Lysates of *Methylococcus capsulatus* Bath induce a lean-like microbiota, intestinal FoxP3⁺RORγt⁺IL-17⁺ Tregs and improve metabolism

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BAHJ, JBH, CRK, KK and TEL conceived and designed the study. BAHJ, JBH, ISL, SBS and MTFD conducted the in vivo experiments. BAHJ, KK and TEL supervised all parts of the study. JTT, AM, LM, WA and CS supervised parts of the study. NvB, AR performed flowcytometry analyses. BAHJ, JBH, ISL, SD, CPÅ, NvB, ALA, AR, SAI, YJA, KS, EF, MTFD, and SIP performed key experiments and analyzed the data. BAHJ integrated the data and wrote the manuscript with inputs from JBH, ISL, KK and TEL. All authors edited, revised and approved the final version of the manuscript.

Conflicts of interest:

BAHJ, JBH, ISL, KK, CRK and TEL are co-inventors of International (PCT) Patent Application No. PCT/EP2018/071076 based on the enclosed data.

Funding:

This work was supported by the Norwegian Research Council (project 267655). B.A.H.J. was supported by Lundbeck Foundation (grant number: R232-2016-2425) and Novo Nordisk Foundation (grant number: NNF17OC0026698). A-L.G. and A.M. were supported by Canadian Institutes for Heart Research and Sentinel North from the Canada First Research Excellence Fund.

Abstract:

Interactions between host and gut microbial communities may be modulated by diets and play pivotal roles in securing immunological homeostasis and health. Here we show that intake of feed based on whole-cell lysates of the non-commensal bacterium *Methylococcus capsulatus* Bath (McB) as protein source reversed high fat high sucrose-induced changes in the gut microbiota to a state resembling that of lean, low fat diet-fed mice, both under mild thermal stress (T_{22°C}) and at thermoneutrality (T_{30°C}). McB feeding selectively upregulated triple positive (Foxp3⁺RORγt⁺IL-17⁺) regulatory T cells in the small intestine and colon, and enhanced mucus production and glycosylation status suggesting improved gut health. Mice receiving McB lysates further exhibited improved glucose regulation, reduced body and liver fat along with diminished hepatic immune infiltration. Collectively, these data points towards profound whole-body effects elicited by the McB lysate suggesting that it may serve as a potent modulator of immunometabolic homeostasis.

Introduction:

Gut microbes shape intestinal immunity¹ and increase the bioavailability of otherwise indigestible nutrients². A well-balanced community structure is therefore essential for immunometabolic homeostasis, whereas aberrant gut microbiota compositions associate with numerous diseases, both within and outside the gastrointestinal tract³.

While therapeutic implications of rebalancing a mistuned gut microbiota appear promising, inconsistent response rates in relation to both probiotics and fecal transfer studies, with occasional adverse events, emphasize the complexity of such approaches. One example relates to the otherwise promising probiotic candidate *Akkermansia muciniphila*⁴, where negative effects have been seen in immunocompromised recipients^{5,6}. Similarly, *Prevotella copri* aids in metabolizing fibers in healthy individuals⁷ and protects against bacterial invasion in high fiber, chow-fed mice⁸, yet associates with insulin resistance in prediabetic obese individuals and precipitates glucoregulatory impairments in diet-induced obese (DIO) mice⁹.

An alternative to administering viable microbes is to utilize whole cell lysates, or selected cell components, of non-living bacteria. Apart from alleviating global energy demands, if used as a nutrient source, such components may also potentially affect host physiology as recently reported for *A. muciniphila*¹⁰ and *Bifidobacterium bifidum*¹¹. In the latter example, cell surface polysaccharides of *B. bifidum* were used to induce peripheral immune-tolerance via generation of regulatory T cells (T_{regs}). The authors reported a pronounced increase in Foxp3⁺RORγt⁺ T_{regs} (pT_{regs}), specifically in lamina propria (LP) of the large intestine (LI)¹¹. This cell type is believed to be induced by commensal microbes and has emerged as a potent T_{reg} subset, exhibiting increased lineage stability and enhanced immunosuppressive capacity during intestinal inflammation compared to conventional Foxp3⁺RORγt⁻ T_{regs} (nT_{regs})¹².

RORγt is the canonical transcription factor controlling IL-17 expression; a pleiotropic cytokine with both proinflammatory and immune-resolving actions depending on the eliciting cell type and physiological context¹³. Unfortunately, the studies describing pT_{reg} function did not measure IL-17 secretion. It therefore remains unknown whether these cells exhibit normal, reduced or increased IL-17 levels, and how this translates to host physiology. The impact of this predominantly colonic cell subset on host metabolism also remains unknown. Still, mounting evidence points towards the importance of intestinal IL-17 for controlling metabolic homeostasis^{14,15}. IL-17⁺ pT_{regs} may

therefore be leveraged as a ‘dual hit’ strategy to curb immunometabolic dysfunction and gastrointestinal disturbances based on the immune-regulatory capacity of pT_{regs} concomitant with the metabolic benefits of gut-delivered IL-17. To this end, we hypothesised that environmental bacteria, who have not been under evolutionary scrutiny for host-microbe interactions, would provide an unexplored reservoir of immunomodulatory stimuli. In support of this hypothesis, the methanotropic non-commensal bacterium *Methylococcus capsulatus* Bath (McB) has previously been shown to reduce inflammation and disease activity in dextran sulfate sodium (DSS)-induced colitis²⁴. However, the impact on host metabolism and intestinal immune cells as well as mucus dynamics was not addressed.

We accordingly explored the effect of using whole-cell lysates from McB as protein source to reshape immunometabolism and the aberrant gut microbiota of DIO mice. We show that McB lysates augment Foxp3⁺RORγt⁺IL-17⁺ triple positive pT_{regs} in both SI- and LI-LP, and resets the obese microbiota concomitant with reversed key disease traits of diet-induced obesity.

Materials and Methods:

Mice and Ethical Statements

All experiments were conducted in accordance with the EU directive 2010/63/EU as approved by the Danish Animal Experiments Inspectorate (#2014-15-2934-01,027). 6-7-week-old male C57BL/6JBomTac and C57BL/6JRj mice were acquired from Taconic Laboratories, Denmark, or Janvier Labs, France, respectively, as detailed below. All mice were allowed to acclimatize on regular chow diet for the first week upon arrival and then fed a LFD for two weeks prior to study initiation. Mice were kept under specific pathogen free conditions at 22 °C (T_{22°C}) or 30 °C (T_{30°C}), as indicated, in 12 h light/dark cycle (6AM–6PM).

Housing, Diets and Experimental setup

Standard pelleted diets as well as protein-free western diet (WD) powder were obtained from Ssniff Spezialdiäten GmbH, Germany, and stored at -20 °C throughout the duration of the experiment. Mice were fed a compositionally defined low fat diet (LFD, S8672-E050) until study initiation. A subgroup remained on LFD, whereas the remaining mice were transferred to a soy oil based reference WD (S8672-E025) (high fat, high sucrose, containing 0.15% cholesterol) for the run-in

period (12 or 21 weeks dependent on the experiment). Experimental WDs were lot-matched and produced without protein (S9552-E021), which was subsequently added, and then pelleted, by the investigators as indicated; WD_{REF} = containing 19.5% (w/w) casein, WD_{CNTL} = containing 16.5% (w/w) casein and 3% Macadamia oil, WD_{McB} = 19.5% (w/w) whole-cell bacterial lysates (predominantly protein but up to ~15% phospholipids¹⁶). Diet composition is more extensively described in Table S1. Detailed description of bacterial lysates is provided in the designated section below. All diets were freshly made for each experiment with independent batches of bacterial lysates to substantiate robustness and reproducibility of the observed findings. Mice were fed *ad libitum*, and feed intake was measured thrice a week. Water was changed weekly.

12+6 protocol T₂₂°C

Experimental setup is outlined in Figure 1A. C57BL/6JRj mice were purchased from Janvier Labs, France, and housed 3 mice per cage, reproduced in two independent experiments. For transparency, all data are depicted in respective graphs with mean ± SEM plus individual dots for the combined results of the two independent experiments. Experiment-dependent shapes enables visual discrimination of respective experiments as well as data distribution within and between experiments.

21+5 protocol T₃₀°C

Experimental setup is outlined in Figure 4A. C57BL/6JBomTac mice were purchased from Taconic Laboratories, Denmark, and initially housed 10 per cage to accelerate weight gain. 12 weeks into the run-in period, mice were single caged to allow accurate assessment of food intake, and also monitor if the more sensitive microbiome of single-housed mice would uniformly change towards a composition typical for LFD-fed mice, or if such phenomenon relied on a few highly responding mice transferring their microbiome to cage mates under group housed conditions. The timing of single housing allowed affected mice to adapt to social isolation and stabilize their weight development before intervention (Figure S4B).

Cultivation of *M. capsulatus* Bath and preparation of bacterial lysate.

Methylococcus capsulatus Bath (NCIMB 11132) was cultivated in nitrate mineral salts (NMS) medium as previously described¹⁷ to produce the single-strain bacterial lysate. McB culture aliquots were frozen in liquid nitrogen and stored at −80°C. Cultivations on agar plates and in shake flasks

(orbital shaker incubator at 200 rpm) were performed at 45°C in an atmosphere of 75% air, 23.25% CH₄, and 1.25% CO₂. Continuous cultivation was carried out in a 3-liter bioreactor (Applikon, The Netherlands) with a working volume of 2 liters. Cells were pre-cultivated in shake flasks and used to inoculate the bioreactor to an optical density at 440 nm (OD₄₄₀) of 0.1. The temperature was maintained at 45°C, stirring set to 650 rpm, and pH maintained at 6.8 by automatic addition of 2.5 M NaOH/2.5 M HCl. A gas mixture of 75% air and 25% methane was sparged into the bioreactor. The continuous culture was started after an initial batch phase, and the dilution rate was set to 0.01 h⁻¹. The OD₄₄₀ at steady state was generally sustained at approximately 10. Culture effluent was collected, and cells were harvested by centrifugation. Bacterial cell walls were disrupted by the use of a French press before freeze-drying of the material.

Glucose and insulin tolerance tests

Mice were subjected to magnetic resonance (MR)-scan using EchoMRI 4in1 (Texas, USA) to determine fat- and lean mass at indicated time points (Figure 1A, 4A). Mice were fasted 5 or 2 hours prior to any oral glucose tolerance (OGTT) or intraperitoneal insulin tolerance test (ITT), respectively. Fasting blood glucose was measured by tail vein bleeding using the Bayer Contour glucometer (Bayer Health Care). Mice were subsequently gavaged with 3 µg glucose/g lean mass (OGTT) or intraperitoneally injected with 0.75 mU insulin/g lean mass (ITT). Blood were sampled at specified time points for blood glucose and insulin measurements to assess glucoregulatory capacity including glucose stimulated insulin secretion (GSIS) as described in detail elsewhere¹⁸.

Short chain fatty acid measurements

Short chain fatty acids (SCFAs) were determined by gas chromatographic analysis. Feces were suspended in MilliQ water (1:1 (w/v)) and subsequently homogenized in a FastPrep-96 (MP Biomedicals) sample preparation unit. The homogenates were diluted with 0.4% formic acid (1:1 (v/v)), transferred to Eppendorf tubes and centrifuged at 13,000 rpm for 10 minutes. 300 µl of the supernatants were applied to spin columns (VWR, 0.2 µm pore size) and centrifuged at 10,000 rpm for 5 minutes. The eluates were transferred to 300 µl GC vials. Split injection mode was used with an injection volume of 0.2 µl. The gas chromatograph was a Trace 1310 (Thermo Scientific) equipped with an autosampler and a flame ionization detector. Helium was used as carrier gas, and the column was a 30 m long Stabilwax (Restek) with polyethylene glycol as stationary phase. Injector temperature: 250 °C, temperature intervals: 2 minutes at 90 °C before a 6 min increase to

150 °C , then a 2 minutes increase to 245 °C and a hold at this temperature for 4.9 minutes. Detector temperature: 275 °C.

RNA extraction and quantitative RT-PCR

Frozen liver tissue was cryo-grinded by mortar and pestle on liquid nitrogen and total RNA was extracted by TRIreagent (Sigma-Aldrich, USA) according to the manufacturer's protocol using PRECELLYS® 24 for homogenization. One µg of RNA was transcribed into cDNA by reverse transcriptase (Invitrogen, USA). Quantitative PCR analyzes were performed using the SYBR Green qPCR Master mix (Thermo Scientific, USA) and the Stratagene Mx3000P qPCR System. Standard curve and no template control (NTC) were included on every 96-well plates. Validation of each target was based on Rsq of standard curve >0.995, amplification efficiency of 100±10% and singular peak. Ct values for each target were related to 18S reference Ct values of the same sample by $2^{\Delta Ct}$ and visualized as fold-change to the LFD reference group mean. Primer sequences are summarized in Table S2.

Histology

Liver tissue was sampled promptly after euthanasia, fixed in 10% formalin and subsequently embedded in paraffin prior to sectioning according to standard procedures for light microscopy. Colon was emptied for content and subsequently rolled around a 27G needle after which the generated Swizz Rolls were carefully preserved in liquid nitrogen (wrapped in aluminum foil) until paraffin embedding. Embedded tissues were cut 2 µm thick, and sections mounted onto glass slides. These sections were processed further and stained either with hematoxylin and eosin (H&E), Oil Red O, PAS, or HID-AB, or labelled with antibody of interest for immunohistochemical investigations, as detailed below. Samples were randomized and blinded to the pathologists performing the histological analyses.

Liver

Non-alcoholic fatty liver disease activity score (NAS)

Liver sections were prepared by standard protocols and subsequently assessed by two independent observers in a blinded fashion, using the established NAFLD activity score (NAS) for evaluation of H&E stained liver sections¹⁹. In short, score of 0 to 2 excludes non-alcoholic steatohepatitis (NASH), a score of 3–4 defines “borderline NASH”, and score ≥ 5 is considered as NASH.

233

234 *Oil Red O staining*

235 Liver biopsies were fixed in paraformaldehyde, cryoprotected in polyvinylpyrrolidone/saccharose,
236 and frozen in liquid nitrogen. Cryosections were prepared and stained using the lipid-specific Oil
237 red O (Sigma Aldrich, Germany) and analyzed under an Axio Imager M2 microscope. Images were
238 captured with the Axiocam 506 color camera (Zeiss, Jena, Germany). Areas of stained lipids were
239 determined using ImageJ-1.50i software (<https://imagej.nih.gov/ij/index.html>).

240

241 Colon

242 The morphological and morphometrical evaluations of the colonic tissues were performed in a
243 blinded fashion, and all measurements were made by the same trained pathologist. One H&E
244 stained section of colon from each individual was evaluated histologically for any sign of
245 pathology. To assess the quality of the mucins, colonic tissues were stained for acidic as well as
246 neutral mucins. Neutral mucins were stained by Periodic Acid Schiff's (PAS). Acidic mucins were
247 stained through a combination of High Iron Diamine (HID), for sulfated mucins (sulfomucins), and
248 Alcian Blue (AB) for carboxylated mucins (sialomucins). Crypt depth (CD) measurements, as well
249 as analyses of mucin type and amount, were performed on HID/AB- and PAS-stained sections in
250 three histologically distinct areas of the colon, i.e. the proximal, middle and distal area. At each of
251 these three locations, three longitudinally sectioned crypts were measured. Each data point is thus
252 presented as an average of three (mucin type and amount) or six (CD) measures. Crypts were
253 selected only when the entire crypt epithelium was visible from the *lamina muscularis mucosae* to
254 the lumen. The histological sections of the colon were examined in an Axio Imager Z2 microscope
255 (Zeiss, Jena, Germany), and digital images were obtained using an Axiocam 506 color camera
256 (Zeiss, Jena, Germany). Micrographs were captured with the same 20× objective magnification.
257 Crypt depth measurements were performed using the software program Image J-Fiji version 1.52e
258 Java 1.8.0_181²⁰. For quantitative evaluation and automated scoring of the different mucin types, a
259 plugin for color deconvolution for the Image J-Fiji program was used²¹.

260

261 **Hepatic lipidomics**

262 Samples were randomized and lipid extracted as previously described. In short, 50 mg of frozen
263 tissue were homogenized in a pre-chilled 2 mL tube added 0.5 mL of ice-cold 50% MeOH with ~40
264 μM D5-tryptophan following standard protocols and stored at -80°C until LCMS analysis. The

peaklist obtained after preprocessing (features defined by their mass/charge value, retention time and peak area) was analyzed in MetaboAnalyst 4. Data were then auto-scaled (mean-centering and division by the square root of standard deviation of each variable) to enforce Gaussian distribution enabling relative comparison. Univariate and multivariate analysis were performed with t-tests, volcano plot, analyses of variance (ANOVA) followed by Tukey's HSD test, and principal component analysis (PCA) to detect significant hits and to visually separate trends between groups. Features showing a similar pattern between LFD and WD_{M_{CB}} were identified by pattern matching function. The top 25 features obtained were then processed for identification using the online database Lipidmaps (<http://lipidmaps.org>) with a mass tolerance between the measured m/z value and the exact mass of 3 ppm.

Microbiome analysis and bioinformatic processing

Fresh feces samples were collected 3-4 hours into the light cycle, snap frozen and stored at -80°C until downstream processing. Bacterial DNA from fecal samples was extracted using a NucleoSpin soil kit (Macherey-Nagel) according to manufacturer's instructions. 16S rRNA gene amplification, library preparation and sequencing were performed as previously described²². Initial data processing was performed as described elsewhere²³. Data was rarefied to 23,107 reads per sample. The principal coordinate analysis (PCoA) was conducted based on the weighted UniFrac distance. To select OTUs and species enriched in different subgroups, DESeq2²⁴ with default settings was used. The relative abundances of OTUs were aggregated to genus level. Genera present in >1/3 of the samples were used in the differential abundance testing. Genera with p.adj. < 0.05 comparing the last sampling point for WD_{REF}/WD_{CNTL} vs WD_{M_{CB}} with DESeq2 differential expression analysis based on the negative binomial distribution were reported. Permutational multivariate analysis of variance (permanova) using weighted UniFrac distance matrices (Adonis, Vegan R package) was used to evaluate overall microbial compositions.

Isolation of small intestine (SI) and large intestine (LI) lamina propria (LP) cells

Feces and mucus from LI were scraped off while SI was flushed with 1x HBSS (Gibco) containing 15 mM HEPES (Thermo Scientific). Peyer's patches were carefully excised from SI. SI and LI were opened longitudinally, cut into approx. 1 cm pieces and washed 3 times with 1x HBSS (Gibco) containing 15 mM HEPES (Thermo Scientific), 5% FCS (ViralexTM, PAA Laboratories), 50 µg/mL gentamycin (Gibco) and 2 mM EDTA (Invitrogen, Life Technologies) for 15 min at 37

°C. During the first incubation step, 0.15 mg/mL DL-dithiothreitol (Sigma-Aldrich) was additionally added to LI samples. LI, but not SI samples, were shaken on an orbital shaker at 350 rpm during incubation. After each incubation step, SI, but not LI samples, were shaken by hand for 10 s. Media containing epithelial cells and debris were discarded by filtration through a 250 µm mesh (Tekniska Precisionsfilter, JR AB). The remaining tissue was digested for 25-28 min at 37 °C under magnetic stirring (500 rpm) in R-10 media (RPMI 1640 (Gibco) with 10% FCS (Viralex, PAA Laboratories), 10 mM HEPES (Thermo Scientific), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 50 µg/mL gentamycin (Gibco), 50 µM 2-mercaptoethanol (Gibco) and 1 mM sodium pyruvate (Gibco)) containing 1 mg/ml collagenase P (Roche) and 0.03 mg/mL DNase I (Roche). After digestion, SI-LP and LI-LP cells were purified by density gradient centrifugation (600 *rcf* for 20 min at 22 °C, acceleration 5 and brake 0) with 40/70 % Percoll (GE Healthcare). Cell suspensions were subsequently filtered through 100 µm cell strainers (BD Biosciences) and re-stimulated *in vitro* prior to staining for flow cytometric analysis.

***Ex vivo* stimulation of SI-LP and LI-LP cells**

SI-LP and LI-LP cells were re-stimulated *in vitro* as previously described²⁵. Briefly, cells were simulated in R-10 medium in the presence of either 20 ng/mL IL-23 (R&D Systems) or 250 ng/mL PMA (Sigma-Aldrich) in combination with 0.5 µg/mL ionomycin (Sigma-Aldrich) for 4 hrs at 37 °C. After 1 hr incubation, 10 µg/mL brefeldin A (BioLegend) was added.

Flow cytometry

Flow cytometry was performed according to standard procedures. Cell aggregates (identified in FSC-H vs. FSC-A scatter plots) and dead cells identified by using Zombie Aqua Fixable Viability Kit (BioLegend) were excluded from analyses. Intracellular staining was performed using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Data was acquired on a LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Antibodies (Abs) and reagents

The following mAbs and reagents were used in the study: anti-CD3ε (17A2), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD11c (N418), anti-CD19 (6D5), anti-CD45 (30F11), anti-CD45R/B220 (RA3-6B2), anti-CD90.2 (30-H12), anti-CD127 (A7R34), anti-Ly-6G/Ly-6C (Gr-1) (RB6-8C5),

anti-Ter119 (TER-119), anti-TCR β (H57-597), anti-TCR $\gamma\delta$ (GL3), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-FoxP3 (FJK-16s), anti-ROR γ t (B2D), anti-T-bet (4B10), anti-IL-17A (TC11-18H10.1), anti-IL-22 (IL22JOP) and anti-IFN γ (XMG1.2). All Abs were purchased from eBioscience, BioLegend or BD Biosciences. PECF594-conjugated streptavidin was purchased from BD Biosciences.

Multiplex cytokine quantification

Liver tissues were crushed with a mortar and pestle on liquid nitrogen. 30-40 mg of tissue were transferred to a clean tube and added 400 μ L T-PER tissue protein extraction reagent (Thermo Scientific) buffer including proteinase inhibitors (Sigma-Aldrich). Protein concentration was assessed by BCA (Thermo Scientific) according to manufacturer's instruction. Cytokines were quantified using xMAP technology with combinations of Bio-Plex Pro (BioRad) premixed mouse cytokine panels, and the analyses were carried out on a Bio-Plex 200 system and the Bio-Plex Manager Software package.

Statistical analysis

Omics data were acquired as described in their appropriate sections. Normality residuals of remaining data were assessed by D'Augostino-Pearson omnibus (k2), Graphpad Prism 8, and subsequently evaluated by parametric (gaussian distributed) or non-parametric (non-gaussian distributed) tests as appropriate. Details of each test including post hoc assessment are specified in figure legends. Data are expressed as mean $\pm$ SEM with individual dots, and all groups are compared to the relevant WD_{REF} or WD_{CNTL} group as indicated.

Results:

McB feeding reverses WD-induced gut microbiota changes and increases cecal SCFA levels

To induce obesity and immunometabolic dysfunctions, C57BL/6JRj mice were initially fed an obesogenic WD. After 12 weeks of WD feeding, the mice were stratified into new groups based on weight, fat mass and glucoregulatory capacity (Figure 1A), and fed experimental WDs for an additional 6 weeks. While dietary fat is known to elicit reproducible and lipid-dependent alterations in the murine gut microbiome across a variety of different diet compositions²⁶, less is known about the microbiome-modulating impact of protein. Thus, to investigate if dietary protein (i.e. casein versus whole-cell bacterial lysates) would affect gut microbiota community structures, we analyzed freshly collected fecal samples before and throughout the dietary intervention. LFD and WD_{REF} fed mice showed distinct gut microbiota profiles after 12 weeks of feeding (intervention baseline, week 12+0; Figure 1B,C), including ~10-fold lower abundance of the health-promoting genera, *Parasutterella*²⁷ and *Parabacteroides*²⁸, countered by an equally increased abundance of the obesity associated genus *Desulfivobrio*^{30,31} (Figure 1D) as well as a ~4-fold increase in the *Firmicutes* to *Bacteroidetes* (F/B) ratio (Figure 1E). Interestingly, WD_{CNTL} fed mice showed negligible changes in the microbiome signature during the 6 weeks of intervention (Figure 1B-F & S1A-B), suggesting that the added lipid source had limited influence on the intestinal ecology. In contrast to this observation, we noted a pronounced shift in bacterial composition in mice fed WD_{McB}. Within the first 2 weeks of treatment, the general community structure in these mice shifted towards that of their LFD-fed counterparts (Figure 1B-F, Table S3). We next asked if the observed taxonomical differences between groups related to alterations in the functional potential. SCFAs are main end products of metabolized fibers, and to a lesser extent amino acids escaping digestion in the SI, with vast impact on host physiology^{32,33}. The highest levels of SCFAs are found in the cecum and proximal colon³⁴. We therefore investigated if cecal SCFA levels were different between groups. We found a consistent increase in the levels of the three major as well as three minor classes of SCFAs in the cecum of WD_{McB} fed mice compared to WD_{CNTL} fed counterparts pointing towards not just taxonomically, but also functionally, discrete microbiota profiles in the two groups of mice, supporting a beneficial health impact on dietary inclusion of McB lysates (Figure 1G-H).

WD_{McB} feeding stimulates induction of gut-specific regulatory T cells

The intricate relationship between gut microbes and host immunity, combined with the immunoregulatory capacity of SCFAs³⁵, prompted us to investigate if the observed changes mediated by WD_{McB} feeding were associated with immune alterations. We accordingly analysed the immune cell profile of SI-LP and LI-LP in a subset of experimental mice (n = 6-10/group) using multicolour flow cytometry focusing on phenotypic characterization of group 3 innate lymphoid cells (ILC3), natural killer (NK) cells and T cells (consult Figure S2A-B for gating strategies). Numbers of ILC3s, NK cells and T cell receptor (TCR)- $\gamma\delta^+$ T cells, were similar between groups (Figure S2C-E). The same was true for the numbers of TCR β^+ CD4 $^+$ T cells, as well as the proportion of T helper (T_H)1-, T_H17- and nT_{regs} cells (Figure 2A-B). Interestingly, the proportion of pT_{regs} was more than 2-fold increased in LI-LP of WD_{McB} fed mice compared to WD_{CNTL} fed counterparts (Figure 2C, $p < 0.001$). Notably, this regulatory T cell subset has been shown to curb intestinal inflammation¹² and mediate immunological tolerance to the gut pathobiont *Helicobacter hepaticus*, thereby protecting against T_H17-mediated barrier dysfunction and subsequent colitis³⁶. Because ROR γt is the hallmark transcription factor for T_H17 cell differentiation and essential for their IL-17 production, we next assessed if the pT_{regs} induced by the different diets were also capable of expressing IL-17. Indeed, *ex vivo* stimulated LI-LP pT_{regs} produced substantial and diet-dependent amounts of IL-17 protein (Figure 2D-G, $p < 0.001$). A similar phenotype was observed in the SI-LP (Figure 2H-N), where pT_{regs} in WD_{McB} fed mice reached a ~3-fold increase compared to WD_{CNTL} fed mice (Figure 2J, $p < 0.001$).

WD_{McB} mitigates diet-induced obesity

The altered immune profile combined with a shift of the gut microbiota towards a state similar to that observed in lean LFD-fed mice, could potentially elicit crosstalk to glucoregulatory organs. To examine if McB lysates could reverse impaired glucose regulation, we performed OGTTs and assessed GSISs concomitant with body mass composition in obese mice fed WD_{REF} for 11 weeks and after 5 weeks of dietary intervention allowing for temporal analyses (Figure 1A). All mice were stratified into experimental groups based on their pre-intervention glucoregulatory capacity (Figure S3A-B). While the response to glucose challenge remained largely unaffected from week 11 to week 12+5, regardless of experimental diets (Figure 3A-C), both 5h fasted insulin levels and glucose stimulated insulin responses were significantly increased in mice fed WD_{CNTL} (Figure 3E, $p < 0.01$ & $p < 0.001$, respectively) in accordance with our previous report on time-dependent alterations in glucose regulation³⁷. LFD- and WD_{McB}-fed mice were fully protected against this

detrimental trajectory (Figure 3D, F & S3C, $p = 0.24$ & 0.68 , respectively), and WD_{M_{CB}}-fed mice further exhibited modestly improved insulin sensitivity based on 5h fasted glycemia (Figure 3C, $p < 0.05$) and intraperitoneal insulin tolerance test (Figure 3G, $p < 0.05$). Overall, weight development mimicked the glucoregulatory capacity. As such, WD_{M_{CB}}-fed mice exhibited stability of weight, fat mass and lean mass when changed to experimental diets, contrasting the continuous weight and fat mass development of WD_{CNTL} fed mice (Figure 3H-I & S3D). The absence of weight gain was not explained by decreased feed intake (Figure 3J), but rather appeared to be associated with enhanced fecal energy secretion (Figure 3J-K). Since obesity and impaired glucose regulation are tightly associated with NAFLD³⁸, we next subjected paraffin-embedded liver sections to histological evaluation. These analyses revealed both diminished steatosis and hepatocellular ballooning in WD_{M_{CB}} fed mice compared to WD_{CNTL}-fed counterparts, where especially hepatocellular ballooning was arrested in (or returned to) a state reminiscent that of lean LFD fed mice (Figure 3L-M, $p < 0.05$). Importantly, hepatocellular ballooning is instrumental in the development of the more severe liver disease, NASH³⁸.

WD_{M_{CB}} feeding resets the hepatic lipidome and decreases hepatic immune infiltration alleviating NAFLD

Based on the decreased NAFLD in WD_{M_{CB}}-fed mice housed at T_{22°C} we designed a new experiment (study outline, Figure 4A) using a recently described³⁹ method where thermoneutral housing (T_{30°C}) potentiates NAFLD in WT C57BL/6J mice fed an obesogenic diet for 20-24 weeks. To more thoroughly investigate the effect of WD_{M_{CB}}-feeding, we also redesigned the diets and omitted macadamia oil in the WD_{CNTL} group, as this might lead to progression of obesity (Figure 3) and related disorders. This new diet design entailed an increased fat/protein ratio in WD_{M_{CB}} compared to WD_{REF}, due to phospholipids inherently present in bacterial lysates¹⁶ (Table S1). Despite the lower protein content in WD_{M_{CB}} compared to both other diets, protein availability was well beyond critical levels, corroborated by similar lean mass to WD_{REF}-fed mice post diet intervention (Figure S4A). Still, WD_{M_{CB}}-fed mice exhibited significantly improved 5h fasting insulin levels and decreased fat mass (Figure 4B-C), despite weight maintenance and significantly increased energy intake compared to both LFD and WD_{REF} fed mice (Figure S4B-D). The decreased body fat mass was accompanied by a diminished NAS, supported by both pathological evaluation of H&E stained liver sections (Figure 4D-E) and hepatocytic lipid content assessed by Oil-Red-O staining (Figure 4F-H). We additionally observed augmented adiponectin secretion (Figure 4I), pointing towards improved

insulin sensitivity in the WD_{McB} group, further supported by the assessment of insulin tolerance and hepatic gene transcription activity of key metabolic enzymes (Figure 4J-K). Of interest, we observed a >10-fold downregulation of *Scd1* in the liver of WD_{McB}-fed mice, the hepatic expression of which is a) regulated by the microbiota⁴⁰ and b) instrumental in *de novo* lipogenesis at the onset of metabolic syndrome⁴¹.

We next assessed the hepatic lipidome by mass spectrometry (MS/MS) to elucidate if diminished NAFLD was associated with an altered lipid profile. Through comparison of WD_{McB} and WD_{REF} we identified 57 and 279 differentially regulated peaks in negative and positive ionization mode, respectively (Figure 5A-B, S5A-B, all FDR < 0.05). Of these, most classified lipids were changed with WD_{McB} in the direction of LFD fed mice (Figure 5C-D). Notably, 57% of upregulated species were odd-chain fatty acids, whereas 80% of down-regulated species represented lipids with even carbon numbers (Figure 5C-D & Table S4), hence supporting previous reports where odd- rather than even-chain fatty acids are inversely associated with human insulin resistance⁹ and type 2 diabetes⁴².

To estimate the functional consequences of an altered lipid profile, we used the Lipidmaps database to identify affected pathways and plotted the observed changes on a log₂ scale comparing both LFD and WD_{McB} to WD_{REF}. The majority of affected pathways were similarly regulated in both direction and magnitude in LFD and WD_{McB} mice compared to WD_{REF} mice (Figure 5E & S5C). Of note, bile acids and ceramides, both of which were significantly downregulated in WD_{McB} fed mice compared to WD_{REF} fed counterparts, have been shown to mediate steatohepatitis by upregulation of IL-6 and TNF- α , respectively^{43,44}. We therefore measured these hepatic cytokines and observed similarly reduced levels in both LFD and WD_{McB} compared to WD_{REF} (Figure 5F).

A key feature of diet-induced liver pathologies, including NAFLD, is recruitment of newly activated immune cells capable of eliciting a proinflammatory immune response. This process is generally hampered in mice housed at mild thermal stress, which therefore fail to phenocopy human pathophysiology. However, thermoneutral housing recapitulates some human disease traits⁴⁵, which combined with HFD-feeding accentuates intrahepatic infiltration of proinflammatory Ly6^{high} monocytes⁴⁶. These monocytes interact with tissue resident T cells and play a central role in the pathogenesis of liver injury, hence representing an attractive therapeutic target to mitigate NAFLD development and to curtail associated pathologies⁴⁴.

We therefore subjected liver tissues from representative mice to immunological evaluation by immunohistochemistry and observed a marked decrease in both CD3⁺ T cells and Ly6^{high}

monocytes in WD_{McB}-fed mice compared to their WD_{REF}-fed counterparts (Figure 5G). Diminished hepatic immune infiltration was mirrored by increased levels of circulating IL-22, IL-18, and IL-17 in WD_{McB}-fed mice compared to their WD_{REF}-fed counterparts (Figure 5H, $p < 0.01$, < 0.05 & < 0.05 , respectively).

WD_{McB} feeding reverses prolonged gut microbial dysbiosis and markedly improves colonic mucus production

The improved hepatic phenotype prompted us to further investigate potential traits in the gut-liver axis. We initially assessed if the observed cytokine responses associated with improved gut health in mice housed at T_{30°C}, where inflammation is expected to be increased⁴⁵. Indeed, WD_{McB}-fed mice were resistant to WD-induced colonic shortening closely associated with colonic inflammation (Figure 6A).

To gain additional insights to the immunomodulating properties of McB lysates, we focused on mucus production and -function. Because mucin production is a constitutive process where both secretion and adherence are constantly ongoing and rapidly adjust to environmental changes, immunohistochemical labelling for various MUC epitopes may not fully recapitulate the physical properties of the mucus. We therefore applied specialized mucin histochemistry staining allowing us to differentiate between neutral and acidic mucins according to the net charge of each molecule. Acidic mucins were further separated into sulfomucins and sialomucins. Sections revealed that neutral mucins in crypt-residing goblet cells were consistently down regulated in WD_{REF}-fed mice compared to LFD-fed counterparts in three well defined segments of the colon; i.e. proximal, middle and distal area (Figure 6B, D). WD_{McB}-feeding not only reversed this pattern in all three segments but even enhanced the production of neutral mucins exceeding the levels found in LFD-fed counterparts. This is a remarkable finding considering the continuous intake of a westernized diet high in fat and sucrose, known to hamper goblet cell function. We next evaluated crypt depth (CD) in stained sections. While the CD of the proximal and distal part of the colon were largely unaffected by diet, we observed increased CD in the middle segment of WD_{McB}-fed mice (Figure 6C). WD_{McB}-feeding further enhanced the glycosylation pattern, particularly in the middle segment of colon, where this group exhibited a 3-fold increase in sulfomucins balanced by a similar (~2-fold) decrease in sialomucins compared to WD_{REF}-fed mice (Figure 6E-G). No differences were observed between WD_{REF} and LFD-fed mice, indicating that the reciprocal regulation of mucin glycosylation status was specific to WD_{McB}-feeding.

With focus on the dynamic interactions between gut immunity, mucin glycosylation and commensal microbes⁴⁷, we next assessed the gut microbiota composition in temporally separated samples. This was done to determine if the changes towards a gut microbiota resembling that of lean LFD-fed mice was recapitulated in this intensified setup. In contrast to the first set of experiments, where we used cohoused mice shown to exhibit resilient microbiota profiles, we now employed single-housed mice to explore if the WD_{McB}-mediated community structures were persistent enough to induce consistent changes in the more dynamic communities of single-housed mice. Similar to our first experiments at T₂₂°C, we observed a normalization of the gut microbiome of WD_{McB}-fed mice, despite prolonged WD-feeding prior to intervention (Figure 6H-K). WD_{McB}-induced changes were surprisingly consistent with the first set of experiments, including a significantly lower F/B ratio (Figure 6J and S5D), and a substantial reduction of *Desulfovibrio* abundance, countered by a similar bloom of the *Parasutterella* and *Parabacteroides* genera (Figure 6K, Table S5). Of note, the age-related increases in *Desulfovibrio* abundances recently reported³⁰ was confirmed in this study where the general magnitude in both WD_{REF}- and LFD-fed mice increased 2-3-fold over the 5 week intervention (Figure 6K). WD_{McB}-feeding fully prevented this trajectory and paired analyses even revealed a diminished relative abundance of *Desulfovibrio* in these mice.

Discussion:

In this report, we explored the relationship between dietary nutrients and host-microbe interactions with a focus on immunometabolic response rates in the context of high fat, high sucrose, WD-feeding. We found whole-cell lysates from the non-commensal methanotrophic bacterium, McB, capable of reversing hallmark signatures of WD-feeding despite continued intake of an obesogenic diet. The signatures included diminished fat mass, improved intestinal immunity and glucoregulatory capacity accompanied by gut microbial community structures resembling that of lean LFD-fed counterparts.

The aberrant microbiota composition associated with obesity was recently shown to reflect HFD intake rather than obesity *per se*⁴⁸. It is therefore worth noting that we were able to normalize the dysregulated gut microbiome in mice remaining on a westernized, high fat, high sucrose diet; especially considering that it remains a clinical challenge to change fatty dietary habits in lifestyle-related obese subjects. This remarkable change was reproduced in two diverse sub-strains of mice originating from different vendors, corroborating a robust phenotype not notably affected by baseline microbiota composition. To this end, a previous report on the resilient microbiome, argued for prolonged normalization⁴⁹. This study found that mice transferred to a low fat, fiber-rich chow diet after 12 weeks of HFD feeding, shifted their microbiota towards age-matched chow-fed control mice within 4 weeks, but only fully converged 10 weeks post diet change. This is particularly interesting as dietary fibers are known to be the most potent dietary regulator of the gut microbiota⁵⁰, by far exceeding that of dietary fat⁵¹. Still, in our hands, WD_{McB}-feeding was able to reverse the obese microbiome at a higher pace than chow diet was in the previous report⁴⁹, despite similar fiber content in the two WDs. We further showed that the reversal of the microbiota traits were reproducible at different temperatures, altered reference diets, changed experimental duration, and in both co-housed and single-housed mice; all of which are prominent modulators of gut microbiota community structures. While many genera were similarly affected between experiments, others were either exclusively regulated at T_{30°C} (e.g., *Bifidobacterium*) or less pronounced affected at T_{30°C} (e.g., *Barnesiella*), suggesting dispensability for these specific microbes in the metabolic disease traits observed in this model. Similar observations were made for *Akkermansia*, which was ~3-fold upregulated at T_{30°C}, but discordantly affected (log2 FC bouncing from +9.3 to -10.4) between the two replication studies at T_{22°C} with pronounced intragroup variation.

Contrasting these discrepancies, there was a consistent downregulation of *Desulfovibrio* accompanied by >10-fold upregulation of *Parabacteroides* and *Parasutterella* suggesting that WD_{McB} feeding nourishes a favorable microbiota, albeit such speculations have yet to be mechanistically described. Still, *Desulfovibrio* was most recently shown to flourish in aged, immunocompromised, obese mice with impaired glucose regulation³⁰. Both T cell loss and *Desulfovibrio* administration *per se*, precipitated obesity-induced insulin resistance³⁰. This bacterium has moreover been shown to thrive in desulfonated colonic mucosa, associated with human⁵² and mouse⁵³ colitis. Combined, these observations lend credence to the hypothesis of a mechanistic link between WD_{McB}-mediated reductions of *Desulfovibrio* and the observed improvements in mucin chemotype and metabolic response rates.

Future studies are warranted to elucidate if the gut microbiota changes observed in WD_{McB}-fed mice were a result of the unique nutrient source, or if it was facilitated by WD_{McB}-induced pT_{regs}. On this note, mucin-producing goblet cells are capable of delivering luminal antigens to LP-residing dendritic cells (DC)⁵⁴ instrumental for T cell polarization⁵⁵. As McB potently induce DC maturation markers *in vitro* – even exceeding the effects of the well-described probiotic strain, *Escherichia coli* Nissle 1917⁵⁶ – we predict a direct link between McB intake and the corresponding immune profile. In addition to the direct link proposed here, McB intake might also indirectly promote T_{reg} polarization through augmented SCFAs⁵⁷. These important metabolites also sustain mucus production and facilitate tissue crosstalk in the gut-liver axis². In keeping with this notion, we observed decreased hepatic bile acids and TNF- α levels combined with a pronounced reduction of intrahepatic CD3⁺ and Ly-6C^{high} cells. Importantly, while Ly-6C^{high} cells represent proinflammatory, fibrogenic macrophages⁵⁸, their *in situ* differentiation to Ly-6C^{low} cells⁵⁹ facilitate tissue repair and improved NASH prognosis⁶⁰. Mentioned targets are all proposed as relevant strategies to curb NAFLD and the more terminal liver disease, NASH⁴⁴.

The substantial, consistent, and global changes in the immunometabolic profile found in this study point towards clinical potential if further developed. Notably, only a single study has previously succeeded in inducing pT_{regs}, and this was limited to LI-LP¹¹. Apart from inducing this cell subset throughout the gastrointestinal tract, we further show that these cells exhibited enhanced secretory capacity of IL-17 in WD_{McB}-fed mice compared to their WD_{CNTL} fed counterparts. To this end, emerging evidence indicates that the non-regulatory counterparts to pT_{regs}, i.e. TH17 cells, are

purged from SI-LP in DIO mice¹⁴ and that reintroducing *ex vivo* differentiated gut tropic T_H17 cells curtail obesity development, hence improving insulin sensitivity¹⁵. That IL-17 in this intervention is produced by T_{regs} and not T_H17 cells might further improve the safety profile of a putative medication, as the physiological impact and properties of T_H17 cells are context-dependent. As such, T_H17 cells may potentiate inflammatory bowel disease (IBD) in individuals with compromised barrier function⁶¹, whereas pT_{regs} have shown enhanced suppressive capacity against the same disease¹². This is a pertinent feature given the relatively high proportion of subjects with metabolic complications co-suffering from IBD⁶². While our study was not designed to assess IBD susceptibility, it is well described that purified diets compromise barrier function^{50,63,64} and thus represent clinical IBD-features (disease-cause) preceding inflammation (symptom)⁶⁵. It is therefore worth noting that in addition to the immunomodulating phenotype, WD_{McB}-feeding augmented neutral mucus production in all segments of the colon while enhancing IBD-protective sulfomucins specifically in the middle segment. In this segment, we also detected increased CD in McB fed mice, collectively pointing towards increased barrier function.

In summary, this study demonstrates a consistent activation of Foxp3⁺RORγt⁺IL-17⁺ triple positive pT_{regs} throughout the gastrointestinal tract and a reversion of gut dysbiosis in response to WD_{McB}-feeding, even in the context of high dietary fat and sucrose intake. The prospects of using bacterial lysates as an alternative to traditional live probiotics merit further investigation, just as future studies are warranted to assess to what extent our results can be translated to humans. Future studies are also urgently needed to identify the biological effector molecule(s) comprised in this bacterial lysate, allowing for subsequent development of a versatile medical product(s) rebalancing intestinal immunity while targeting gut-related dysbiosis and metabolic abnormalities.

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Figure 1: McB feeding reverses WD-induced gut microbiota changes and increases cecal SCFA levels: A) Study outline of two independent experiments with initial Western diet (WD_{REF}) feeding for 12 weeks at 22°C and oral glucose tolerance test (OGTT), glucose-stimulated insulin secretion (GSIS), and body composition by MR scan assessed after 11 weeks to stratify groups (□) followed by dietary intervention for 6 weeks of feeding either WD including McB lysate (WD_{McB}) or a matched WD control diet (WD_{CNTL}). During the experimental period body composition, OGTT, GSIS, and fresh fecal sampling was carried out at the indicated intervals. An insulin tolerance test (ITT) was carried out in the first of two experiment at the indicated timepoint. The second experiment included a reference group of fed low fat diet (LFD) fed mice. Groups or procedures featured uniquely in one experiment are italicized. **B)** Principle coordinate analysis (PCoA) using Weighted UniFrac distances of fecal microbiota sampled from first and second experiment biweekly during the dietary intervention period, as indicated by numbers post dietary intervention in centroids. The WD_{CNTL} and WD_{McB} groups were similar in microbiota composition prior to dietary intervention week 12+0 (PERMANOVA $p = 0.88$ and 0.43 in Exp1 and Exp2, respectively). At the end of each experiment, the microbiota composition was significantly different between these groups (PERMANOVA $p = 0.001$ and 0.002 in Exp1 and Exp2, respectively). **C)** Taxasummary of most abundant bacterial genera showing mean relative abundance in % of indicated family and genera in each group at indicated time points **D)** Deseq analysis of fecal bacterial genera abundances significantly regulated by McB intervention compared to the WD_{CNTL} ($p_{adj.} < 0.05$). Relative abundance in % in each group and variation are shown for each regulated genus at the sampled time points. Fold-change and adjusted p values of individual are indicated in Table S3. **E)** Firmicutes/Bacteroidetes ratio of fecal samples of individual mice before (12+0) and after 6 weeks of dietary intervention (12+6) in Exp1 (◆) and Exp2 (●). $n = 6$ (LFD) to 15 (WD_{McB} and WD_{CNTL}). Statistical significance is indicated by * on paired t-test. **F)** Weighted UniFrac distance (instability test) between paired samples from indicated two-weeks' interval post dietary intervention in Exp1 (◆) and Exp2 (●). $n = 6$ (LFD) to 15 (WD_{McB} and WD_{CNTL}). Statistical significance is indicated by * on paired t-test. **G-H)** Major short-chained fatty acids (SCFAs) (acetate, propionate, and butyrate) and minor SCFAs (isobutyrate, isovalerate, and valerate) in cecal content as fold change pmol per cecum in Exp1 (◆) and Exp2 (●). $n = 6$ (LFD) and 10-11(WD_{CNTL} and WD_{McB}, respectively). Statistical significance compared to WD_{CNTL} by one-way ANOVA with multiple comparison and Dunnett post hoc is indicated by *. **E-H)** * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 2: WD_{McB} feeding stimulates induction of gut-specific regulatory T cells: A-D) Number of indicated cells in colon from Exp1 (◆) and Exp2 (●). E-G) Representative plots of colonic TCRb⁺CD4⁺ FoxP3⁺ RORγt⁺ pT_{regs} (left) and IL17⁺ pT_{regs} (right) in LFD (E), WD_{CNTL} (F), and WD_{McB} (G) group. **H-K)** Number of indicated cells in small intestine from Exp1 (◆) and Exp2 (●). **L-N)** TCRb⁺CD4⁺ FoxP3⁺ RORγt⁺ pT_{regs} (left) and IL17⁺ pT_{regs} (right) in LFD (L), WD_{CNTL} (M), and WD_{McB} (N) group. **A-N)** n = 6 (LFD) to 10 (WD_{McB} and WD_{CNTL}). **A-D, H-K)** Statistical significant differences to WD_{CNTL} group by one-way ANOVA with multiple comparisons and Dunnett post hoc is indicated by *. * = p < 0.05; ** = p < 0.01, and *** = p < 0.001.

Figure 3: Dietary intervention with McB blunts progression of insulin resistance and fat mass accumulation: A-C) Oral glucose tolerance test (OGTT) and 5h fasting blood glucose prior to dietary intervention (Week 11) and 5 weeks post intervention (Week 12+5) of LFD (A), WD_{CNTL} (B), and WD_{McB} (C) groups showing mean ± SEM of two independent experiments. **D-F)** Glucose-stimulated insulin concentration during OGTT and 5h fasting insulin levels prior to dietary intervention (Week 11) and 5 weeks post intervention (Week 12+5) of LFD (D), WD_{CNTL} (E), and WD_{McB} (F) groups showing mean ± SEM of two independent experiments. **G)** Intraperitoneal insulin tolerance test (ITT) 3 weeks post dietary intervention showing mean ± SEM. **H)** Body weight development. Mice were fed either LFD or WD_{REF} the first 12 weeks followed by 6 week dietary intervention period showing mean ± SEM of two independent experiments. Dotted vertical line depicts intervention start. **I)** Fat mass in gram through the dietary intervention period (Week 12+0 to 12+6) measured by MR scan showing mean ± SEM of two independent experiments. **J)** Feed intake per cage as average grams per mouse during 48h in Exp1 (◆) and Exp2 (●). **K)** Fecal content of fat and protein in Exp1 (◆) and Exp2 (●). **L)** NAFLD activity score based on hepatic steatosis grade (0-3), inflammation (0-2) and hepatocellular ballooning (0-3) graded by blinded histological assessment of liver tissue in Exp1 (◆) and Exp2 (●). **M)** Representative H&E stain of liver tissue from indicated experimental group. **A-F)** n = 6 (LFD) to 15 (WD_{McB} and WD_{CNTL}). Statistical significance within each time point is indicated by * at paired two-way ANOVA-RM with Bonferroni post hoc test. Fasting glucose and insulin levels were evaluated by paired t-tests. **G)** n = 8 (WD_{McB}) to 9 (WD_{CNTL}). Statistical significance within each timepoint is indicated by * during two-way ANOVA-RM with Bonferroni post hoc test. **H-I)** n = 6 (LFD) to 15 (WD_{McB} and WD_{CNTL}). Statistical significance is indicated by *. LFD and WD_{McB} were compared to WD_{CNTL} by

two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett's post hoc. **J-K**) Each data point resembles the average of one cage. $n = 2$ (LFD) to 5 (WD_{McB} and WD_{CNTL}). Statistical significance is indicated by *. LFD and WD_{McB} were compared to WD_{CNTL} by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett's post hoc. **L**) $n = 6$ (LFD) to 13 (WD_{McB} and WD_{CNTL}). Statistical significance is indicated by *. LFD and WD_{McB} were compared to WD_{CNTL} by Kruskal-Wallis, adjusted for multiple comparisons by Dunn's post hoc. **A-L**) * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 4: Dietary McB intervention improves diet-induced metabolic and hepatic phenotype after prolonged WD feeding: **A**) Study outline with initial Western diet (WD_{REF}) feeding for 21 weeks at 30°C. Insulin tolerance test (ITT) and body composition by MR scan were assessed after 20 weeks to stratify groups ($\square$) followed by dietary intervention for 5 weeks of feeding either WD_{REF} or WD including McB lysate (WD_{McB}). A reference group fed low fat diet (LFD) was included throughout the experiment (week 0 to week 21+5). Body composition, ITT, and fresh fecal sampling was carried out at the indicated intervals. Mice were co-housed the first 12 weeks and then separated to individual cages. **B**) 5h fasting plasma insulin levels before (Week 20) and after (Week 21+5) dietary intervention. **C**) Fat mass in grams measured by MR scan at indicated time points from dietary intervention. **D**) NAFLD activity score separated in hepatic steatosis grade (0-3), inflammation (0-2) and hepatocellular ballooning (0-3) graded by blinded histological assessment of H&E stained liver tissue. **E**) Representative H&E stain of liver tissue from indicated experimental group. **F**) Number of lipid droplets in each of 3-4 liver samples per experimental group quantified by Oil Red O staining. **G**) Average size of lipid droplets in each of 3-4 liver samples per experimental group quantified by Oil Red O staining. **H**) Distribution of lipid droplet size in % of all lipid droplets within each experimental group. **I**) Plasma adiponectin concentration in 5h fasted mice before (Week 20) and after (Week 21+5) dietary intervention. **J**) ITT after four weeks of dietary intervention (Week 21+4). **K**) Expression of key metabolic enzymes in liver tissue after five weeks of dietary intervention (Week 21+5) by RT-qPCR. **B+I**) $n = 6-9$ per group. Statistical significance is indicated by * on paired t-test. **D+K**) $n = 8-10$ per group. Statistical significance compared to WD_{REF} by Kruskal-Wallis test, adjusted for multiple comparisons by Dunn's post hoc. **F-H**) $n = 3$ randomly selected samples per group. Statistical significance compared to WD_{REF} by Kruskal-Wallis test, adjusted for multiple comparisons by Dunn's post hoc. **C+J**) $n = 9-10$ per group. Statistical significance compared to WD_{REF} by two-way ANOVA-RM,

adjusted for multiple comparisons by Dunnett post hoc. * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 5: WD_{McB} feeding resets the hepatic lipidome and decreases hepatic immune infiltration alleviating NAFLD: **A)** Principle coordinate analysis (PCoA) of hepatic lipid species identified in negative ionization mode. **B)** As in A but depicted in a volcano plot with significantly regulated lipid species (FDR < 0.05) presented on a log₂ scale in either green (WD_{McB} > WD_{REF}) or blue (WD_{McB} < WD_{REF}). **C-D)** Identified lipid species differentially expressed (FDR < 0.01) between WD_{McB} and WD_{REF} as indicated. Fold-change and adjusted p values of individual lipid species are indicated in Table S4. **E)** Lipid pathways identified by fold-change analysis separating LFD (grey) and WD_{McB} (green) groups from WD_{REF} in negative ionization mode. **F)** Hepatic cytokine levels of TNF- α and IL-6 as indicated. **G)** Ly6G⁺ and CD3⁺ immune cell revealed by immunohistochemistry. **H)** Cytokine levels in plasma of IL-22, IL-18, and IL-17. **F+H)** n = 8-10. Statistical significance compared to WD_{REF} group by Kruskal-Wallis test, adjusted for multiple comparisons by Dunn's post hoc. **G)** n = 3 per group (randomly selected). Statistical significance compared to WD_{REF} by one-way ANOVA, adjusted for multiple comparisons by Dunnett post hoc. * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 6: WD_{McB} treatment improves colonic mucus production and reverses obesity-induced gut microbiota changes to resemble the composition found in lean LFD-fed mice: **A)** Colon length in centimeters. **B)** Area of neutral mucins determined by Periodic Acid Schiffs (PAS) staining in three discrete locations, i.e. proximal, middle, and distal colon segments. Three longitudinally sectioned crypts were measured in each sample (one data point) at each location. **C)** Colon crypt depth (CD) in proximal, middle, and distal colon segments, measured in histological sections. Measurements are given in μm , and results are given as means of six crypt measurements per location per individual mice (3 longitudinally PAS stained and HID-AB stained crypts, respectively, per data point). **D)** PAS staining for mucus in colon middle segments, representative histological sections from the three experimental groups as indicated. **E)** Area of sulfomucins in proximal, middle, and distal colon segments by HID staining. Three longitudinally sectioned crypts were measured in each sample and at each location. **F)** Area of sialomucins in proximal, middle, and distal colon segments by AB staining. Three longitudinally sectioned crypts were measured in each sample and at each location. **G)** Representative HID-AB staining of middle segment of

907 indicated experimental group. **H)** PCoA of fecal microbiota composition of indicated group before
 908 (Week 21+0) and after (21+5) dietary intervention with group mean indicated as centroids.
 909 Microbiota composition was significantly different between the WD_{REF} and WD_{McB} groups at the
 910 end of the experiment week 21+5 (PERMANOVA $p = 0.001$) **I)** Taxasummary of most abundant
 911 bacterial genera showing mean relative abundance of indicated genera in each group at indicated
 912 time point. **J)** Firmicutes/Bacteroidetes ratio of fecal samples of individual mice at termination
 913 (Week 21+5). **K)** Deseq analysis of fecal bacterial genera abundances significantly regulated by
 914 McB intervention ($p_{\text{adj.}} < 0.05$). Relative abundance in % in each group and variation are shown
 915 for each regulated genera at the sampled time points. Fold-change and adjusted p values of
 916 individual genera are indicated in Table S5. **A-K)** $n = 8-10$. **A-C, E-J)** Statistical significance
 917 compared to the WD_{REF} group by one-way ANOVA or Kruskal-Wallis test (depending on Gaussian
 918 distribution) with multiple comparisons by Dunnett or Dunn's post hoc, respectively. * = $p < 0.05$;
 919 ** = $p < 0.01$, and *** = $p < 0.001$.

920

921 **Supplementary figure S1: A)** Taxasummary of most abundant bacterial genera showing mean
 922 relative abundance in % of indicated family and genera in each group at indicated time point. **B)**
 923 Deseq analysis of fecal bacterial genera abundances significantly regulated by McB intervention
 924 compared to the WD_{CNTL} ($p_{\text{adj.}} < 0.05$). Relative abundance in % in each group and variation are
 925 shown for each regulated genus at the sampled time points. Fold-change and adjusted p values of
 926 individual are indicated in Table S3.

927

928 **Supplementary figure S2: A)** Gating strategy for group 3 innate lymphoid cells (ILC3).
 929 Representative flow cytometry plots of *ex vivo* stimulated small intestine (SI) lamina propria (LP) cells.
 930 ILC3s were gated as single, live cells, lineage1⁻lineage2⁻CD90.2⁺CD127^{int/+}ROR γ δ ⁺ cells.
 931 Representative data of ROR γ δ ⁺ ILC3s after stimulation with or without (w/o) IL-23 are shown.
 932 Lineage1: CD11c, CD19, CD45R/B220, Ly-6G/Ly-6C (Gr-1), Ter119, NK1.1; Lineage2: CD3 ϵ , CD8 α ,
 933 TCR β , TCR γ δ . **B)** Gating strategy for NK cells and T cells. Representative flow cytometry plots of *ex*
 934 *vivo* stimulated colon lamina propria cells. NK cells were gated as single, live cells, CD8 α ⁻
 935 CD45⁺NK1.1⁺; TCR γ δ ⁺ T cells were gated as single, live cells, CD8 α ⁻CD45⁺TCR γ δ ⁺CD4⁻; CD4⁺ T_H1
 936 cells were gated as single, live cells, CD8 α ⁻CD45⁺TCR β ⁺CD4⁺T-bet⁺IFN γ ⁺; CD4⁺ T_H17 cells were
 937 gated as single, live cells, CD8 α ⁻CD45⁺TCR β ⁺CD4⁺ROR γ δ ⁺IL-17A⁺. Natural regulatory T cells (nT_{regs})
 938 were gated as single, live cells, CD8 α ⁻CD45⁺TCR β ⁺CD4⁺FoxP3⁺ROR γ δ ⁻ and peripheral-induced

regulatory T cells (pT_{regs}) were gated as single, live cells, $CD8\alpha^{-}CD45^{+}TCR\beta^{+}CD4^{+}FoxP3^{+}ROR\gamma\delta^{+}$. Representative data of $TCR\beta^{+}CD4^{+}$ T cells after stimulation with or without (w/o) PMA and Ionomycin (PMA/Iono) are shown. **C)** ILC3 cell number in large intestine (LI)-LP and SI-LP. **D)** NK1.1 cell number in LI-LP and SI-LP. **E)** $TCR\gamma\delta^{+}CD4^{-}$ cell number in LI-LP and SI-LP. **C-E)** Individual values from Exp1 (◆) and Exp2 (●) are shown and statistical significant differences to WD_{CNTL} by one-way ANOVA, adjusted for multiple comparisons by Dunnett's post hoc. * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Supplementary figure S3: A-B) Blood glucose and plasma insulin values, respectively, during oral glucose tolerance test (OGTT) before dietary intervention (week 11). Mean $\pm$ SEM is shown for two, independent experiments. **C)** Plasma insulin values from OGTT week 12+5 depicted as mean $\pm$ SEM of two, independent experiments. **D)** Lean mass in grams during the dietary intervention period as mean $\pm$ SEM of two, independent experiments. **A-D)** Statistical significance compared to WD_{CNTL} by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post hoc. * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Supplementary figure S4: A) Lean mass in grams during the dietary intervention period as mean $\pm$ SEM. Statistical significance compared to WD_{REF} by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post hoc. **B)** Body weight development; mice were fed either LFD or WD_{REF} for the first 21 weeks, as indicated. WD_{REF} -fed mice were subsequently stratified into experimental groups, fed indicated diets for additional 5 weeks. **C)** Final body weight week 21+5. **D)** Cumulative energy intake in kcal per mouse during dietary intervention (week 21+0 to 21+5). **C-D)** Statistically significant differences to WD_{CNTL} by one-way ANOVA, adjusted for multiple comparisons by Dunnett's post hoc. * = $p < 0.05$; ** = $p < 0.01$, and *** = $p < 0.001$.

Supplementary figure S5: A) Principle coordinate analysis (PCoA) of hepatic lipid species identified in positive ionization mode. **B)** As in A but depicted in a volcano plot with significantly regulated lipid species ($FDR < 0.05$) presented on a $\log_2$ scale in either green ($WD_{McB} > WD_{Ref}$) or blue ($WD_{McB} < WD_{Ref}$). **C)** Lipid pathways identified by fold-change analysis that segregated LFD- (grey) and WD_{McB} (green) groups from WD_{Ref} in positive ionization mode. **D)** Firmicutes/Bacteroidetes ratio of fecal bacteria sampled prior to dietary intervention in week 21+0.

970 Statistically significant differences to WD_{Ref} by one-way ANOVA, adjusted for multiple
 971 comparisons by Dunnett's post hoc. * = p < 0.05; ** = p < 0.01, and *** = p < 0.001.

972

973 **Table S1: Experimental diet compositions**

		WD _{No Protein}	WD _{REF}	WD _{CNTL}	WD _{McB}	LFD
Corn starch	%	5,0000	5,0000	5,0000	5,0000	52,0000
Maltodextrin	%	10,0000	10,0000	10,0000	10,0000	-----
Sucrose	%	31,6300	31,6300	31,6300	31,6300	9,7270
Cellulose powder	%	5,0000	5,0000	5,0000	5,0000	5,0000
L-Cystine	%	0,3000	0,3000	0,3000	0,3000	0,3000
DL-Methionine	%	0,1600	0,1600	0,1600	0,1600	0,1600
Vitamin premix	%	1,0000	1,0000	1,0000	1,0000	1,0000
Mineral premix	%	6,0000	6,0000	6,0000	6,0000	3,5000
Choline chloride	%	0,2500	0,2500	0,2500	0,2500	0,2500
Vitamin K3	%	-----	-----	-----	-----	0,0005
Vitamin B12	%	-----	-----	-----	-----	0,0025
Butylated hydroxytoluene	%	0,0100	0,0100	0,0100	0,0100	0,0100
Cholesterol	%	0,1500	0,1500	0,1500	0,1500	-----
Na CMC	%	-----	-----	-----	-----	1,0000
Soybean oil	%	21,0000	21,0000	21,0000	21,0000	7,0000
Casein	%	-----	19,5000	16,5000	-----	20,0000
McB whole-cell lysate	%	-----	-----	-----	19,5000	-----
Macadamia oil	%	-----	-----	3,0000	-----	-----
Total	%	80,5	100,0	100,0	100,0	100,0

974

975 Table S1: Composition of experimental diets used in the studies as % weight. Experimental diets
 976 were based on lot-matched WD_{No Protein}, and subsequently added casein, macadamia oil and McB
 977 whole-cell lysate as indicated..

978

979 **Table S2: Primer sequences and annealing temperature for qPCR**

Target gene	Forward primer sequence	Reverse primer sequence	Annealing temp. (°C)
<i>Scd1</i>	ACA CCT GCC TCT TCG GGA TT	TGA TGC CCA GAG CGC TG	58
<i>Acaca</i>	TGC TGC CCC ATC CCC GGG	TCG AAC TCT CAC TGA CAC G	58

<i>Acox1</i>	GGG TCA TGG AAC TCA TCT TCG A	GAA TGA ACT CTT GGG TCT TGG G	58
<i>Pparg1</i>	GTG TGA CAG ACA AGA TTT GAA AG	GCT TGA TGT CAA AGG AAT GCG	60
<i>Pparg2</i>	ACA GCA AAT CTC TGT TTT ATG	TGC TGG AGA AAT CAA CTG TGG	60
<i>Ppara</i>	AGA GAG GAC AGA TGG GGC TC	CGT TTG TGG CTG GTC AAG TT	60

980

981 **Table S3: Deseq analysis from 12+6 week experiments**

Related to Figure 1				
Genus	Fold change	Log2FC	p value	Adj p value
g:Chelatococcus	1573,07	10,62	4,43E-25	2,88E-23
g:Barnesiella	27,46	4,78	3,07E-20	6,65E-19
g:Parabacteroides	23,89	4,58	2,22E-20	6,65E-19
g:Vampirovibrio	1401,99	10,45	3,08E-18	5,01E-17
g:Anaerofilum	408,29	8,67	6,55E-18	8,52E-17
g:Parasutterella	109,80	6,78	8,74E-16	9,47E-15
g:Microvirga	268,76	8,07	1,35E-14	1,25E-13
g:Brevibacillus	88,56	6,47	9,74E-09	7,36E-08
g:Pseudoxanthomonas	84,23	6,40	1,02E-08	7,36E-08
g:Clostridium_IV	0,24	-2,06	5,16E-08	3,35E-07
g:Isoptricola	51,65	5,69	5,17E-07	3,05E-06
g:Eubacterium	7,88	2,98	3,56E-06	1,93E-05
g:Akkermansia	632,32	9,30	7,69E-06	3,84E-05
g:Clostridium_XIVa	2,64	1,40	2,23E-05	0,0001
g:Staphylococcus	7,28	2,86	0,0002	0,001

g:Papillibacter	0,25	-2,01	0,0002	0,001
g:Acetatifactor	0,44	-1,19	0,001	0,002
g:Lachnospiracea_incertae_sedis	0,16	-2,61	0,001	0,002
g:Odoribacter	8,16	3,03	0,001	0,002
g:Bacteroides	2,81	1,49	0,002	0,01
g:Alistipes	2,83	1,50	0,003	0,01
g:Porphyromonas	4,77	2,25	0,004	0,01
g:Haloplasma	5,41	2,44	0,01	0,02
g:Desulfovibrio	0,44	-1,17	0,01	0,03
g:Blautia	0,24	-2,05	0,01	0,03
g:Escherichia/Shigella	0,14	-2,88	0,01	0,04
g:Murimonas	0,34	-1,57	0,02	0,04
g:Anaerovorax	3,76	1,91	0,02	0,04
g:Shuttleworthia	0,27	-1,87	0,02	0,05
g:Alkaliphilus	0,15	-2,77	0,03	0,06
g:Dorea	0,41	-1,28	0,03	0,07
g:Allobaculum	2,17	1,12	0,04	0,07
g:Christensenella	8,11	3,02	0,04	0,07
g:Streptococcus	0,34	-1,57	0,03	0,07
g:Clostridium XVIII	0,39	-1,36	0,04	0,07
g:Clostridium III	2,16	1,11	0,04	0,07
g:Ruminococcus	0,42	-1,24	0,04	0,07
g:Pseudoflavonifractor	0,55	-0,86	0,05	0,08
g:Saccharofermentans	2,46	1,30	0,05	0,09
g:Lutispora	4,23	2,08	0,06	0,10
g:Butyrivibrio	1,75	0,81	0,08	0,12
g:Aestuariispira	3,27	1,71	0,09	0,15
g:Hydrogenoanaerobacterium	3,16	1,66	0,11	0,16
g:Anaerotruncus	0,55	-0,87	0,12	0,18
g:Enterorhabdus	0,46	-1,13	0,13	0,19
g:Flavonifractor	0,59	-0,76	0,14	0,19
g:Roseburia	0,54	-0,88	0,14	0,20
g:Sporobacter	1,95	0,96	0,15	0,21
g:Coproccoccus	0,32	-1,62	0,17	0,22
g:Romboutsia	0,26	-1,95	0,26	0,34
g:Intestinimonas	0,59	-0,77	0,29	0,36
g:Butyricicoccus	0,34	-1,55	0,30	0,38
g:Marvinbryantia	1,69	0,75	0,35	0,44
g:Clostridium XIVb	0,78	-0,35	0,38	0,46
g:Clostridium sensu stricto	0,51	-0,97	0,50	0,59

g:Gracilibacter	1,44	0,53	0,52	0,60
g:Catabacter	1,22	0,29	0,56	0,64
g:Anaerosporebacter	0,69	-0,53	0,60	0,67
g:Bilophila	0,79	-0,33	0,67	0,73
g:Enterococcus	1,46	0,54	0,67	0,73
g:Lactobacillus	1,17	0,23	0,73	0,78
g:Alloprevotella	1,17	0,23	0,75	0,79
g:Olsenella	1,13	0,17	0,84	0,86
g:Senegalimassilia	0,86	-0,21	0,85	0,86
g:Oscillibacter	1,01	0,01	0,99	0,99
Related to Figure S1				
Genus	Fold change	Log2FC	p value	Adj p value
g:Akkermansia	0,0007	-10,44	5,67E-35	4,08E-33
g:Bacillus	83,30	6,38	4,04E-15	1,45E-13
g:Acetatifactor	0,20	-2,30	9,26E-12	2,22E-10
g:Lactobacillus	32,59	5,03	1,43E-11	2,46E-10
g:Parasutterella	15,59	3,96	1,71E-11	2,46E-10
g:Clostridium_IV	0,17	-2,53	3,98E-11	4,78E-10
g:Nocardioides	52,24	5,71	9,07E-11	9,33E-10
g:Clostridium XVIII	0,09	-3,54	3,02E-09	2,72E-08
g:Clostridium_XIVa	2,25	1,17	1,36E-08	1,09E-07
g:Sporobacter	0,21	-2,22	1,77E-08	1,28E-07
g:Brevibacillus	22,94	4,52	6,24E-08	4,08E-07
g:Eubacterium	4,77	2,25	9,72E-08	5,83E-07
g:Parabacteroides	4,21	2,07	4,94E-07	2,74E-06
g:Porphyromonas	3,77	1,92	5,48E-07	2,82E-06
g:Barnesiella	3,31	1,73	1,09E-06	5,25E-06
g:Enterococcus	26,51	4,73	1,43E-06	6,42E-06
g:Sphingopyxis	21,41	4,42	1,72E-06	7,28E-06
g:Anaerovorax	5,93	2,57	2,10E-05	8,41E-05
g:Haloplasma	3,38	1,76	9,88E-05	0,0004
g:Arthrobacter	9,18	3,20	0,0003	0,001
g:Rhizobium	8,90	3,15	0,0003	0,001
g:Vampirovibrio	6,25	2,64	0,0003	0,001
g:Allobaculum	4,86	2,28	0,0004	0,001
g:Neorhizobium	8,18	3,03	0,001	0,002
g:Paenibacillus	7,85	2,97	0,001	0,002
g:Romboutsia	4,91	2,29	0,001	0,002
g:Clostridium_sensu_stricto	12,44	3,64	0,001	0,002

g:Anaerotruncus	0,48	-1,07	0,001	0,004
g:Butyricicoccus	0,22	-2,21	0,001	0,004
g:Staphylococcus	6,33	2,66	0,002	0,01
g:Catenisphaera	7,26	2,86	0,002	0,01
g:Catabacter	0,47	-1,10	0,003	0,01
g:Papillibacter	0,43	-1,23	0,003	0,01
g:Coprobacillus	0,06	-4,00	0,01	0,01
g:Dorea	0,45	-1,16	0,01	0,02
g:Lutispora	2,96	1,56	0,02	0,04
g:Shuttleworthia	0,17	-2,54	0,02	0,04
g:Alloprevotella	0,51	-0,98	0,03	0,05
g:Pseudoflavonifractor	0,63	-0,67	0,03	0,05
g:Hydrogenoanaerobacterium	2,49	1,32	0,03	0,06
g:Clostridium XIVb	0,74	-0,44	0,08	0,14
g:Intestinimonas	1,79	0,84	0,08	0,14
g:Turicibacter	3,32	1,73	0,08	0,14
g:Prevotella	3,25	1,70	0,08	0,14
g:Erysipelotrichaceae incertae sedis	3,62	1,86	0,12	0,19
g:Roseburia	0,54	-0,88	0,14	0,21
g:Christensenella	2,02	1,01	0,18	0,27
g:Desulfovibrio	0,80	-0,33	0,23	0,34
g:Lachnospiracea incertae sedis	1,35	0,43	0,22	0,34
g:Murimonas	1,78	0,83	0,23	0,34
g:Anaerosporeobacter	0,59	-0,76	0,25	0,35
g:Odoribacter	1,55	0,63	0,31	0,42
g:Enterorhabdus	1,47	0,56	0,33	0,45
g:Blautia	0,61	-0,71	0,37	0,47
g:Butyrivibrio	0,70	-0,51	0,37	0,47
g:Oscillibacter	1,23	0,30	0,37	0,47
g:Streptococcus	0,69	-0,54	0,36	0,47
g:Olsenella	0,53	-0,91	0,46	0,58
g:Bilophila	1,15	0,20	0,53	0,64
g:Coproccoccus	1,32	0,40	0,54	0,64
g:Aestuariaispira	0,73	-0,46	0,57	0,67
g:Bacteroides	0,84	-0,26	0,58	0,67
g:Ruminococcus2	2,00	1,00	0,58	0,67
g:Saccharofermentans	1,16	0,22	0,64	0,72
g:Gracilibacter	0,88	-0,18	0,75	0,83
g:Alkaliphilus	0,84	-0,25	0,77	0,84
g:Alistipes	0,94	-0,09	0,85	0,89

g:Flavonifractor	1,04	0,06	0,86	0,89
g:Gemella	0,90	-0,15	0,88	0,89
g:Ruminococcus	1,05	0,07	0,87	0,89
g:Senegalimassilia	0,90	-0,15	0,87	0,89
g:Clostridium III	1,01	0,01	0,96	0,96

982

983 Results from deseq analysis of bacterial genera of the two experiments presented in Figure 1 & S1
 984 featuring genus name, Fold change and Log2 fold change, comparing WD_{McB} to WD_{CNTL}.

985 Unadjusted p-values as well as p-values adjusted by Benjamini-Hochberg method are reported and
 986 statistically significantly affected genera (Adj. p < 0.05) are marked in bold.

987

988 **Table S4: Lipid species altered by McB:**

Lipid	Adjusted p-value between WD _{REF} and LFD	Adjusted p-value between WD _{REF} and WD _{McB}
Upregulated by McB:		
lysoPC 17:1	0,7072	0,0022
PC 33:1 B	0,5237	<0,0001
PC 33:5 D	0,0001	<0,0001
PC 38:4 C	<0,0001	<0,0001
PC 39:6	0,0021	<0,0001
PE 38:5	0,0004	0,0081
plasmeyl-PC 36:3	0,0002	0,0004
Downregulated by McB:		
lysoPC 16:1	0,9977	0,0023
lysoPC 18:1	0,4575	0,0065
lysoPC 20:1	0,0003	0,0001
lysoPC 20:2	0,0027	0,0028
lysoPC 20:3	0,0019	0,0005
lysoPC 20:4 A	0,9830	0,0018
PC 32:1	0,0004	0,0012
PC 33:5 C	0,0933	<0,0001
PC 34:3	0,4656	0,0001
PC 35:4	<0,0001	0,0003
PC 36:3 A	0,0003	0,0002
PC 36:3 B	<0,0001	<0,0001
PC 36:5 B	0,9151	<0,0001
PC 37:4 A	0,0009	0,0085
PC 38:4 A	0,0001	0,0004
PC 38:4 B	<0,0001	<0,0001
PC 38:5 A	0,2436	<0,0001
PC 38:6 B	0,0006	<0,0001

PC 39:7	0,0022	0,0033
PC 40:7 B	0,0006	0,0001

Reported p-values compared to the WD_{REF} are obtained by mixed-effects analysis with multiple comparisons and Dunnett's post hoc.

Table S5: Deseq analysis from 21+5 week experiment

Related to Figure 6				
Genus	Fold change	Log2FC	p value	Adj p value
g:Parasutterella	2066,66	11,013	1,4E-38	9,3E-37
g:Parabacteroides	94,91	6,568	7,6E-33	2,6E-31
g:Brevibacillus	138,42	7,113	9,1E-18	2,1E-16
g:Pseudoflavonifractor	0,09	-3,418	3,4E-14	5,9E-13
g:Odoribacter	19,74	4,303	1,9E-13	2,6E-12
g:Desulfovibrio	0,18	-2,501	4,8E-09	5,5E-08
g:Haloplasma	13,78	3,785	7,0E-09	6,9E-08
g:Dorea	0,11	-3,168	1,3E-08	9,9E-08
g:Flavonifractor	0,11	-3,153	1,3E-08	9,9E-08
g:Acetatifactor	0,16	-2,621	2,3E-07	1,6E-06
g:Rhodococcus	40,07	5,325	3,5E-07	2,2E-06
g:Romboutsia	0,04	-4,556	2,9E-06	1,7E-05
g:Alistipes	3,95	1,982	4,3E-06	2,3E-05
g:Porphyromonas	8,17	3,031	1,5E-05	7,4E-05
g:Shuttleworthia	0,09	-3,499	1,6E-05	7,4E-05
g:Allobaculum	2,74	1,454	1,0E-04	4,3E-04
g:Barnesiella	2,88	1,524	1,1E-04	4,7E-04
g:Anaerotruncus	0,24	-2,039	2,0E-04	7,5E-04
g:Akkermansia	8,07	3,013	3,5E-04	1,3E-03
g:Mucispirillum	0,14	-2,789	4,8E-04	1,7E-03
g:Cohnella	15,91	3,992	9,6E-04	3,1E-03
g:Clostridium_IV	0,51	-0,966	1,2E-03	3,6E-03
g:Clostridium_XIVa	0,54	-0,893	1,3E-03	3,8E-03
g:Eubacterium	4,77	2,253	1,6E-03	4,5E-03
g:Papillibacter	0,27	-1,874	2,1E-03	5,8E-03
g:Coproacter	13,09	3,710	3,0E-03	7,8E-03
g:Oscillibacter	0,38	-1,415	3,2E-03	8,2E-03
g:Bifidobacterium	7,60	2,926	4,4E-03	1,1E-02
g:Sporobacter	0,39	-1,364	1,0E-02	2,5E-02

g:Clostridium XVIII	0,36	-1,481	1,2E-02	2,8E-02
g:Ruminococcus	0,44	-1,178	1,6E-02	3,6E-02
g:Catabacter	0,59	-0,768	2,1E-02	4,6E-02
g:Christensenella	4,25	2,086	2,3E-02	4,9E-02
g:Clostridium XIVb	2,63	1,395	0,025	0,050
g:Hydrogenoanaerobacterium	0,25	-2,005	0,029	0,058
g:Streptococcus	0,38	-1,406	0,033	0,064
g:Butyrivibrio	1,64	0,715	0,041	0,077
g:Lactobacillus	2,02	1,018	0,046	0,083
g:Olsenella	2,39	1,260	0,069	0,123
g:Butyricicoccus	0,31	-1,678	0,079	0,137
g:Blautia	0,47	-1,096	0,113	0,182
g:Pedobacter	3,78	1,918	0,112	0,182
g:Roseburia	0,52	-0,954	0,111	0,182
g:Murimonas	0,28	-1,855	0,129	0,203
g:Fodinicurvata	3,54	1,822	0,140	0,214
g:Holdemania	1,96	0,970	0,150	0,225
g:Clostridium III	1,42	0,506	0,184	0,264
g:Intestinimonas	0,54	-0,877	0,183	0,264
g:Anaerofilum	0,44	-1,168	0,191	0,269
g:Anaerosolibacter	0,32	-1,630	0,208	0,287
g:Coproccoccus	2,06	1,042	0,242	0,327
g:Erysipelotrichaceae incertae sedis	0,44	-1,194	0,323	0,428
g:Bilophila	0,68	-0,546	0,329	0,428
g:Anaerovorax	1,79	0,843	0,352	0,449
g:Enterococcus	2,38	1,251	0,366	0,459
g:Staphylococcus	0,61	-0,707	0,379	0,468
g:Escherichia/Shigella	0,42	-1,250	0,411	0,498
g:Bacteroides	0,82	-0,293	0,420	0,500
g:Lachnospiraceae incertae sedis	0,75	-0,408	0,469	0,548
g:Lutispora	1,83	0,871	0,493	0,567
g:Alkaliphilus	1,55	0,632	0,521	0,589
g:Clostridium sensu stricto	1,60	0,680	0,562	0,625
g:Enterorhabdus	1,27	0,346	0,573	0,625
no match	2,30	1,202	0,579	0,625
g:Gracilibacter	1,23	0,304	0,591	0,627
g:Saccharofermentans	1,12	0,167	0,773	0,808
g:Anaerosporeobacter	0,97	-0,047	0,938	0,952
g:Tissierella	1,06	0,078	0,938	0,952
g:Spiroplasma	1,03	0,038	0,969	0,969

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997 Results from deseq analysis of bacterial genera of the 21+5 week experiment featuring genus name,
 998 Log2 fold change, comparing WD_{McB} to WD_{REF} . Unadjusted p-values as well as p-values adjusted
 999 by Benjamini-Hochberg method are reported and statistically significantly affected genera (Adj. $p <$
 1000 0.05) are marked in bold.

1001

1002

Fig. 1

Fig. 2

Fig. 3

Oral glucose tolerance

Glucose-stimulated insulin concentration

Fig. 4

Fig. 5

A Hepatic lipodomics

B

C

Upregulated in McB

D

Downregulated in McB

Downregulated in McB

E

F

Liver cytokines

G

Hepatic immune infiltration

H

Plasma cytokines

Fig. 6