# Supplementary material and methods section

## METHODS

### *Animals*

C57BL/6J male mice (4 weeks old, Janvier Labs, Le Genest St Isle, France) were housed in groups of 3 per cage in a controlled environment (12-h daylight cycle) with free access to food and water. The experiment was approved by and performed following the guidelines of the local ethics committee for animal care of the Health Sector of Université catholique de Louvain under the specific agreement number 2017/UCL/MD/005. Housing conditions were as specified by the Belgian Law of 29 May 2013 regarding the protection of laboratory animals (Agreement no LA 1230314). Every effort was made to minimize animal pain, suffering, and distress. As shown in the figure 1A, mice were divided into nine groups: one control SPF group and eight groups of mice that were humanized by inoculating gut microbiota from obese patients.

*Antibiotic therapy*: According to previous procedures[1 2], we depleted the intestinal microbiota in the eight groups of humanized mice, by using eight days of antibiotic treatment. The antibiotic mixture consisting of ampicillin (10 mg/mL), neomycin (10 mg/mL), metronidazol (8 mg/mL), vancomycin (5 mg/mL) was administered by daily gavage. The first three days of antibiotic gavage was also supplemented with amphotericin B (30 µL/mL). Antibiotic treatment was then followed a bowel cleansing with polyethylene glycol (PEG). SPF mice were daily gavage with water during the antibiotic treatment.

*FMT:* following the antibiotic treatment and PEG administration (except for the SPF), all groups of mice were inoculated three times with a gavage of stool samples from four obese patients (SPF group received the vehicle). The inoculum containing stool samples from donors was diluted in an anoxic ringer-cystein buffer. The four groups of mice inoculated with the stool samples from the four donors are identified as *hum-ob* mice (for humanized obese mice). SPF mice were force-fed with the same volume of vehicle buffer.

*Experiment:* after the first inoculation with stool samples, all groups of mice including the SPF mice were fed a high-fat diet (45% kcal fat, E15744-347, Ssniff, Soest, Germany) for four weeks. For each donor, one subgroup of *hum-ob* mice was supplemented or not with 0.2 g/day per mouse of native inulin (Fibruline®, Cosucra, Pecq, Belgium) in the drinking water for the four weeks. Food intake and water consumption were recorded twice a week.

### *Human cohort*

The clinical intervention was a 3-month, multicentric, single-blind, placebo-controlled randomized intervention in male and female obese patients. The protocol was performed in three university hospitals in Belgium (Cliniques Universitaires of St-Luc from Brussels, ULB Erasme Hospital from Brussels and Centre Hospitalier Universitaire from Liège). The inclusion criteria were: BMI >30 kg/m$^2$, age 18-65 years, Caucasian ethnicity, presence of at least one metabolic disorder associated with obesity (prediabetes/diabetes, dyslipidemia, hypertension, non-alcoholic fatty liver disease). The exclusion criteria included use of antibiotics, pro/prebiotics, fibers as a dietary supplement, or any molecule that modifies intestinal transit time within 6 weeks before starting the study. 106 patients were randomized to consume either 16 g/day of native inulin (Cosucra, Belgium) or 16 g/day of maltodextrin (Cargill, Belgium). 55 patients were randomized in the placebo group and 51 patients were assigned to the inulin group. In addition, patients were asked to consume, at least once a day, a recipe based on vegetables enriched or not in inulin-type fructans. A dietician performed a one-week recall questionnaire to evaluate energy intake at baseline and at the end of the intervention. Physical activity was evaluated by IPAQ questionnaire. Anthropometric measures were assessed at baseline and after three months of intervention, i.e. weight, height, waist and hip circumference, blood pressure, body composition (using bioimpedance devices BIA 101, Akern, Italy; Biocorpus, Medi Cal, Germany; Tanita BC-418 MA, Tanita,

UK). Fibroscan assessed liver stiffness (fibrosis) and controlled attenuation parameter (steatosis). This study was approved by the "Comité d'éthique Hospitalo-facultaire de Saint-Luc". Written informed consent was obtained from all participants before inclusion in the study. This trial was registered at clinicaltrial.gov as NCT03852069.

***Metabolic measurements***

Lipid content was measured in the liver and gastrocnemius muscle tissues after extraction with chloroform-methanol according to the Folch method [3]. Blood glucose levels were determined, after 6 hours of fasting, using a glucose meter (Roche Diagnostics) on 3.5 µl of blood collected from the tail vein. Blood sample was also harvested at the same time to assess plasma insulin concentrations. Plasma insulin concentrations were determined using an ultrasensitive ELISA kit (Mercodia, Uppsala, Sweden).

***RNA Extraction and real-time quantitative PCR***

Total RNA was isolated from tissues using the TriPure isolation reagent kit (Roche Diagnostics, Penzberg, Germany). Complementary DNA was prepared by reverse transcription of 1 µg total RNA using the Kit Reverse Transcription System (Promega,Madison,WI). Real-time polymerase chain reaction (PCR) was performed with a CFX96 Touch Real-Time PCR Detection System and software (Biorad Laboratories Ltd, UK) using SYBR Green (Applied Biosystems, The Netherlands and Eurogentec, Verviers, Belgium) for detection. All samples were run in duplicate in a single 96-well reaction plate, and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. The purity of the amplified product was verified by analyzing the melting curve performed at the end of amplification. The ribosomal protein L19 (RPL19) gene was chosen as a reference gene.

***Histology***

Measurement of mean adipocytes area: adipocytes area were measured after hematoxylin and eosin staining of the subcutaneous adipose tissue slices. Adipocytes area were quantified using the ImageJ software. At least 500 adipocytes were analyzed per mouse.

Hepatic lipid staining: frozen liver sections were sliced at 5 µm, stained with oil red O and scanned (Leica SCN400; Leica, Wetzlar, Germany). The lipid area was determined on whole sections using the imaging software TissueIA (version 2.0.3, Leica Biosystems, Dublin, Ireland). Pixels corresponding to the oil red O staining were selected to create a color profile. Total tissue area was defined by setting the tissue intensity threshold at 210 (grey value). Results were expressed as stained area (below threshold)/tissue area (below threshold). Two representative tissue pieces were analyzed for each mouse.

***Protein extraction and immunoblotting***

*For total protein extraction:* 50mg of liver or gastrocnemius muscle were placed in ice-cold buffer [20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM 1,4-dithiothreitol (DTT), and 10% protease inhibitor cocktail 10X (Roche Applied Science, Vilvoorde, Belgium)] and then homogenized using a TissueLyser device (Qiagen). Homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatants were collected and stored at -80°C.

*For nuclear protein extraction:* nuclear proteins were extracted following manufacturer's instruction (NE-PER, Thermo Scientific,Waltham, MA, USA) from 50 mg of liver.

50µg of total proteins were denaturated by mixing with Laemmli buffer and linearization was achieved by heating the samples for 5 min at 100°C. The samples were loaded with prestained molecular mass markers (Thermo Fisher Scientific). The proteins were then separated on SDS–polyacrylamide gels and transferred to a PVDF membrane. Membranes were then blocked in Tris-buffered saline with 0.1% v/v Tween 20 (TBST) containing 5% of non-fat dried milk. Membrane were incubated overnight with the following primary antibodies (1:1000 dilution in TBST containing 1% of bovine serum albumin): phospho-

ACC (Ser79), phospho-Akt (Ser473), Akt, phospho-mTOR (Ser2448) from Cell Signaling, Srebp1c from Thermo Scientific, Srebp2 and β-actin from Abcam. Membranes were then incubated with a secondary antibody (1:1000 diluted in TBST containing 5% of non-fat dried milk) conjugated to horseradish peroxidase from Cell Signaling Technology. β-actin, α-tubulin or Ponceau staining were used as loading control and all results were expressed relative to the SPF conventional mice. Signals were revealed using ECL Western blotting substrates (SuperSignal West Pico Substrate, Thermo Scientific). Gels are analyzed and quantified by ImageQuant TL instrument and software version 8.1 (GE Healthcare, Buckinghamshire, England).

***DNA Extraction and 16S rRNA Gene Sequencing***

*Mice and donor subset:*

DNA was extracted from the mouse cecal content and donor stool samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hildren, Germany) including a bead-beating step.

*Cohort subset:*

Stool samples were available for 47 patients on 51 assigned to the inulin group, during the clinical intervention. Stool samples were collected at baseline and after 3 months of intervention and stored at room temperature with a DNA stabilizer (Stratec biomolecular, Berlin, Germany) for maximum three days, then transferred to -80°C for the analysis of the gut microbiota composition. Genomic DNA was extracted from feces using a PSP® spin stool DNA kit (Stratec biomolecular, Berlin, Germany).

Amplicon sequencing of the microbiome was done at the University of Minnesota Genomics Center. Briefly, the V5-V6 region of the 16S rRNA gene was PCR-enriched using the primer pair V5F_Nextera (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**RGGATTAGATACCC**) and V6R_Nextera (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CGACRRCCATGCANCACCT**) in a 25 µl PCR reaction containing 5 µl of template DNA, 5 µl of 2X HotStar PCR master mix, 500 nM of final concentration of primers and 0.025 U/µl of HostStar Taq+ polymerase (QIAGEN). PCR-enrichment reactions were conducted as follow, an initial denaturation step at 95°C for 5 min followed by 25 cycles of denaturation (20 s at 98°C), annealing (15 s at 55°C), and elongation (1 min at 72°C), and a final elongation step (5 min at 72°C). Next, the PCR-enriched samples were diluted 1:100 in water for input into library tailing PCR. The PCR reaction was analogous to the one conducted for enrichment except with a KAPA HiFi Hot Start Polymerase concentration of 0.25 U/ µl, while the cycling conditions used were as follows, initial denaturation at 95°C for 5 min followed by 10 cycles of denaturation (20 s at 98°C), annealing (15 s at 55°C), and elongation (1 min at 72°C), and a final elongation step (5 min at 72°C). The primers used for tailing are the following: F-indexing primer AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC and R-indexing primer CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGG GCTCGG, where [i5] and [i7] refer to the index sequence codes used by Illumina. The resulting 10µl indexing PCR reactions were normalized using a SequalPrep normalization plate according to the manufacturer's instructions (Life Technologies). 20 µl of each normalized sample was pooled into a trough, and a SpeedVac was used to concentrate the sample pool down to 100 µl. The pool was then cleaned using 1X AMPureXP beads and eluted in 25 µl of nuclease-free water. The final pool was quantitated by QUBIT (Life Technologies) and checked on a Bioanalyzer High-Sensitivity DNA Chip (Agilent Technologies) to ensure correct amplicon size. The final pool was then normalized to 2 nM, denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20% PhiX, and heat denatured at 96°C for 2 minutes immediately prior to loading. A MiSeq 600 cycle v3 kit was used to sequence the pool.

Subsequent bioinformatics and biostatistics analyses were performed *in house*. Initial quality filtering of the reads was performed with the Illumina Software, yielding an average of 168312 (for the mice and donor subset) and 111507 (for the human cohort subset) pass-filter reads per sample. Quality scores were visualized with the FastQC software (http://www.bioinformatics.babraham.ac.uk/ publications.html), and reads were trimmed to 220 bp (R1) and 200 bp (R2) with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Next, reads were merged with the merge-illumina-pairs application v1.4.2 (with P = 0.03, enforced Q30 check, perfect matching to primers which are removed by the software, and otherwise default settings including no ambiguous nucleotides allowed)[4]. For mice

and donor analysis, for all the samples but two, a subset of 29000 reads was randomly selected using Mothur v1.25.0 [5], to avoid large disparities in the number of sequences. In the human cohort subset, a subset of 25000 reads was randomly selected. Subsequently, the UPARSE pipeline implemented in USEARCH[6] was used to further process the sequences. Amplicon sequencing variants (ASVs) were identified using UNOISE3. The analysis allowed the identification of 1937 ASVs for the mouse and donor subset and 3305 ASVs for the cohort subset. Taxonomic prediction was performed using the *nbc_tax* function, an implementation of the RDP Naive Bayesian Classifier algorithm[7]. The phylotypes were computed as percent proportions based on the total number of sequences in each sample. Alpha diversity indexes and beta diversity indexes were calculated using QIIME [8]. PCoA plot of the beta-diversity indexes were visualized using R software.

For the mice subset, significantly affected taxa by inulin were identified using a Welch's t-test in R, for each donor. The p-value of the Welch's t-test was then adjusted (q-value) to control for the false discovery rate (FDR) for multiple tests according to the Benjamini and Hochberg procedure[9].

Raw sequences can be accessed in SRA database (accession numbers PRJNA594535, PRJNA595949).

**LEGENDS**

**Supplemental Figure 1. Dietary intake and metabolic parameters.**
(A) Daily food intake in grams per mouse. (B) Daily water intake per mouse in milliliters. (C) Daily inulin intake per mouse in grams. (D) Fasted plasma glucose ($mg.dl^{-1}$). (E) Fasted plasma insulin ($mg.L^{-1}$).

**Supplemental Figure 2. Gut microbiota composition analysed by qPCR in DNA extracted from caecal content.**
(A) Total bacteria (B) *Bifidobacterium* spp. (C) *Roseburia* spp. (D) *Faecalibacterium* spp. (E) *Lactobacillus* spp. (F) *Akkermansia muciniphila*. A circle is used for each mouse, a square represents the level of bacteria found in the feces of the human obese donor. For each analysis, results are expressed as mean ± SEM., *p<0,05 and ***p<0,001 for untreated *hum-ob* mice versus SPF mice., $^{\$}$p<0,05, $^{\$\$}$p<0,01 and $^{\$\$\$}$p<0,001 for comparison between the group receiving inulin and their counterpart for each donor.

**Supplemental Figure 3. Several ASVs are correlated with metabolic features in *hum-ob* mice.**

Heatmap of Spearman's correlations between ASVs significantly modified by inulin treatment (FDR correction, q value) and the most significant metabolic changes observed with inulin. *q<0,05, **q<0,01 and ***q<0,001 for significant correlations between parameters. Analysis was performed using ASVs present at 0.1% of relative abundance in at least one sample.

**Supplemental Figure 4. Schematic representation of the microbial characteristics of inulin-responders in both *hum-ob* mice and human cohort.**
In *hum-ob* mice model, inulin decreased *Victivallis*, *Butyricimonas*, *Bilophila*, *Barnesiella* and *Hungatella,* five genera correlated with detrimental metabolic features, in one group of inulin-responder mice.
In the second inulin-responder group in *hum-ob* mice, inulin supplementation increased the abundance of *Blautia*, *Akkermansia*, *Clostridium XIVa* and *Raoultella,* four genera negatively correlated with hepatic lipid accumulation. *Akkermansia*, *Clostridium XIVa* and *Raoultella* also negatively correlated with the expansion of SAT mass. The increase of *Blautia* and *Akkermansia* are also associated with the reduced intramuscular lipids accumulation.
Finally, in the human cohort of obese individuals, the responder group in terms of BMI improvement was characterized by a higher abundance of *Akkermansia muciniphila* and *Butyricicoccus* at baseline.

## REFERENCES

1. Le Roy T, Debedat J, Marquet F, et al. Comparative Evaluation of Microbiota Engraftment Following Fecal Microbiota Transfer in Mice Models: Age, Kinetic and Microbial Status Matter. *Front Microbiol* 2018;9:3289. doi: 10.3389/fmicb.2018.03289 [published Online First: 2019/01/30]
2. Reikvam DH, Erofeev A, Sandvik A, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS One* 2011;6(3):e17996. doi: 10.1371/journal.pone.0017996 [published Online First: 2011/03/30]
3. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226(1):497-509. [published Online First: 1957/05/01]
4. Eren AM, Vineis JH, Morrison HG, et al. A filtering method to generate high quality short reads using illumina paired-end technology. *PLoS One* 2013;8(6):e66643. doi: 10.1371/journal.pone.0066643 [published Online First: 2013/06/27]
5. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75(23):7537-41. doi: 10.1128/AEM.01541-09 [published Online First: 2009/10/06]
6. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;10(10):996-8. doi: 10.1038/nmeth.2604 [published Online First: 2013/08/21]
7. Wang Q, Garrity GM, Tiedje JM, et al. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73(16):5261-7. doi: 10.1128/AEM.00062-07 [published Online First: 2007/06/26]
8. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7(5):335-6. doi: 10.1038/nmeth.f.303 [published Online First: 2010/04/13]
9. Benjamini Y HY. Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing. . *Journal of the Royal Statistical Society* 1995;57:289-300.