhed F. Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. Cell Metab. 2015;22:658–68. https://doi.org/10.1016/j.cmet.2015.07.026.
[15] Cui C, Li Y, Gao H, Zhang H, Han J, Zhang D, et al. Modulation of the gut microbiota by the mixture of fish oil and krill oil in high-fat diet–induced obesity mice. PLoS One. 2017;12:1–18. https://doi.org/10.1371/journal.pone.0186216.
[16] Lee R, Hagen W, Kattner G. Lipid storage in marine zooplankton. Mar Ecol Prog Ser. 2006;307:273–306. https://doi.org/10.3354/meps307273.
[17] Cook CM, Larsen TS, Derrig LD, Kelly KM, Tande KS. Wax ester rich oil from the marine crustacean, *Calanus finmarchicus*, is a bioavailable source of EPA and DHA for human consumption. Lipids. 2016;51:1137–44. https://doi.org/10.1007/s11745-016-4189-y.
[18] Pedersen AM, Salma W, Höper AC, Larsen TS, Olsen RL. Lipid profile of mice fed a high-fat diet supplemented with a wax ester–rich marine oil. Eur J Lipid Sci Technol. 2014;116: 1718–26. https://doi.org/10.1002/ejlt.201400052.
[19] Gurr MI, Harwood JL, Frayn KN, Murphy DJ, Michell RH. Lipids: biochemistry, biotechnology and health. John Wiley & Sons; 2016.
[20] Hargrove JL, Greenspan P, Hartle DK. Nutritional significance and metabolism of very long chain fatty alcohols and acids from dietary waxes. Exp Biol Med. 2004;229:215–26. https://doi.org/10.1177/153537020422900301.
[21] Carey MC, Small DM, Bliss CM. Lipid digestion and absorption. Annu Rev Physiol. 1983;45:651–77. https://doi.org/10.1146/annurev.ph.45.030183.003251.
[22] Cowey CB, Sargent JR. Lipid nutrition in fish. Comp Biochem Physiol Part B Comp Biochem. 1977;57:269–73. https://doi.org/10.1016/0305-0491(77)90051-7.
[23] Verschuren PM, Nugteren DH. Evaluation of jojoba oil as a low-energy fat. 2. Intestinal transit time, stomach emptying and digestibility in short-term feeding studies in rats. Food Chem Toxicol. 1989;27:45–8. https://doi.org/10.1016/0278-6915(89)90091-4.
[24] Della Vedova MC, Muñoz MD, Santillan LD, Plateo-Pignatari MG, Germanó MJ, Rinaldi Tosi ME, et al. A mouse model of diet-induced obesity resembling most features of human metabolic syndrome. Nutr Metab Insights. 2016;9:93–102. https://doi.org/10.4137/NMI.S32907.
[25] Echeverriá F, Valenzuela R, Bustamante A, Álvarez D, Ortiz M, Espinosa A, et al. High-fat diet induces mouse liver steatosis with a concomitant decline in energy metabolism: attenuation by eicosapentaenoic acid (EPA) or hydroxytyrosol (HT) supplementation and the additive effects upon EPA and HT co-administration. Food Funct. 2019;10:6170–83. https://doi.org/10.1039/c9fo01373c.
[26] Höper AC, Salma W, Sollie SJ, Hafstad AD, Lund J, Khalid AM, et al. Wax esters from the marine copepod *Calanus finmarchicus* reduce diet-induced obesity and obesity-related metabolic disorders in mice. J Nutr. 2014;144:164–9. https://doi.org/10.3945/jn.113.182501.
[27] Höper AC, Salma W, Khalid AM, Hafstad AD, Sollie SJ, Raa J, et al. Oil from the marine zooplankton *Calanus finmarchicus* improves the cardiometabolic phenotype of diet-induced obese mice. Br J Nutr. 2013;110:2186–93. https://doi.org/10.1017/S0007114513001839.
[28] Mack CM, Moore CX, Jodka CM, Bhavsar S, Wilson JK, Hoyt JA, et al. Antiobesity action of peripheral exenatide (exendin-4) in rodents: effects on food intake, body weight, metabolic status and side-effect measures. Int J Obes (Lond). 2006;30: 1332–40. https://doi.org/10.1038/sj.ijo.0803284.
[29] Fliegerova K, Tapio I, Bonin A, Mrazek J, Callegari ML, Bani P, et al. Effect of DNA extraction and sample preservation method on rumen bacterial population. Anaerobe. 2014;29: 80–4. https://doi.org/10.1016/J.ANAEROBE.2013.09.015.
[30] Milani C, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, et al. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS One. 2013;8: e68739. https://doi.org/10.1371/journal.pone.0068739.
[31] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Alghalith GA, et al. QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science; 2018. https://doi.org/10.7287/peerj.preprints.27295v2.
[32] Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. PeerJ. 2016; 2016. https://doi.org/10.7717/peerj.2584.
[33] Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31:814–21. https://doi.org/10.1038/nbt.2676.
[34] Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles.

Bioinformatics. 2014;30:3123–4. https://doi.org/10.1093/bioinformatics/btu494.
[35] Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;71:8228–35. https://doi.org/10.1128/AEM.71.12.8228-8235.2005.
[36] Jansen KM, Moreno S, Garcia-Roves PM, Larsen TS. Dietary Calanus oil recovers metabolic flexibility and rescues postischemic cardiac function in obese female mice. Am J Physiol Circ Physiol. 2019;317:H290–9. https://doi.org/10.1152/ajpheart.00191.2019.
[37] Konikoff T, Gophna U. Oscillospira: a central, enigmatic component of the human gut microbiota. Trends Microbiol. 2016;24:523–4. https://doi.org/10.1016/j.tim.2016.02.015.
[38] Tims S, Derom C, Jonkers DM, Vlietinck R, Saris WH, Kleerebezem M, et al. Microbiota conservation and BMI signatures in adult monozygotic twins. ISME J. 2013;7:707–17. https://doi.org/10.1038/ismej.2012.146.
[39] Cândido FG, Valente FX, Grześkowiak ŁM, Moreira APB, Rocha DMUP, de CG Alfenas R. Impact of dietary fat on gut microbiota and low-grade systemic inflammation: mechanisms and clinical implications on obesity. Int J Food Sci Nutr. 2018;69:125–43. https://doi.org/10.1080/09637486.2017.1343286.
[40] Schots PC, Pedersen AM, Eilertsen KE, Olsen RL, Larsen TS. Possible health effects of a wax ester rich marine oil. Front Pharmacol. 2020;11:961. https://doi.org/10.3389/fphar.2020.00961.
[41] Mujico JR, Baccan GC, Gheorghe A, Díaz LE, Marcos A. Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice. Br J Nutr. 2013;110:711–20. https://doi.org/10.1017/S0007114512005612.
[42] Echeverría F, Ortiz M, Valenzuela R, Videla LA. Long-chain polyunsaturated fatty acids regulation of PPARs, signaling: relationship to tissue development and aging. Prostaglandins Leukot Essent Fat Acids. 2016;114:28–34. https://doi.org/10.1016/j.plefa.2016.10.001.
[43] Danneskiold-Samsøe NB, Dias de Freitas Queiroz Barros H, Santos R, Bicas JL, CBB Cazarin, Madsen L, et al. Interplay between food and gut microbiota in health and disease. Food Res Int. 2019;115:23–31. https://doi.org/10.1016/J.FOODRES.2018.07.043.
[44] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505:559–63. https://doi.org/10.1038/nature12820.
[45] Kyriachenko Y, Falalyeyeva T, Korotkyi O, Molochek N, Kobyliak N. Crosstalk between gut microbiota and antidiabetic drug action. World J Diabetes. 2019;10:154. https://doi.org/10.4239/WJD.V10.I3.154.
[46] Montandon SA, Jornayvaz FR. Effects of antidiabetic drugs on gut microbiota composition. Genes (Basel). 2017;8. https://doi.org/10.3390/genes8100250.
[47] Wang L, Li P, Tang Z, Yan X, Feng B. Structural modulation of the gut microbiota and the relationship with body weight: compared evaluation of liraglutide and saxagliptin treatment. Sci Rep. 2016;6:1–10. https://doi.org/10.1038/srep33251.
[48] Zhao L, Chen Y, Xia F, Abudukerimu B, Zhang W, Guo Y, et al. A glucagon-like peptide-1 receptor agonist lowers weight by modulating the structure of gut microbiota. Front Endocrinol (Lausanne). 2018;9:233. https://doi.org/10.3389/fendo.2018.00233.
[49] Moreira G, Azevedo F, Ribeiro L, Santos A, Guadagnini D, Gama P, et al. Liraglutide modulates gut microbiota and reduces NAFLD in obese mice. J Nutr Biochem. 2018;62:143–54. https://doi.org/10.1016/J.JNUTBIO.2018.07.009.
[50] Basso N, Soricelli E, Castagneto-Gissey L, Casella G, Albanese D, Fava F, et al. Insulin resistance, microbiota, and fat distribution changes by a new model of vertical sleeve gastrectomy in obese rats. Diabetes. 2016;65:2990–3001. https://doi.org/10.2337/db16-0039.
[51] Caesar R. Pharmacologic and nonpharmacologic therapies for the gut microbiota in type 2 diabetes. Can J Diabetes. 2019;43:224–31. https://doi.org/10.1016/J.JCJD.2019.01.007.
[52] De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut. 2016;65:1812–21. https://doi.org/10.1136/gutjnl-2015-309957.
[53] Brown JM, Hazen SL. Targeting of microbe-derived metabolites to improve human health: the next frontier for drug discovery. J Biol Chem. 2017;292:8560–8. https://doi.org/10.1074/JBC.R116.765388.
[54] Wesolowska-Andersen A, Bahl M, Carvalho V, Kristiansen K, Sicheritz-Pontén T, Gupta R, et al. Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. Microbiome. 2014;2:19. https://doi.org/10.1186/2049-2618-2-19.
[55] Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the gut microbiome using 16S or shotgun metagenomics. Front Microbiol. 2016;7:459. https://doi.org/10.3389/fmicb.2016.00459.
[56] Costea PI, Zeller G, Sunagawa S, Pelletier E, Alberti A, Levenez F, et al. Towards standards for human fecal sample processing in metagenomic studies. Nat Biotechnol. 2017;35:1069. https://doi.org/10.1038/nbt.3960.