Supplementary Online Material

Title:

Changes in gut microbial metagenomic pathways associated with clinical outcomes after elimination of malabsorbed sugars in an IBS cohort.

Authors:

Mack, A.[1]*, Bobardt, J.S.[1]*, Haß, A.[1], Nichols, K.B.[2], Schmid, R.M.[1], Stein-Thoeringer, C.K.[1,3]

*both authors contributed equally

Affiliations:

[1]Klinik und Poliklinik fuer Innere Medizin II, Klinikum rechts der Isar, Technical University of Munich, Germany

[2]Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center (MSKCC), New York, USA

[3]German Cancer Research Center (DKFZ), Heidelberg, Germany

MATERIAL and METHODS

Study cohort

37 adult patients (20 female, 15 male) fulfilling the Rome IV criteria for IBS were recruited from June 2016 to August 2017 at a tertiary care outpatient clinic (Department of Internal Medicine II, Klinikum rechts der Isar, TUM) through a gastroenterologist to ensure inclusion and exclusion criteria were met (Supplementary Table 1 for demographics). Exclusion criteria were pregnancy, lactation, another diagnosis / disease explaining the gastrointestinal problems (including celiac disease), antibiotic treatments in the last 3 months. All patients reported current IBS symptoms with a symptom history longer than 2 years. Patients were permitted to use their IBS-related medication, but it had to be stable for 2 weeks before inclusion and throughout the study. Written informed consent was obtained from all patients. The study protocol was approved by the ethics committee of the Technical University of Munich [AZ-09/16S].

Study protocol and dietary intervention

In the screening phase, participants not previously diagnosed with a carbohydrate malabsorption (except one patient with an existing diagnosis of lactose malabsorption) were referred to carbohydrate malabsorption assessment using H2-breathing tests (HBT). An H2-lactulose breath test preceded all other tests for assessing proper testing conditions. HBTs were performed for lactose, fructose and sorbitol within a 1-3 day interval (for details see recent study by Klee et al.) [1]. H2 concentrations were recorded in parts per million (p.p.m.) prior to and every 30 minutes after ingestion of the test carbohydrate for a total of 180 minutes (50 g lactose, 25 g fructose, 10 g sorbitol; 10 g lactulose) using an HBT device (Hydrocheck, Neomed Medizintechnik, Uechtelhausen, Germany). Test doses for lactose and fructose corresponded to doses previously used in a study of 1372 patients assessing the association between intolerances and symptoms in functional gastrointestinal disorders [2], or in a dose finding study for sorbitol [3]. Increases of H2 concentrations by at least 20 p.p.m after test carbohydrate delivery were classified as malabsorption [1]. Patient symptoms, such as nausea, abdominal pain or meteorism, and diarrhea were recorded during the testing procedure.

Upon a new diagnosis of carbohydrate malabsorption, study patients were further asked to participate in a 7-week prospective clinical study for diet intervention, assessment of clinical symptoms, and microbiome changes.

The study contained 4 visits and three phases (see also Figure 1A): (I), a 1-week baseline period (visit 1 at the beginning and visit 2 at the end); (II), a 2-weeks elimination phase (visit 3 at the end of the period; EL) followed by (III), a 4-weeks tolerance phase (visit 4 at the end; TO). During the

baseline phase, participants were asked to maintain their regular diet and to daily record each food item using the Freiburger food-frequency questionnaire [4]. During the EL phase patients were asked to continue recording their diet daily, in addition to avoiding/minimizing consumption of any food containing the sugar that was found to be malabsorbed by the HBT. In the TO phase, patients were asked to re-introduce the malabsorbed sugar into their diet under the guidance of a dietician. Changes in diet and food components were individually discussed in order to achieve an increase in the daily intake of lactose or fructose by 2 g per day or of sorbitol by 0.1 g. This increase was carried out approximately every third day until an individually tolerated sugar dose was reached. Diet was recorded only in the last 7 days in this phase of the study.

Each phase was introduced by detailed nutritional counseling by a dietitian. Diet analyses were performed with the software package Prodi Expert® (Nutri-Science, Freiburg, Germany) containing the German Ministry of Agriculture and Nutrition - database of 15,000 foods with full nutritional values (Bundeslebensmittelschluessel) and expanded with additional items from certified resources.

In this study, seven IBS patients tested negative in HBTs were recruited as controls and completed a baseline study phase with a 7-day nutrition recording, clinical symptom monitoring, and microbiome analyses.

Subject characterization

Demographic information, body mass index, and current and past use of medications were obtained from all patients. A standard physical examination, including measurements of body temperature, heart rate, blood pressure, and blood testing for standard hematological and serum parameters, was performed at every study visit.

Gastrointestinal symptoms were assessed at every visit using the 100-mm visual analogue scale (VAS) as described by Halmos et al. [5], where 0 indicated no symptoms and 100 represented the worst symptoms ever experienced. This score was used as primary endpoint and measured overall gastrointestinal symptoms, abdominal pain, meteorism, and dissatisfaction with bowel habits. A reduction in VAS score greater than 25 mm (baseline - visit 4 at the end of the tolerance phase) was considered as clinical response to the diet intervention. Furthermore, Bristol Stool Scale scores [6] and the frequency and intensity of abdominal pain and bloating were recorded at each visit. Pain and bloating frequency were determined by the number of pain or bloating episodes per week. Pain intensity was assessed as maximum pain intensity during an episode on a scale of 0 to 10 (0, no pain and 10, worst pain ever experienced), while bloating intensity was rated from 0 to 4 (0, no bloating and 4, maximal bloating). Health-related quality of life (QoL)

was determined using the Short Form 36 (SF-36) questionnaire to evaluate an individual's health on the level of physical, social, and emotional functioning.

Fecal microbiome analysis

16S rRNA sequencing. Fecal samples for 16S ribosomal RNA (rRNA) gene amplicon analyses were collected from all subjects at study visits 2, 3 and 4. Fecal material was aliquoted and stored at -80C until DNA extraction using a bead-beating, phenol-chloroform protocol [7] followed by purifications on columns (OneStep-96 PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA). 16S rRNA gene amplicons spanning the V4 – V5 hypervariable region were sequenced using a MiSeq system (Illumina, San Diego, CA) as described previously [7]. Sequence data were compiled and processed using mothur, then screened and filtered for quality. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using NCBI blastn classifier and the alignment hit with the highest score. In order to correct for very rare OTUs, which could bias downstream statistical analyses, we excluded OTUs with less than 10 counts in less than 5% of all samples.

Metagenome sequencing. Extracted and purified DNA was sheared to a target size of 650 bp with a Covaris ultrasonicator and prepared for sequencing with the Illumina TruSeq DNA library preparation kit according to the Illumina protocol. Sequencing was performed on a HiSeq system (Illumina, San Diego, CA) targeting ~ 10-20x10^6 reads per sample with 100 bp, paired-end reads. Metagenome sequences were further analyzed using the HUMAnN2 tool developed for the shotgun metagenome analyses of the Human Microbiome Project 2 as described by Lloyd-Price et al. [8]. Prior to the HUMAnN2 workflow, shotgun sequences were filtered with quality control and removal of host reads using KneadData (http://huttenhower.sph.harvard.edu/kneaddata). Taxonomic profiling was done by MetaPHLAn2, and genes were annotated to UniRef90 and metabolic pathways to the MetaCyc database.

Statistical analysis

Depending on the nature of clinical data and their comparisons, Mann-Whitney or Kruskal-Wallis tests (for not normally distributed values), Wilcoxon matched-pairs signed rank test or Student's t-tests (for normally distributed values; a Shapiro-Wilks test was used to estimate normality) were used for parametric or non-parametric group comparisons with Bonferroni's correction for multiple testing in case of two-group comparisons (e.g., responders vs. non-responders). Regarding the analyzes of microbiota data, alpha-diversity was calculated on the basis of species richness and Shannon effective counts. Taxonomic differences were computed using the LEfSe algorithm following a previous publication [9] and the guidelines given by the authors

(https://bitbucket.org/nsegata/lefse). LEfSe is an algorithm for high-dimensional biomarker discovery and explanation that allows for the identification of taxonomic composition differences between two or more treatment conditions [9]. A correction for multiple testing is not included in this algorithm. We also used t-SNE as implemented in the R package Rtsne with generalized UniFrac distances as the distance measure as described previously [10]. As described for the Rhea analysis tool in R [11], we also computed NMDS (non-metric multi-dimensional scaling) as a second clustering method to assess beta-diversity on the basis of generalized UniFrac distances, and followed by a permutational multivariate analysis of variance (PERMANOVA) to assess significant differences between the groups. ANOVAs were applied for normally distributed data between several groups or for repeated measures, whereas Friedman tests were performed in case of ordinal, non-normal distributions. False discovery rate (FDR) corrections were applied as mentioned in the text. P-values less than 0.05 were considered significant.

SUPPLEMENTARY TABLES

Table 1.

Demographics of study cohort at baseline

	Diet intervention cohort (n=22)	Controls (n=7)	*P* value
Gender (F/M)	15 / 7	3 / 4	0.375
Age, years[1]	33 (19-77)	36 (22-54)	0.409
Body mass index[2] (kg/m^2)	21.8 (6.64)	26.5 (7.61)	**0.021**
VAS score[2] (0-100)	65 (20)	60 (10)	0.752
Abdominal pain[2] (0-10)	5.5 (4.8)	5 (3)	0.236
Meteorism[2] (0-4)	2.5 (1.8)	3 (1.5)	0.170
BSS[2] (1-7)	4 (1)	4 (1.5)	0.695
BSS subtypes, n (IBS-C/IBS-U/IBS-D)	5 / 14 / 3	1 / 5 / 1	1
Sugar malabsorptions, n (%)			
Lactose	1 (4.6)		
Fructose	2 (9.0)		
Sorbitol	10 (45.4)		
Lactose + Sorbitol	2 (9)		
Fructose + Sorbitol	2 (9)		
Lactose + Fructose + Sorbitol	5 (22.7)		

Data presented as median with [1]min-max range or [2]IQR; *P* value calculated by Mann-Whitney tests, except for gender and BSS subtypes by Fisher's exact tests.

Table 2.

Quality of life – parameters assessed by SF-36 in the study cohort at baseline

	Diet intervention cohort (n=22)	Controls (n=7)	*P* value
Physical functioning	95 (15)	95 (10)	0.67
Physical role	87.5 (68.8)	100 (50)	0.61
Bodily pain	51 (30.8)	51 (25)	0.93
General health	50 (35.3)	62 (29.5)	0.52
Vitality	50 (23.8)	45 (32.5)	0.94
Social functioning	62.5 (50)	87.5 (37.5)	0.56
Emotional role	100 (58.3)	100 (50)	0.91
Mental health	68 (33)	52 (18)	0.49

Data presented as median (IQR) with scores from 0 to 100; higher scores indicated better QoL. *P* value calculated by Mann-Whitney tests.

Table 3.

Quality of life – parameters assessed by SF-36

Symptom Score	Baseline		Elimination		Tolerance	
	Responder	Non-responder	Responder	Non-responder	Responder	Non-responder
Physical functioning	**95 (6.25)**	87.5 (33.8)	**100 (1.3)**	95.5 (23.8)	**100 (5) ***	85 (17.5)
Physical role	100 (31.3)	75 (87.5)	100 (0)	75 (56.3)	100 (31.3)	75 (81.3)
Bodily pain	**56 (13.5)**	41.5 (50.3)	**78 (29.5)**	46 (27.3)	**62 (51.5) ***	46 (28.5)
General health	**54.5 (40)**	46 (27.3)	**82 (43.3)**	42.5 (28.8)	**67 (55.5) *****	61 (34)
Vitality	60 (11.3)	37.5 (20)	65 (22.5)	42.5 (28.8)	65 (30)	62 (34)
Social functioning	87.5 (37.5)	50 (25)	87.5 (25)	56.3 (34.4)	93.8 (37.5)	37.5 (37.5)
Emotional role	100 (0)	66.7 (58.3)	100 (0)	66.7 (66.7)	100 (0)	66.7 (33.3)
Mental health	72 (33)	58 (28)	78 (23)	64 (37)	84 (25)	66 (14)

Data are presented as median (IQR); * $p<0.05$, *** $p<0.001$ responder baseline vs. elimination and tolerance, highlighted (Friedman test for non-parametric repeated measures).

Table 4.

Baseline dietary intake in malabsorbers / intervention cohort vs. controls

	Diet intervention cohort (n=22)	Controls (n=7)	*P* value
Energy [kcal/d]	2225.7 (537)	2131.5 (442.7)	0.42
Fat [g/d]	91.9 (19.5)	103.7 (22.6)	0.35
Carbohydrates [g/d]	199.5 (87)	214.6 (37.2)	0.39
Protein [g/d]	79.5 (24.8)	85.1 (10.8)	0.34
Fiber [g/d]	15.5 (10.5)	15.4 (2.8)	0.55
Fructose [g/d]	14.8 (10.2)	17.9 (2.9)	0.25
Lactose [g/d]	5.5 (5.8)	5.4 (5.2)	0.41
Sorbitol [g/d]	0.4 (0.7)	0.6 (0.4)	0.40

Data presented as median (IQR). *P* value calculated by Mann-Whitney tests.

Table 5.

IBS symptoms of diet responders vs. non-responders at baseline.

Symptom Score	Responder	Non-responder	*P* value *
Abdominal pain [0-10]	6.5 (10)	5.0 (8)	0.461
Meteorism [0-4]	2.0 (3)	3.0 (3)	0.174
BSS [1-7]	4.0 (6)	3.0 (5)	0.495
VAS [0-100]	70 (50)	55 (20)	0.063

Data are presented as median (IQR); responder, n = 12; non-responder, n = 10;

* Mann-Whitney tests.

Table 6.

Pathway	Ctrl	Intv	p value	FDR
PWY.5103..L.isoleucine.biosynthesis.III	0.0027	0.0014	0.0002	0.049
BRANCHED.CHAIN.AA.SYN.PWY..superpathway.of.branched.amino.acid.biosynthesis	0.0029	0.0016	0.0002	0.049
PWY.6737..starch.degradation.V	0.0045	0.0025	0.0003	0.049
PWY.1296..purine.ribonucleosides.degradation	0.0026	0.0016	0.0022	0.306
TRNA.CHARGING.PWY..tRNA.charging	0.0030	0.0017	0.0034	0.335
PWY.5005..biotin.biosynthesis.II	1.2302	8.3744	0.0041	0.335
PWY.3001..superpathway.of.L.isoleucine.biosynthesis.I	0.0021	0.0013	0.0049	0.336
THRESYN.PWY..superpathway.of.L.threonine.biosynthesis	0.0018	0.0011	0.0071	0.413
CALVIN.PWY..Calvin.Benson.Bassham.cycle	0.0028	0.0018	0.0084	0.430
ILEUSYN.PWY..L.isoleucine.biosynthesis.I..from.threonine.	0.0040	0.0026	0.0137	0.476
PWY.7111..pyruvate.fermentation.to.isobutanol..engineered.	0.0040	0.0026	0.0137	0.476
VALSYN.PWY..L.valine.biosynthesis	0.0040	0.0026	0.0137	0.476
PWY.6317..galactose.degradation.I..Leloir.pathway.	0.0021	0.0011	0.0160	0,476
PWY.66-422..D.galactose.degradation.V..Leloir.pathway.	0.0021	0.0011	0.0160	0.476
PWY.5918..superpathay.of.heme.biosynthesis.from.glutamate	0.6003	2.9823	0.0179	0.476
COBALSYN.PWY..adenosylcobalamin.salvage.from.cobinamide.I	0.0013	0.0007	0.0186	0.476
HISTSYN.PWY..L.histidine.biosynthesis	0.0015	0.7954	0.0186	0.476
PWY.1042..glycolysis.IV..plant.cytosol.	0.0033	0.0021	0.0215	0.519
PWY.6609..adenine.and.adenosine.salvage.III	0.0018	0.0011	0.0248	0.562
PANTO.PWY..phosphopantothenate.biosynthesis.I	0.0016	0.0024	0.0256	0.562
PWY.6700..queuosine.biosynthesis	0.0017	0.0021	0.0286	0.562
NONOXIPENT.PWY..pentose.phosphate.pathway..non.oxidative.branch.	0.0025	0.0016	0.0327	0.562
DTDPRHAMSYN.PWY..dTDP.L.rhamnose.biosynthesis.I	0.0026	0.0017	0.0373	0.562
PWY.5100..pyruvate.fermentation.to.acetate.and.lactate.II	0.0013	0.6646	0.0373	0.562
PWY.6121..5.aminoimidazole.ribonucleotide.biosynthesis.I	0.0032	0.0022	0.0373	0.562
PWY.6527..stachyose.degradation	0.0016	0.0001	0.0373	0.562
PWY.724..superpathway.of.L.lysine..L.threonine.and.L.methionine.biosynthesis.II	0.0024	0.0017	0.0373	0.562
SER.GLYSYN.PWY..superpathway.of.L.serine.and.glycine.biosynthesis.I	0.0019	1.1378	0.0373	0.562
PWY.6122..5.aminoimidazole.ribonucleotide.biosynthesis.II	0.0032	0.0021	0.0425	0.562
PWY.621..sucrose.degradation.III..sucrose.invertase.	0.0014	0.0082	0.0425	0.562
PWY.6277..superpathway.of.5.aminoimidazole.ribonucleotide.biosynthesis	0.0032	0.0021	0.0425	0.562
PWY.7237..myo...chiro..and.scillo.inositol.degradation	5.0444	8.1743	0.0425	0.562
PWY.7219..adenosine.ribonucleotides.de.novo.biosynthesis	0.0054	0.0038	0.0482	0.613
HEME.BIOSYNTHESIS.II..heme.biosynthesis.I..aerobic.	0.8163	2.9537	0.0493	0.613

Median relative abundances of microbial pathways in feces at baseline of patients without intolerances (Ctrl) vs. all patients with sugar malabsorptions combined undergoing diet intervention (Intv). *P values* obtained by Kruskal-Wallis testing (p value) with FDR-correction (FDR). Presented are only pathways with significant difference in the Kruskal-Wallis test (ranking according to magnitude of difference).

Table 7.

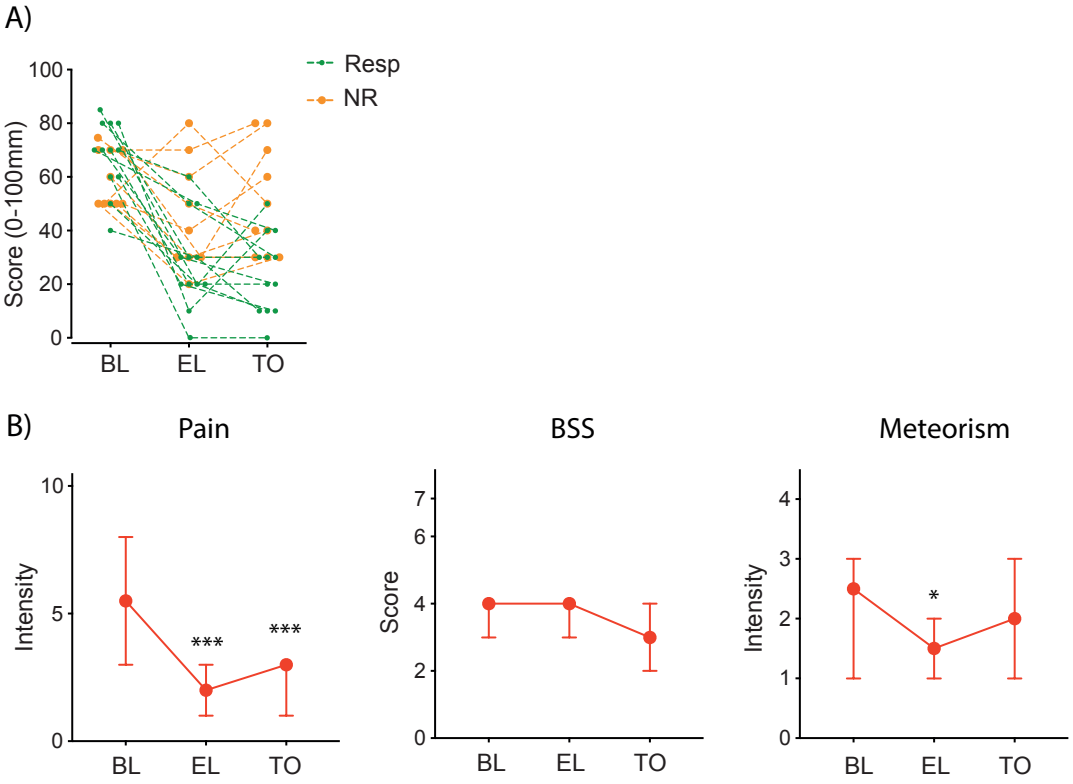
Pathway	Coefficient	p value	FDR
PWY-5103__L-isoleucine_biosynthesis_III	-0.00874076398645614	9,11E+07	0.0002
BRANCHED-CHAIN-AA-SYN-PWY__superpathway_of_branched_amino_acid_biosynthesis	-0.00835718718166046	1,68E+08	0.0002
TRNA-CHARGING-PWY__tRNA_charging	-0.00699489258702028	5,91E+09	0.0046
PWY-3001__superpathway_of_L-isoleucine_biosynthesis_I	-0.00610279379153467	6,00E+08	0.0046
PWY0-1296__purine_ribonucleosides_degradation	-0.00567944882974878	0.000454374	0.0241
CALVIN-PWY__Calvin-Benson-Bassham_cycle	-0.00600477116082881	0.000483234	0.0241
THRESYN-PWY__superpathway_of_L-threonine_biosynthesis	-0.00496125107728647	0.000542351	0.0241
ILEUSYN-PWY__L-isoleucine_biosynthesis_I_from_threonine	-0.00565731921105798	0.002068167	0.0505
PWY-7111__pyruvate_fermentation_to_isobutanol_engineered	-0.00565731921105798	0.002068167	0.0505
PWY-1042__glycolysis_IV_plant_cytosol	-0.00616461215364823	0.002195504	0.0505
PWY-6737__starch_degradation_V	-0.00583219197845164	0.002266796	0.0505
PWY-6277__superpathway_of_5-aminoimidazole_ribonucleotide_biosynthesis	-0.00459309440699081	0.003407574	n.sig.
PWY-724__superpathway_of_L-lysine_L-threonine_and_L-methionine_biosynthesis_II	-0.00404526918371397	0.004963728	n.sig.
PWY-6121__5-aminoimidazole_ribonucleotide_biosynthesis_I	-0.00439104389674386	0.005355247	n.sig.
PANTO-PWY__phosphopantothenate_biosynthesis_I	-0.00464052347852179	0.008774361	n.sig.
PWY-6609__adenine_and_adenosine_salvage_III	-0.005302199910653	0.009186205	n.sig.
NONOXIPENT-PWY__pentose_phosphate_pathway_non-oxidative_branch	-0.004382109580736	0.010026873	n.sig.
ARO-PWY__chorismate_biosynthesis_I	-0.00343281041124071	0.023599402	n.sig.
PWY-7219__adenosine_ribonucleotides_de_novo_biosynthesis	-0.00418996739315033	0.040762263	n.sig.
DTDPRHAMSYN-PWY__dTDP-L-rhamnose_biosynthesis_I	-0.00466903879630791	0.045017881	n.sig.

Table with results from a multivariate analysis using a general linear model to find associations between clinical data and metagenomic pathways. Pathways are ranked according to the magnitude of statistical difference between non-malabsorbing IBS controls, malabsorbing responders and non-responders of the intervention cohort (BL and TO time points combined). The model was adjusted for the intake of fiber in gram per day. *P values* are FDR-adjusted according to the Benjamini-Hochberg method.

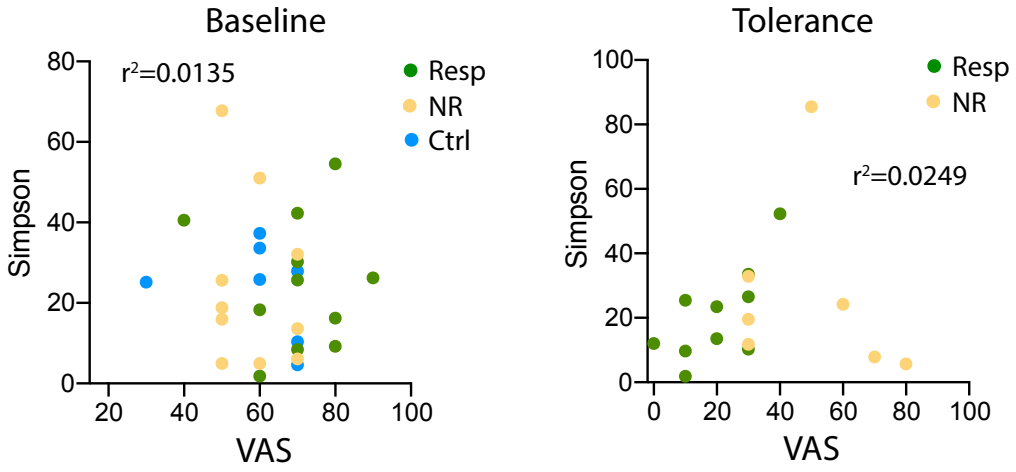
Table 8.

	Species							
Pathway	Eubacterium rectale	Eubacterium sireum	Akkermansia muciniphila	Faecalibact. prausnitzii	Ruminococcus bromii	Ruminococcus obeum	Bifidbacterium longum	Odoribacter splanchnicus
PWY 5103: L-isoleucine biosynthesis III	r2 = 0.0009, p > 0.05	r2 = 0.027, p > 0.05	r2 = 0.0003, p > 0.05	r2 = 0.55, p < 0.0001	r2 = 0.153, p = 0.0049	r2 = 0.163, p = 0.0036	r2 = 0.146, p = 0.006	r2 = 0.0005, p > 0.05
Branched chain amino acid synthesis	r2 = 0.011, p > 0.05	r2 = 0.025, p > 0.05	r2 = 0.0009, p > 0.05	r2 = 0.53, p < 0.0001	r2 = 0.144, p = 0.0066	r2 = 0.160, p = 0.0045	r2 = 0.152, p = 0.005	r2 = 0.0005, p > 0.05
PWY 6737: starch degradation V	r2 = 0.034, p > 0.05	r2 = 0.051, p > 0.05	r2 = 0.014, p > 0.05	r2 = 0.29, p < 0.0001	r2 = 0.047, p > 0.05	r2 = 0.067, p > 0.05	r2 = 0.104, p = 0.022	r2 = 0.0007, p > 0.05

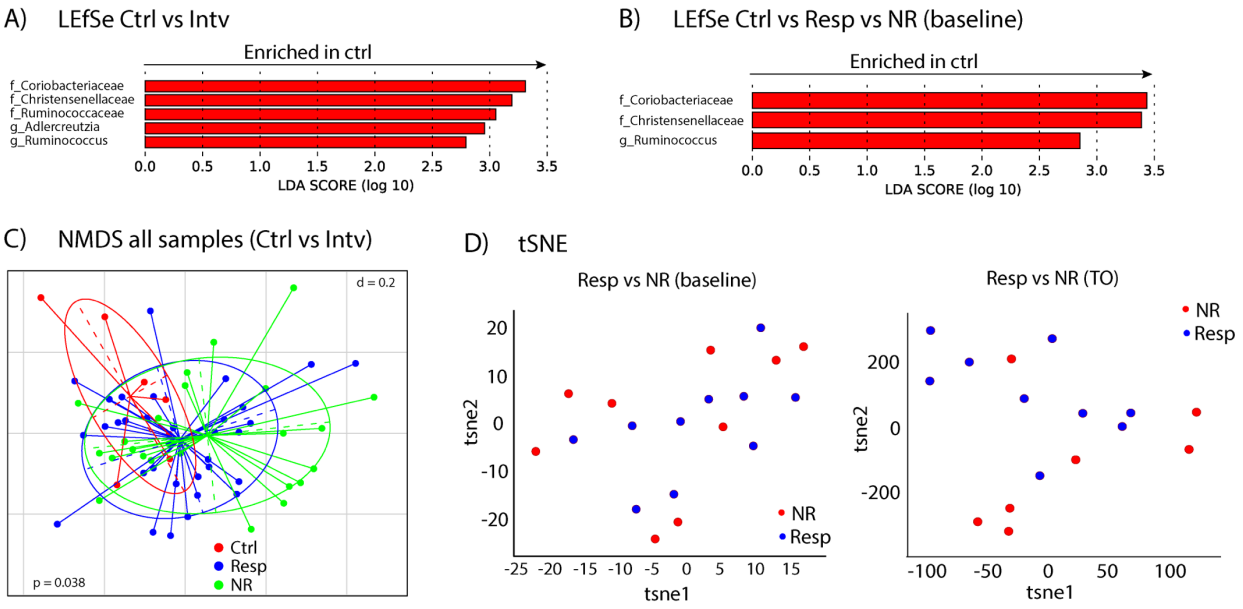
Tabulated results of correlation analyses of abundances of different species from Figure 3C vs. abundances of selected microbial metagenomic pathways (including control subjects and non-responders/responders of the intervention cohort). R squared and p-value are applied to describe the goodness and comparisons of fits. P-values are not corrected for multiple-hypothesis testing.



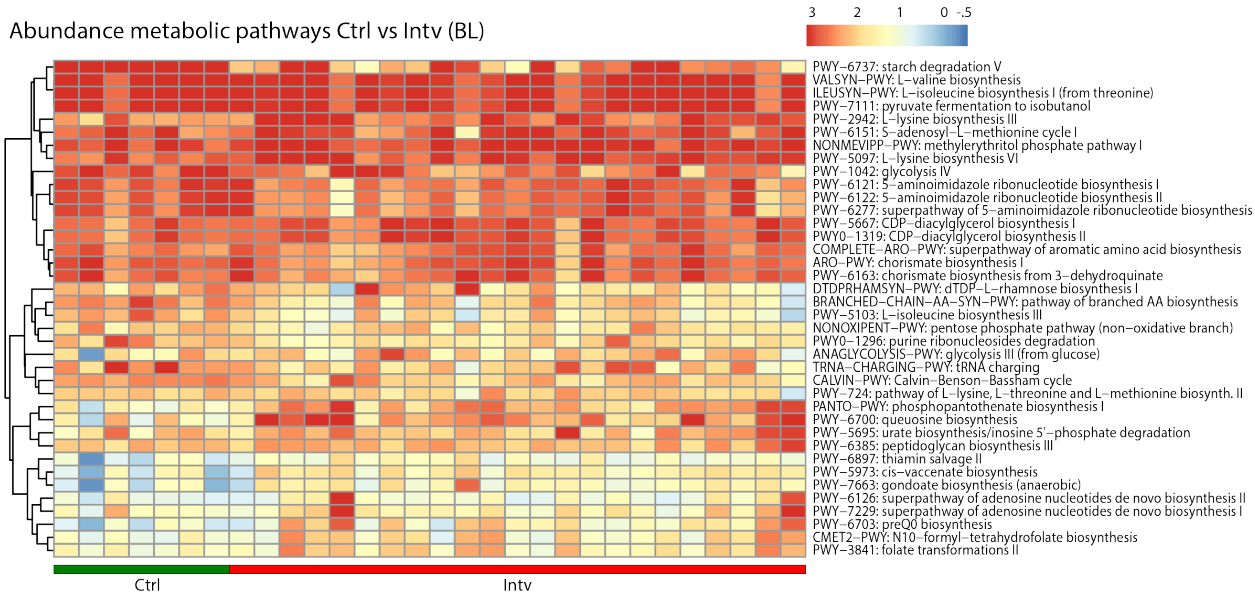
Supplementary Figure 1. (A) VAS scores displayed for each study patient individually in the intervention group and color-coded into responders (Resp) and non-responders (NR). (B) Changes of pain intensity, BSS scores and meteorism intensity during elimination (EL) and tolerance periods (TO) in the total (non-dichotomized) intervention cohort. *p<0.01, ***p<0.001 (Wilcoxon matched-pairs signed rank test; uncorrected); data presented as median with 95% CI in (B).



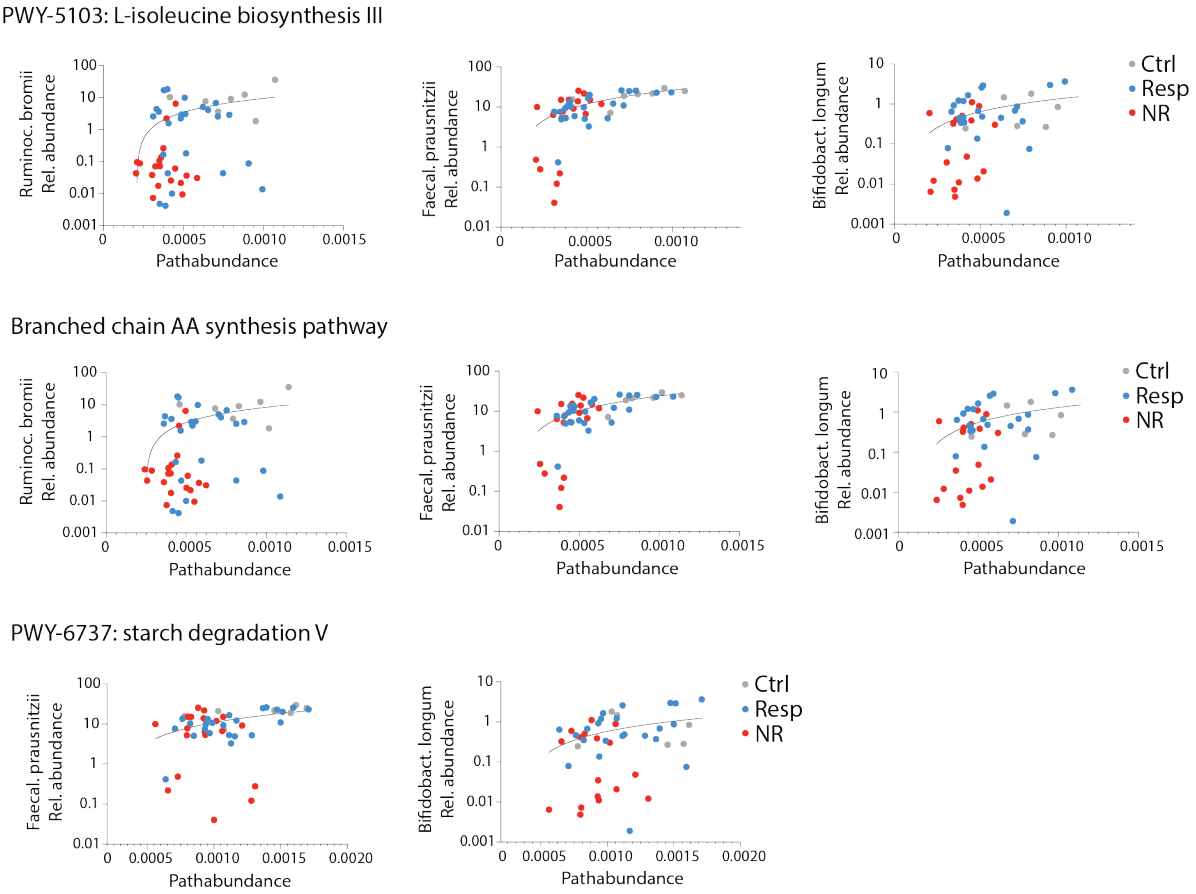
Supplementary Figure 2. Correlation of individual alpha diversity indices measures by Simpson's score at baseline (left) and after tolerance phase (right) with the different groups color-coded into controls (Ctrl), responder (Resp) and non-responder (NR). r^2, correlation coefficient determined by a linear regression.



Supplementary Figure 3. Microbiome changes during diet intervention. (A) The linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed to explore differences in the relative abundance of fecal 16S rRNA – associated taxa in controls vs. patients with malabsorptions at baseline. (B) LEfSe to compare differences between controls vs. VAS responder and non-responder study subjects at baseline. (C) Non-metric multidimensional-scaling (NMDS) plot of fecal microbiota beta-diversity indices (generalized Unifrac distances) from control (ctrl) patients and diet intervention (intv) – patients (latter not separated into different time points); p-value obtained by a permutational multivariate analysis of variance. (D) Visualization of beta-diversity (Unifrac distances) by tSNE of fecal s16S rRNA from intervention patients color-coded as responder (Resp) and non-responder (NR) at baseline (left) or at TO phase (right).



Supplementary Figure 4. Representation of highly abundant metabolic pathways annotated from MetaCyc in the gut microbiota of controls (blue bar) vs. malabsorbing / intervention patients (green bar at the bottom of the heat map) at baseline; clustering of pathways by h-clust.



Supplementary Figure 5. Correlation of metagenomic microbial pathways identified by LefSe analysis to be overrepresented in controls and/or diet responders (Fig. 3) with individual taxa (see also Supplementary Table 7 for statistical details). Color-coding of non-malabsorbing controls (Ctrl), diet responders (Resp) and non-responders (NR) of the intervention cohort.

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